Influence of Seed Treatment on Tobacco Mosaic Virus Incidence in Tobacco Seedlings and Virus Distribution in Greenhouse Transplant Production

Rachel E. L. Loveday

Thesis submitted to the Faculty of the
Virginia Polytechnic Institute and State University
in partial fulfillment of the requirements for the degree of

Master of Science
in
Crop and Soil Environmental Sciences

Carol A. Wilkinson, Co-Chair

Sue A. Tolin, Co-Chair

T. David Reed

February 7, 2007

Blacksburg, Virginia

Keywords: Seed Transmission, Chemical Seed Treatment, Greenhouse Float Production

Influence of Seed Treatment on Tobacco Mosaic Virus Incidence in Tobacco Seedlings and Virus Distribution in Greenhouse Transplant Production

Rachel E.L. Loveday

(ABSTRACT)

Tobacco mosaic virus (TMV) is an economically important pathogen that has been studied for over one hundred years. Seedlings, seed coats, and nutrient solution were assayed for the presence of the virus and seed treatments were tested on seeds. Double antibody sandwich enzyme-linked immunosorbent assay (DAS ELISA) and biological local lesion assay data were collected. Seed coats from seed collected from TMV infected plants were always positive for TMV regardless of chemical treatment. Seed from infected source plants have lower germination than seed from healthy plants. Trisodium phosphate and hydrochloric acid treatments reduced virus infection of seedlings when grown under controlled conditions. Virus particles were serologically and biologically detected in both the leaves and roots of seedlings mechanically inoculated with TMV. Nutrient solution collected from 28 day old seedlings, 12 days post inoculation, tested positive for biologically active TMV by ELISA and infectivity assay. Infected water in float bed production could facilitate viral movement to all seedlings sharing nutrient solution. Seed transmission of TMV was shown to occur at a rate of 0.2%. This is in contrast to other research attempting to demonstrate seed transmission where visual symptoms on seedlings have been used to assess seed transmission.

ACKNOWLEDGEMENTS

I thank Dr. Carol Wilkinson for her support and help. We saw it through and I am grateful. I also thank Dr. Sue Tolin for her assistance and the knowledge that she shared. Thank you for opening your home, heart and office to a very nervous young lady with a big dream. Your patience and guidance were invaluable! I thank Dr. David Reed for his unwavering support. You always seemed interested. You trusted me and I thank you. I gratefully acknowledge and express thanks to Philip Morris USA for their financial support that allowed me to pursue my graduate degree.

I thank Mac Tilson for all of his suggestions, jokes, criticism, entertainment, and support. When I needed to be reminded of certain things, I could always count on you for honest answers and straight talk. I wish I knew half of what you have forgotten.

I also thank the past and present field crew, Bud, Roger, Calvin, Bill W., Sandy, Walter, Margaret, Randy, Billy Wayne, Danny, Jeff, Ned, and William. You all have seen me at my best and worst, my cleanest and dirtiest, in the sweltering summers, cold winters, and cleansing rains, and there was always energy and laughter in your eyes. I have appreciated the support and laughter you ALL have given me. I will always be grateful for your friendships.

Thank you to the office staff for their kind friendship and support. I thank Sherry for sharing some of her short time with me. Your sweet temperament and calm manner were greatly appreciated.

To The Old Crow, I thank you immensely. It is hard to express the love, support, and drama you have given me and I would not have it any other way. You are a precious friend and dear confidant. Your perspective and guidance were and will be greatly appreciated.

I thank James Green for his laughter and entertainment. You never let me stay sad for long and you know how to make those around you feel important. I also thank you for your excellent ability to always listen, even if it was truly ridiculous.

The author also thanks a hillbilly from Southwest Virginia, Seth Mullins, for his constant corrections, irritation, and unfaltering evil spirit. You were a big, degenerate brother to me for way too long. You were, however, someone who forced me improve myself and taught me how to toughen up. I appreciate your honest friendship and thank you for your help and guidance.

I thank Corey Loveday for all of his help while Josh and I lived in Blacksburg. Thank you for watching our home and caring for our animals. We would not have been able to survive with out you.

The author offers Peta-Gaye Chang gratitude and thanks for her honest, open friendship. By having you as a friend, you made Blacksburg feel like home. Your presence, charisma, and optimistic personality were a blessing and pleasure for me. You will always be part of me and I now realize Jamaica has paved roads.

I thank Cecilia and Sebastian for opening up there home and hearts for a wonderful Peruvian meal, the likes of which make your taste buds sing. The best part of the meal was your honest friendship. I will always be grateful. (Oh, who could forget wallyball and Hunan?)

I acknowledge the special contributions of my family, especially my mom and dad, Linda and Danny Loveday, and Betty and Roscoe Lewis. You remained supportive of me throughout this journey and instilled a passion for agriculture and conservation. I will always be grateful, thank you.

Last but not least, the author acknowledges the person, whom without his support, none of this would be possible, Joshua. Undeniable, he loved, pushed, reminded, struggled, and

physically and emotionally sacrificed for me to pursue my long carried dream. Most importantly, he trimmed his hunting season to live in Blacksburg while I attended classes. Thank you, Joshua, for promising to love me unconditionally, supporting my goals, honoring and respecting me, laughing with me, crying with me, and cherishing me for as long as we both shall live. I hope I can give you the beautiful gift you have given me. I love you.

TABLE OF CONTENTS

Chapter **Page**

Abstract ii
Acknowledgements iii
List of tables vii
List of figures x
I. Introduction 1
II. Literature Review
Tobacco mosaic virus 5
Physical characteristics and properties 5
Replication and movement 6
Virus survival and dissemination 7
Evidence for a role of seed transmission 8
Location of TMV in the seed 10
Location of TMV in the root 11
TMV in water 12
Control 14
Literature Cited 21
III. Seed treatment of flue-cured tobacco seed collected from tobacco mosaic virus infected plants
Abstract 28
Introduction 30
Materials and Methods 32
Results and Discussion 37
Conclusions 42
Literature Cited 44
IV. Evaluation of tobacco mosaic virus movement in greenhouse float production of K 326 flue-cured tobacco
Abstract 54
Introduction 55
Materials and Methods 57
Results and Discussion 63
Conclusions 66
Literature Cited 67
V. Summary 76
VI. Appendix 78
VII. Vita 103

LIST OF TABLES

Chapter III. Seed treatment of flue-cured tobacco seed collected from tobacco mosaic virus infected plants

		Page
Table 3-1.	Analysis of variance from two growth chamber tests evaluating effectiveness of chemicals and treatment time on germination and seed transmission of tobacco mosaic virus in K326 flue-cured tobacco seedlings and seed coats.	46
Table 3-2.	Means from two growth chamber tests evaluating effectiveness of chemicals on germination and seed transmission of tobacco mosaic virus in K326 flue-cured tobacco seedlings and seed coats.	47
Table 3-3.	Means from two growth chamber tests evaluating effectiveness of treatment times on germination and seed transmission of tobacco mosaic virus in K326 flue-cured tobacco seedlings and seed coats.	48
Table 3-4.	Analysis of variance from two growth chamber tests evaluating effectiveness of chemicals on germination and seed transmission of tobacco mosaic virus in K326 flue-cured tobacco seedlings and seed coats.	49
Table 3-5.	Means from two growth chamber tests evaluating effectiveness of chemicals on germination and seed transmission of tobacco mosaic virus in K326 flue-cured tobacco seedlings and seed coats.	50

Chapter IV. Evaluation of tobacco mosaic virus movement in greenhouse float production of K 326 flue-cured tobacco

Table 4-1.	Analysis of variance for three greenhouse tests investigating the presence of tobacco mosaic virus (TMV) in plant tissue and the nutrient solution.	70
Table 4-2.	Means from three greenhouse tests investigating the presence of tobacco mosaic virus (TMV) in plant tissue and the nutrient solution.	71
Table 4-3.	Analysis of variance for three greenhouse tests evaluating distribution of tobacco mosaic virus (TMV) via seed transmission.	72
Table 4-4.	Means from three greenhouse tests evaluating distribution of tobacco mosaic virus (TMV) via seed transmission.	73

Chapter VI. Appendix

		Page
Table A-1.	Analysis of variance from the first of two growth chamber tests evaluating effectiveness of chemicals and treatment time on germination and seed transmission of tobacco mosaic virus (TMV) in flue-cured tobacco seedlings and seed coats.	79
Table A-2.	Means from the first of two growth chamber tests evaluating effectiveness of chemicals on germination and seed transmission of tobacco mosaic virus (TMV) in flue-cured tobacco seedlings and seed coats.	80
Table A-3.	Means from the first of two growth chamber tests evaluating effectiveness of treatment time on germination and seed transmission of tobacco mosaic virus (TMV) in flue-cured tobacco seedlings and seed coats.	81
Table A-4.	Analysis of variance from the second of two growth chamber tests evaluating effectiveness of chemicals and treatment time on germination and seed transmission of tobacco mosaic virus (TMV) in flue-cured tobacco seedlings and seed coats.	82
Table A-5.	Means from the second of two growth chamber tests evaluating effectiveness of chemicals on germination and seed transmission of tobacco mosaic virus (TMV) in flue-cured tobacco seedlings and seed coats.	83
Table A-6.	Means from the second of two growth chamber tests evaluating effectiveness of treatment time on germination and seed transmission of tobacco mosaic virus (TMV) in flue-cured tobacco seedlings and seed coats.	84
Table A-7.	Analysis of variance from the first of two growth chamber tests evaluating effectiveness of selected chemicals and treatment time combinations on germination and seed transmission of tobacco mosaic virus (TMV) in flue-cured tobacco seedlings and seed coats.	85
Table A-8.	Means from the first of two growth chamber tests evaluating effectiveness of selected chemicals and treatment time combinations on germination and seed transmission of tobacco mosaic virus (TMV) in flue-cured tobacco seedlings and seed coats.	86
Table A-9.	Analysis of variance from the second of two growth chamber tests evaluating effectiveness of selected chemicals and treatment time combinations on germination and seed transmission of tobacco mosaic virus (TMV) in flue-cured tobacco seedlings and seed coats.	88

Table A-10. Means from the second of two growth chamber tests evaluating effectiveness of selected chemicals and treatment time combinations on germination and seed transmission of tobacco mosaic virus (TMV) in flue-cured tobacco seedlings and seed coats. 89

Table A-11. Analysis of variance for the first of three greenhouse tests investigating distribution of tobacco mosaic virus (TMV) in plant tissue and the nutrient solution. 91

Table A-12. Means for the first of three greenhouse tests investigating distribution of tobacco mosaic virus (TMV) in plant tissue and the nutrient solution. 92

Table A-13. Analysis of variance for the second of three greenhouse tests investigating distribution of tobacco mosaic virus (TMV) in plant tissue and the nutrient solution. 93

Table A-14. Means for the second of three greenhouse tests investigating distribution of tobacco mosaic virus (TMV) in plant tissue and the nutrient solution. 94

Table A-15. Analysis of variance for the third of three greenhouse tests investigating distribution of tobacco mosaic virus (TMV) in plant tissue and the nutrient solution. 95

Table A-16. Means for the third of three greenhouse tests investigating distribution of tobacco mosaic virus (TMV) in plant tissue and the nutrient solution. 96

Table A-17. Analysis of variance for the first of three greenhouse tests evaluating distribution of tobacco mosaic virus (TMV) via seed transmission. 97

Table A-18. Means for the first of three greenhouse tests evaluating distribution of tobacco mosaic virus (TMV) via seed transmission. 98

Table A-19. Analysis of variance for the second of three greenhouse tests evaluating distribution of tobacco mosaic virus (TMV) via seed transmission. 99

Table A-20. Means for the second of three greenhouse tests evaluating distribution of tobacco mosaic virus (TMV) via seed transmission. 100

Table A-21. Analysis of variance for the third of three greenhouse tests evaluating distribution of tobacco mosaic virus (TMV) via seed transmission. 101

Table A-22. Means for the third of three greenhouse tests evaluating distribution of tobacco mosaic virus (TMV) via seed transmission. 102

LIST OF FIGURES

Chapter III. Seed treatment of flue-cured tobacco seed collected from tobacco mosaic virus infected plants

		Page
Figure 3-1.	Germination box and blue blotter paper used in seed treatment tests.	52
Figure 3-2.	Seed treated with 9.1% ultra bleach solution (left) and healthy control seed treated with deionized water (right) showing the color difference.	53

Chapter IV. Evaluation of tobacco mosaic virus movement in greenhouse float production of K 326 flue-cured tobacco

Figure 4-1.	Clay's Seeder used to seed float trays with raw 'K326' flue-cured tobacco seed.	74
Figure 4-2.	Comparison of various seedlings in the evaluation of tobacco mosaic virus in seedlings via seed transmission.	75

Chapter I

INTRODUCTION

Tobacco mosaic virus (TMV) occurs worldwide in all areas where tobacco (*Nicotiana tabacum* L.) is grown. Early season infection causes the most extreme loss in quality, yield and average price, while infection after the topping stage has less effect on yield, quality and average price (Gooding, 1981; Chaplin and Mann, 1978). A tobacco leaf with TMV produces a thin, brittle cured leaf, which is undesirable for production. TMV is an economically important pathogen because of its impact on cured tobacco and subsequent sales.

Resistance to TMV is limited. The first case of resistance was observed in the variety 'Ambalema' by Nolla and Roque (1933). After the initial exposure to TMV, the plant remained symptomless. The mechanism for resistance was found to be two recessive genes coupled with modifying genes (Clayton et al., 1938). This type of resistance was of limited use due to severe wilting on hot days, which resulted in some plant fatality (Valleau, 1952).

A second source of TMV resistance is the N-gene found in *Nicotiana glutinosa* as a single, dominant gene (Holmes, 1938). A tobacco plant containing the N-gene exhibits a hypersensitive local response, otherwise known as a necrotic local lesion, to inoculation with TMV (Hull 2002). Holmes (1938) incorporated the N-gene into *N. tabacum* burley and flue-cured genotypes. While burley tobacco N-gene derived resistance was successful, the first flue-cured tobacco cultivars developed with N-gene derived resistance had reduced yield and quality (Chaplin and Mann, 1978). Elimination and reduction of viral inoculum could provide better control for this pathogen.

Although TMV is located in the tobacco seed coat (Benoit and Maury, 1976), it has not been considered as seed transmitted. Several studies were conducted to demonstrate seed transmission (Taylor, 1962; Benoit, 1977; Lusso et al., 2002) based on suggestions by Allard (1915), but none of these tests were successful. Recent research has demonstrated seed transmission of TMV to 14-day-old flue-cured tobacco seedlings (C. A. Wilkinson, personal communication, 2007). Seed surface sterilization methods have been successful in eliminating the virus in tomato seed (Alexander, 1960; Nitzany, 1960; Niemyski, 1963; Alekseev et al., 1975; Gooding, 1975) and could be used to eliminate the potential of seed transmission in tobacco.

Surface waters are known to contain TMV (Tosic and Tosic, 1984; Piazolla et al., 1986). Park et al. (1999) suggested that the potential infection of plants in closed hydroponic systems was likely to be root grafting rather than uptake of viral particles out of the nutrient solution. Yarwood (1960) collected drainage water from TMV infected plants and found that the water contained biologically active virus particles. Smith et al. (1969) concluded that undamaged, nonnecrotic roots released the viral particles found in water.

The occurrence of TMV in greenhouse float bed tobacco production is an increasing concern. While there are several sources of inoculum, such as contaminated tobacco products, infected weeds grown in or around the greenhouse, or from workers hands with contagion, transmission from seeds or water has not been investigated.

The objectives of this study were to determine the effectiveness of chemical seed surface sterilization treatments on reducing or eliminating seed transmission of TMV in tobacco and to detect and test viability of TMV in shoots, roots, and water in a greenhouse float production system.

LITERATURE CITED

Alekseev, R. V., Shcherbinin, B. M., Tokareva, N. N. 1975. Control of tobacco mosaic virus (TMV) in tomato seed crops. Ovoshchevodstva i Bakhchevodstva 3/4:82-87.

Alexander, L. J. 1960. Inactivation of TMV from tomato seed. Phytopathology 50:627.

Allard, H. A. 1915. Distribution of the virus of the mosaic disease in capsules, filaments, anthers and pistils of affected tobacco plants. Journal of Agricultural Research 5:251-256.

Benoit, M. A. 1977. Does tobacco seed transmit tobacco mosaic virus? Planter 53: 197-201.

Benoit, M. A. and Maury, Y. 1976. The location of tobacco mosaic virus in tobacco seed. Planter 52:392-396.

Chaplin, J. F. and Mann, T. J. 1978. Evaluation of tobacco mosaic resistance factor transferred from burley to flue-cured tobacco. Journal of Heredity 69:175-178.

Clayton, E. E., Smith, H. H., and Foster, H. H. 1938. Mosaic resistance in *Nicotiana tabacum* L. Phytopathology 28:286-288.

Gooding, G. V., Jr. 1975. Inactivation of tobacco mosaic virus on tomato seed with trisodium orthophosphate and sodium hypochlorite. Plant Disease Reporter 59:770-772.

Gooding, G. V., Jr. 1981. Control of tobacco mosaic virus on flue-cured tobacco by crop protection. Tobacco Science 40:30-31.

Holmes, F. O. 1938. Inheritance of resistance to tobacco mosaic disease in tobacco. Phytopathology 28:553-561.

Hull, R. 2002. Matthew’s Plant Virology. Elsevier Academic Press 1001 pp.

Lusso, M., Davis, G., and Barker, H. 2002. The route of tobacco mosaic virus to *Nicotiana tabacum* seeds and evaluation of its seed transmission. Paper presented at the 40th Tobacco Workers Conference, Jan 14-17, Pinehurst, NC.

Niemyski, K. 1963. Inactivation of tobacco mosaic virus in tomato seed. Ada agrobot 14:233-244.

Nitzany, F. E. 1960. Transmission of mosaic virus through tomato seed and virus inactivation methods of seed extraction and seed treatments. Ktavim 2:59-61.

Nolla, J. S. B. and Roque, A. 1933. A variety of tobacco resistant to ordinary tobacco mosaic. Journal of the Puerto Rico Department of Agriculture 17:301-303.

Park, W. M., Lee, G. P., Ryu, K. H., and Park, K. W. 1999. Transmission of tobacco mosaic virus in recirculating hydroponic system. Scientia Horticulture 79:217-226.

Piazolla, P., Castellano, M. A. and Stradis, A. 1986. Presence of plant viruses in some rivers of southern Italy. Journal of Phytopathology 116:244-246.

Smith, P. R., Campbell, R. N. and Fry, P.R. 1969. Root discharge and soil survival of viruses. Phytopathology 59:1678-1687.

Taylor, R. H. 1962. The location of tobacco mosaic virus in tobacco seed. Australian Journal of Experimental Agriculture and Animal Husbandry 2:86-89.

Tosic, M. and Tosic, D. 1984. Occurrence of tobacco mosaic virus in water of the Danube and Sava rivers. Phytopathologische Zeitschrift 110:200-202.

Valleau, W. D. 1952. Breeding tobacco for disease resistance. Economic Botany 6:69-102.

Yarwood, C. E. 1960. Release and preservation of virus by roots. Phytopathology 50:111-114.

Chapter II

LITERATURE REVIEW

Tobacco mosaic virus (TMV) is commonly used as an ideal model to study host-pathogen reactions and cellular trafficking in *Solanaceous* plants (Scholthof, 2004). The initial analysis of TMV began as an investigation for the cause of the detrimental mosaic disease affecting tobacco production (Scholthof, 2004). Much is known about the structure, composition, symptoms, and host range of TMV (Hull, 2002), while some aspects of transmission, specifically seed transmission, remain unclear (Taylor, 1962; Benoit and Maury, 1976; Benoit, 1977).

Tobacco Mosaic Virus

TMV is distributed worldwide in all areas where tobacco (*Nicotiana tabacum* L.) is grown. TMV infects all classes of tobacco. The virus is known to infect at least 199 species from 30 plant families (Shew and Lucas, 1991). Symptoms appear three to four days post infection, beginning with vein clearing on young, systemically invaded leaves. The typical light and dark green mosaic pattern subsequently develops. Leaf curling, blistering, yellowing, and necrosis are also symptoms of progressed disease.

Physical Characteristics and Properties

TMV belongs to a large group of positive-sense single-stranded ribonucleic acid (RNA) viruses in the genus *Tobamovirus*. The small, rigid rod-shaped virus particle measures 300 nm x 15 nm with an axial radius of 2 nm. Each particle contains about 6,400 nucleotides and the

single genomic RNA encodes four proteins, two for replication, one for a capsid protein (CP) and one for a movement protein (MP) (Fauquet et al., 2005; Hull, 2002). The RNA is encapsidated in a helical coat of 2,160 subunits of coat protein (Heinlein, 2002).

TMV is highly stable. It is known to remain infectious for 50 yr in unpreserved plant sap (Silber and Burk, 1965). The thermal inactivation point is 93°C for 10 min, and the dilution end point is 1×10^{-6} (Smith, 1972). Virus may be preserved by freezing fresh leaves, by freeze–drying tissue, and in dried tissue (Zaitlin, 2000).

Replication and Movement

Replication of TMV occurs only after the virus enters the plant cell through a minor wound. After entry, the virus undergoes cotranslational disassembly in which the virus uncoats at the 5’ end within 2 to 3 min (Heinlein, 2002). As replication proceeds from the resultant negative strand RNA template, positive-strand, full-length genomic RNA is produced, as well as subgenomic 30 kDa MP and CP (Heinlein, 2002). Ishikawa et al. (1991) noted that the synthesis of negative strand RNA stops 6 to 8 h post inoculation (pi), while positive strand synthesis continues beyond its maximum accumulation at 14 to 18 h pi. Gradually, the progeny virus is encapsidated by the accumulated capsid protein and new infectious virions are formed.

There are two forms of viral movement within an infected plant, short-distance and long-distance (Samuel, 1934; Leisner and Howell, 1993). Short-distance movement throughout a leaf is limited to the adjacent cells by way of the plasmodesmata, a discrete plasma membrane-lined intracellular cytoplasmic strand (Lucas et al., 1993). The MP facilitates travel through the cell walls by altering the size exclusion limit (SEL) of the plasmodesmata (Wolf et al., 1989; Atkins et al., 1991; Citovsky, 1999). While the normal SEL is within 700 to 800 Da, TMV MP permits

movement of molecules with a molecular mass of about 9400 Da (Wolf et al., 1989). When the virus enters the adjacent plant cell, the disassembly, transcription and replication begins again. The initial invaded cell enters a late infection stage, where the virus particles begin to accumulate on the microtubules and slows movement out of the cell (Heinlein, 2002). The estimated rate of short-distance movement from the upper epidermis to the lower is 0.8 µm/h (Uppal, 1934).

Long-distance movement of TMV occurs in the conducting cells of the phloem that are interconnected by sieve plates with large pores (Hull, 2002). There are many different estimates of the time required for TMV to travel from the parenchyma into the phloem (Mandahar, 1990). Factors that impact this rate of movement include host species and virus, age of host, method of inoculation, and temperature. SEL of plasmodesmata in sink tissues, those which import photoassimilates and nutrients, appear to be much larger than that in source tissues, those which export bulk photoassimilates (Santa Cruz, 1999; Hull, 2002). These openings may be large enough for the advancement of the viral infection. Once in the phloem, long-distance movement of TMV can be very rapid. Capoor (1949) recorded movement rates of 8 cm/h in tobacco stems. While the MP is involved in short-distance movement, CP is used in long-distance movement to encapsidate and protect the virus particle while in the vasculature. The direction of travel for TMV depends on the flow of photoassimilates, size and shape of the vasculature, and the arrangement of the leaves (Hull, 2002). Sink tissue, which is importing photoassimilates and nutrients, may draw in more virus particles.

Virus Survival and Dissemination

The primary source of inoculum for TMV includes perennial weeds, such as horse nettle (*Solanum carolinense* L.), ground cherry (*Physalis* spp.), and overwintering crop debris in the

fields. Primary infection, the original infection resulting from a dormant pathogen, only accounts for about 15% of the disease incidence (Gooding and Todd, 1976). Therefore, secondary infection of the virus accounts for most of the diseased plants. Secondary infection, which primarily results from mechanical transmission, can originate from people's hands, equipment, leaf to leaf contact, infected crop residue and debris, or tobacco products, any of which have come in contact with infected tissue. Soil-borne virus particles can also serve as sources of infection (Noordam, 1973; Gooding, 1986).

Evidence for a Role of Seed Transmission

TMV is primarily transmitted through mechanical inoculation from an infected or contaminated article. While the general concurrence is that TMV is not seed transmitted, there have been studies indicating TMV is located on or in the seed and may play a role in TMV dissemination. In 1915, Allard suggested that TMV may occur in tobacco seed. During an investigation into the distribution of TMV in affected tobacco plants, Allard (1915) macerated seed collected from infected plants to find, when inoculated to a healthy seedling, TMV infection occurred. Chamberlain (1937) observed seed transmission in seven out of 5,628 tobacco seedlings grown from seed collected from mosaic infected plants. He was not, however, convinced that the infection of these seedlings was not from accidental inoculation. In more recent research, Taylor (1962) investigated the location of the virus within the seed. Seed were collected from TMV infected plants and allowed to dry for 1 to 9 mo. Testa, endosperm plus embryo, and whole seeds were tested at 1, 6, and 9 mo intervals. Each sample was ground and assayed on *Nicotiana glutinosa* L., *N. tabacum* cv. Xanthi NN, and *Datura stramonium* L, which produce local, necrotic lesions when TMV is present. Taylor (1962) found virus present in every

replicate of seed tested. The concentration of the virus was higher in the endosperm plus embryo than the testa group. While this research did not report the exact location of TMV in the seed due to the difficulty of separating the endosperm from the embryo, Taylor (1962) proposed the virus was located within the endosperm. Taylor (1962) also noted that storing seed for periods of up to nine months at room temperature before dissection decreased the viability of the virus.

To demonstrate seed transmission, seedlings were grown from seeds harvested from TMV infected parent plants, and none were demonstrated to have TMV using local lesion assays (Taylor, 1962). The lack of TMV infected seedlings may have been due to large sample volume and sub-sampling technique employed. Taylor (1962) conducted two additional experiments where 100 seed were sown in sand to biologically assay for the presence of virus using TMV resistant plants. Plantlets were harvested in groups of fifty before the first true leaf appeared. Leaf and root samples were taken. Because the buffer dilution was not given, one might conclude that one seedling out of fifty could not be detectable and was below the local lesion assay threshold.

Taylor (1962) also selected 500 seedlings to be grown from seed collected from an infected source. Some were grown in beakers until six leaves appeared and the others were grown in the field. All plants were visually healthy. Again, no dilution was given.

Lusso et al. (2002) performed enzyme linked immunoassays (ELISA) on TMV-infected plant parts, including leaves, petals, pollen, ovaries, anthers, styles, and seeds. TMV was detected in all plant tissue evaluated. They also performed reciprocal crosses between healthy and infected plants. Plants infected with TMV, when pollinated by a healthy plant produced infected seed, while healthy plants pollinated with TMV-infected plants resulted in non-infected seedlings (Lusso et al., 2002). They also evaluated seed transmission of TMV by growing out

seed from infected parent plants. The results suggested that all of the 11,268 nine-week-old plants were healthy, but the only parameter for gauging health was a visual inspection. No serological or biological assays were conducted to demonstrate absence of TMV in the plants.

Chatzivassiliou et al. (2004) surveyed viruses in tobacco crops, seed beds, fields, and native flora of Greece, and pointed out that among the destructive viruses in Greece, TMV was the only virus that is seed-borne. Zaitlin and Israel (1975) reported that TMV is not transmissible by seed or pollen. Zaitlin's revised Descriptions of Plant Viruses (2000) pointed out that TMV could be present in the seed coat of tomato seed.

Research conducted at Virginia Tech has demonstrated that TMV is seed transmitted to 14-day-old flue-cured tobacco seedlings (C. A. Wilkinson, personal communication, 2007). Transmission of TMV to seedling samples was highest from raw seed (31%) followed by melt away coat (13%) and split coat (8%). A higher rate of transmission was observed in growth chamber tests as compared to greenhouse tests.

Location of TMV in the Seed

Benoit and Maury (1976) utilized an immunochemical technique, fluorescent antibody, to determine the precise location of the virus within the seed. They showed that the virus is located inside the seed, and is localized in the nucellar layer, not the endosperm, of the infected tobacco seed. Benoit and Maury (1976) noted that the location of the virus in the seed is not an indication of its transmission. TMV seed transmission in tobacco seed is not dependant on the virus alone nor the host, but a virus-host interaction (Benoit and Maury, 1976). Conversely, Benoit (1977) stated that the virus in the seed coat does not transmit to the seedling before or

after transplanting. Benoit (1977) also mentioned that external contamination by associated plant debris could be the source of seedling infection.

Location of TMV in the Roots

Virus is known to reside in the root systems of plants infected with TMV (Gooding and Todd, 1976; Zaitlin, 2000; Valentine et al., 2002). While the roots of a plant are developing, they are a huge sink for assimilate (Waisel et al., 2002). Thus, virus would be delivered to the roots. Despite the knowledge that several viruses can be found in roots, very little is known about invasion characteristics, due in part to the difficulty of working with roots in situ (Waisel et al., 2002). Valentine et al. (2002) studied the noninvasive movement of TMV expressing the green-fluorescent protein (GFP) into the roots of *N .benthamiana*. Plants were sown directly into Murashige and Skoog medium in Petri dishes and then inoculated in two age groups, 21 and 25 days old (Valentine et al. 2002). Inoculum was a 5-fold dilution of sap of plants infected with TMV expressing GFP, plus carborundum, applied with a cotton swab on to the first true leaf (Valentine et al., 2002). Virus could be detected in the primary root system within 5 to 10 d after inoculation. As the lateral root primordium emerged, strong florescence signals were visible due to the presence of replicating virus (Valentine et al., 2002). As the root began to elongate, Valentine et al. (2002) noticed that they gradually lost fluorescence, while unaltered levels of fluorescence remained in the primary roots. Even though lateral roots lost fluorescence, labeled virus particles were detected in the cells at the base of the lateral root, within the root meristem, and within cells of the root (Valentine et al., 2002). The root systems of these seedlings were shown to be heavily infected with TMV at 12 dpi and, gradually replication was inhibited in the lateral roots by 20 dpi (Valentine et al., 2002). Valentine et al. (2002) hypothesized that, in the

case of the lateral primordial, the high titer of viral RNA during meristem activation gives rise to a strong, mobile RNA silencing mechanism that is transmitted back along the lateral root, thus inhibiting the replication of the virus. Concurrently, a functional meristem must be present to initiate the inhibition of viral replication (Valentine et al., 2002).

While there are two main functions of roots, nutrient acquisition and anchorage, they are involved in various other required and unnecessary activities. Soil-borne viral particles are not thought to arise from root exudates which are excretions plants produce to aid in movement of roots through drying soils (Waisel, 2002). Viral particles are likely released from injured, decaying, or dead roots to cause infection in the soil (Yarwood, 1960). In a study by Yarwood (1960), healthy plants were inoculated with tobacco necrotic virus (TNV) or TMV under greenhouse conditions. Drainage water of mechanically infected plants was collected and biological assays were performed on cowpea (*Vigna sinensis*), bean (*Phaseolus vulgaris*), cucumber (*Cucumis sativus*), or tobacco. Drainage water from TMV infected plants produced infections in assay plants by 15 d post inoculation until March 15 of Yarwood's (1960) study. Afterwards, there was a reduction in infections due to unknown reasons. In contrast to Yarwood (1960), Smith et al. (1969) concluded that plant viruses were released by undamaged, nonnecrotic roots.

TMV in Water

The occurrence of TMV in surface waters was established by Tosic and Tosic (1984) and Piazolla et al. (1986). Tosic and Tosic (1984) collected four water samples in Yugoslavia in November of 1982. Two samples of one liter each were collected from the Danube River; one in the Porto of Zemun, and the other near the village of Visnjica. Two more samples were

collected from the Sava River; one near Belgrade and the other near Sremska Mitrovica. The control used by Tosic and Tosic (1984) was tap water from the laboratory of the Plant Pathology Division of the Facility of Agriculture in Zemun. The bottles were allowed to stand to allow coarse suspended material to sediment. The water was then decanted and filtered. Tosic and Tosic (1984) then centrifuged the filtrates for 15 min at 1200 g and followed up with a centrifugation of the supernatant 90 min at 60,000 g. The pellets were resuspended in 1 mL of 0.001 M phosphate buffer, pH 7.0 and clarified by centrifugation for 10 min at 1200 g. The supernatant was tested for viruses by infectivity assay tests and electron microscopy. Tosic and Tosic (1984) mechanically inoculated the samples to the leaves of *Chenopodium amaranticolor, C. murale, N. glutinosa,* and *N. tabacum* cv. Samsun. Local lesion response was seen in *C. amaranticolor, C. murale,* and *N. glutinosa* whereas mosaic symptoms developed in *N. tabacum* cv. Samsun. These responses are typical of TMV. By using plant juices from tobacco cv. Samsun, slide agglutination and gel diffusion tests with TMV antiserum confirmed the presence of TMV in the two samples from the Danube River and the two samples from Sava River.

Piazolla et al. (1986) collected water samples from four rivers which are used for crop irrigation in southern Italy. The 1 L samples were centrifuged and the resulting sediments were resuspended in 3 mL of phosphate buffer. Biological assays were performed on *Chenopodium quinoa* to determine that two of the rivers were carrying virus, with TMV in one river and CMV in the other.

Plant viruses in water are postulated to be more stable when they have aggregated and/or adsorbed to particulate matter and in the presence of plant debris (Koenig, 1986). The protection of viruses adsorbed to clay particles allows them to withstand inactivation from physical and chemical pressures in both soil and water (Keonig, 1986).

The possibility of long-range atmospheric travel of plant viruses without a biological host was investigated by Castello et al. (1995). While TMV has not been detected in such travel, tomato mosaic virus (ToMV), a closely related virus in the *Tobamovirus* genus, was detected in cloud and fog (Castello et al., 1995). Like TMV, ToMV is also a RNA virus which is stable in water and soil and is a concern to agricultural production (Koenig, 1986; Hull, 2002). Castello et al. (1995) collected samples from two locations. Cloud samples were collected from the summit of Whiteface Mountain, located in New York and fog samples were collected off the coast of Maine (Castello et al., 1995). Direct double-antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) and reverse transcription-polymerase chain reaction-blot hybridization (RT-PCR-BH) were used to detect the virus, followed up by an infectivity bioassay and nucleotide sequencing (Castello et al., 1995). Castello et al. (1995) found that 25 of 44 cloud and fog samples tested positive for ToMV by RT-PCR-BH. Similar results were seen in the DAS-ELISA following an ultracentrifugation and resuspension of the samples in a much smaller volume (Castello et al., 1995). Castello et al. (1995) suggested that a cloud/fog mechanism may serve to disseminate other stable viruses to plant communities.

Control

Tobacco mosaic virus continues to be a difficult virus to control and the disease is often very costly. Spread can be very rapid, due in part to many susceptible hosts and because the virus is mechanically transmitted. Inoculation of Maryland tobacco cultivar, MD 609, four weeks after transplanting reduced yield by 25.8% compared to virus-free controls (Diallo and Mulchi, 1981). TMV can also cause quality of cured tobacco to be reduced. The mosaic pattern on the leaf of light and dark green blotches causes off color of the cured leaf. Time of infection,

either early or at topping, is an important determinate of whether or not the quality will be affected (Gooding, 1981; Chaplin and Mann, 1978).

Exclusion

TMV can be prevented or controlled in a variety of ways. The first control method farmers or producers can employ is exclusion. Exclusion methods eliminate the entry of the virus into the cropping ecosystem by controlling the entry of infected or contaminated articles, such as plant tissues or tools. While this method works for small-scale practices through testing individual plants, it is nearly impossible on large scale. Testing hundreds of thousands of seeds, seedling, and plants becomes time consuming and expensive.

Sanitation

Traditionally, certain sanitation efforts have been devised that reduce the probability of early infection. They include (i) crop rotation, (ii) thorough destruction of the roots and stalks with cultivation, (iii) culling infected plants prior to the first cultivation, (iv) controlling weeds in the plant bed and field, (v) sanitation of the plant bed and field areas, (vi) avoidance of susceptible crops in the plant bed and the field, and (vii) using TMV resistant cultivars of tobacco (Gooding, 1981). It is also suggested that anyone coming into direct contact with tobacco should wash their hands with soap and warm water often to reduce the potential for viral spread.

These recommendations need modification to include greenhouse float bed systems because it is just as important to keep greenhouses sanitized and free of weeds as it would be in plant beds. Clipping plants in the greenhouse may facilitate virus spread to all the plants in the

greenhouse. Collecting plant clippings and cleaning the mower after each use with 50% sodium hypochlorite (NaClO) and water solution have been proposed to reduce the likelihood that a TMV infection will develop and spread to the entire greenhouse (Reed et al., 2004).

While sanitation practices are implemented to reduce the likelihood that TMV will spread among plants in an entire greenhouse, it is not known if TMV is present in the water used in float bed production of flue-cured tobacco seedlings. Results from the proposed experiments would provide evidence that infected roots and/or water are potential sources of TMV inoculum for seedling infection in a greenhouse setting. Gosalvez et al. (2003) studied melon necrotic spot virus (MNSV), a member of the genus *Carmovirus* in the family *Tombusviridae* of plant viruses, to detect its presence in water samples. They successfully detected the virus in water samples and also noted that the highest concentration of the virus, excluding the inoculated cotyledons, was in the roots (Gosalvez et al., 2003). In 1999, Park et al. investigated the transmission of TMV in two recirculating hydroponic systems, deep flow culture and container culture, in Korea. The plants used in the study were tobacco (*N. tabacum* cv. Samsun-nn and cv. Xanthi), tomato (*Lycopersicon esculentum* cv. Alchan), and hot pepper (*Capsicum annuum* cv. Dahong) (Park et al., 1999). They found that in closed systems root-tip grafting transmitted the virus readily, while TMV was rarely transmitted via renewal of the hydroponic nutrient solution. Zen et al. (2004), in China, contaminated float beds and trays with TMV in order to test chemical disinfection treatments. High disease incidences of TMV, 70% to 73.5%, were found in control treatments where water was used as a mock disinfectant (Zen et al., 2004).

Additional Sanitation

While TMV readily infects tobacco and other solanaceous plants, there are upwards of 200 other species that serve as hosts (Scholthof, 2004). Horsenettle and ground cherry should be actively removed from the vicinity of tobacco greenhouses and plant beds (Reed et al., 2004). Herbicides should be used in combination with sanitation practices to rid fields of host plants. Proper herbicide use and performance are vital to the elimination of weeds around tobacco.

Resistance

Resistance is the most cost-effective and environmentally friendly source of control for TMV. The first source of resistance was observed in a Columbian tobacco variety 'Ambalema.' After infection, Ambalema showed no signs of infection commonly seen in other varieties of tobacco. Nolla and Roque (1933) crossed the Columbian tobacco with *N. tabacum* in an attempt to produce a resistant tobacco cultivar. The cross produced plants that were high yielding and resistant to TMV. But, the quality was poor due to the severe wilting on hot days and plant mortality was high following wilting. Repeated backcrosses failed to produce an acceptable plant so researchers began to look for other sources of resistance.

Holmes (1938) incorporated resistance from *N. glutinosa* into *N. tabacum* through interspecific hybridization, a cross made between two species. He also discovered the mechanism for resistance in *N. glutinosa* was a single dominate gene that he called 'N'. While Ambalema showed no signs of infection, *N. glutinosa* had a local necrosis response (Holmes, 1938). When the virus enters a tiny wound on the plant the surrounding tissue dies. This necrotic response seals off the virus and prevents its spread to other cells or plants. The N gene was successfully incorporated into *N. tabacum* through backcrossing. According to Holmes

(1938) this gene provides complete resistance to TMV by causing a local lesion necrotic reaction with no significant loss in yield, nor decrease in quality. There are some disadvantages to the N gene function within a plant including lack of hypersensitive response at temperatures above 28°C, severe systemic necrosis following inoculation when returned to temperatures below 28°C, and incomplete N gene resistance in heterozygous hybrids (Erickson et al., 1999).

Most burley tobacco cultivars are resistant to TMV due to the successful addition of the N gene. Resistance in flue-cured tobacco, on the other hand, has not been as successful because of a negative affect on agronomic characteristics such as yield and value. Research conducted at North Carolina State University suggests that it is not the N gene that causes the reduced yield and quality in flue-cured tobacco but the carry over of linked genes on the *N. glutinosa* chromosome (Linger et al., 2000).

The N gene from *N. glutinosa* was successfully cloned and transferred into the tomato cultivar VF36 (Erickson et al., 1999). Erickson et al. (1999) found that the N gene effectively mediated resistance to TMV by the formation of necrotic local lesion response and inhibition of TMV movement in transgenic tomatoes. Demonstrating that resistance genes can be transferred across species barriers provides resistance conventional breeding methods cannot (Erickson et al., 1999). Furthermore, genetic screenings of the N transgenic tomatoes have provided many mutant lines (Erickson et al., 1999) which could lead to successful resistance lines available for other species, including tobacco.

Chemical inactivation

As an alternative approach to control TMV, chemical inactivation has been tested. Various chemicals, such as methyl bromide (Wiggs and Lucas, 1962; Johnson and Chapman,

1963; Van Den Broeck et al., 1967; Winckel, 1974,), ascorbic acid (Lojkin, 1937), ozone (Shinriki et al., 1988), milk (Apple et al., 1963; Reilly, 1979; Reilly and Komm, 1983), formaldehyde (Cartwright et al., 1956), sodium hypochlorite (Demski, 1981; Miner et al., 1986), hydrochloric acid (Alexander, 1960; Niemyski, 1963; Alekseev, 1975), and trisodium phosphate (Gooding, 1975) have been used to inactivate TMV. All of the chemicals were effective to a degree, whether used for inactivation of purified virus or virus infected tissue. In particular, inactivation by milk, trisodium phosphate, and formaldehyde are of interest. Reilly (1979) noted a reduction in disease incidence from 60.6% to 21.4% when 120 g/L powdered milk solution was used to sanitize hands prior to planting. Reilly (1979) and Reilly and Komm (1983) focused their research on inactivation at the point of transplanting. Apple et al. (1963) focused on preventing infection 23 to 26 d after transplantation. Susceptible varieties of Hicks and C139 and their backcrossed derived resistant counterparts were sprayed with a non-fat dry milk solution of 120 g/L and inoculated 3 h after treatment (Apple et al., 1963). Resistant plants treated with the milk solution showed fewer lesions than the non-milk treated resistant plants (Apple et al., 1963). In Apple et al. (1963) the potential of the virus to be infective at the leaf surface was reduced by 80% to 90%.

Cartwright et al. (1956) used a 2% formaldehyde (CH_2O) solution in the pH range of 4.1 to 8.4 to inactivate TMV infected plant pulp from *N. tabacum* L. at 30, 35, and 40°C. Their findings indicate that inactivation was not dependant on temperature and only slightly dependant on pH, but was most likely dependant on a specific reaction with a single formaldehyde particle at one site on the virus particle (Cartwright et al. 1956).

Since the common belief is that TMV is not seed transmitted, studies of inactivation of TMV on tobacco seed are virtually nonexistent. There are studies, however, on the successful

treatment of tomato seeds to eliminate TMV (Alexander, 1960; Nitzany, 1960; Niemyski, 1963; Alekseev et al., 1975; Gooding, 1975). Seed from an infected fruit were extracted by acidification and treated with a 1% potassium permanganate ($KMnO_4$) solution for 15 min by Niemyski (1963). Reduction, but not elimination, in transmission to the seedlings was observed. Alexander (1960), Niemyski (1963), and Alekseev (1975) found different results using hydrochloric acid (HCl). Following treatments of 20% HCl (Alekseev et al., 1975) or 10 mL HCl per 11 kg of tomato pulp until seed separated from the pulp (Alexander, 1960), no transmission of TMV from tomato seeds to seedlings was observed. Conversely, Niemyski (1963) used 1% HCl for 15 min with reduction in infection but not elimination. A 10% trisodium phosphate (Na_3PO_4) solution had no effect in viral incidence in one study (Taylor, 1962), reduced infection in others (Alexander, 1960; Niemyski, 1963), and eliminated the virus in some (Nitzany, 1960; Gooding, 1975). Alexander (1960) treated the seed for 30 min with Na_3PO_4 to reduce infection, while Niemyski (1963) and Nitzany (1960) treated seed for 15 min with Na_3PO_4 with dissimilar results. Remarkably, Taylor (1962) treated seed in 10% Na_3PO_4 for 1 h without viral inactivation. Gooding (1975) inactivated TMV in tomato seed using a 15 min treatment of 10% Na_3PO_4 followed by a 30 min soak in 0.525% sodium hypochlorite (NaClO) solution.

Literature Cited

Alekseev, R. V., Shcherbinin, B. M., Tokareva, N. N. 1975. Control of tobacco mosaic virus (TMV) in tomato seed crops. Ovoshchevodstva i Bakhchevodstva 3/4:82-87.

Alexander, L. J. 1960. Inactivation of TMV from tomato seed. Phytopathology 50:627.

Allard, H. A. 1915. Distribution of the virus of the mosaic disease in capsules, filaments, anthers and pistils of affected tobacco plants. Journal of Agricultural Research 5:251-256.

Apple, J. L., Chaplin, J. F., and Mann, T. J. 1963. Comparison of the Nicotiana glutinosa resistance factor and milk in controlling mosaic in flue-cured tobacco. Tobacco Science 7:46-53.

Atkins, D., Hull, R., Wells, B., Roberts, K., Moore, P. and Beachy, R. N. 1991. The tobacco mosaic virus 30K movement protein in transgenic tobacco plants is localized to plasmodesmata. Journal of General Virology 72:209-211.

Benoit, M. A. 1977. Does tobacco seed transmit tobacco mosaic seed? Planter 53: 197-201.

Benoit, M. A. and Maury, Y. 1976. The location of tobacco mosaic virus in tobacco seed. Planter 52:392-396.

Castello, J. D., Lakshman, D. K., Tavantzis, S. M., Rogers, S. O., Bachand, G. D., Jagels, R., Carlisle, J. and Liu, Y. 1995. Detection of infectious tomato mosaic tobamovirus in fog and clouds. Phytopathology 85:1409-1412.

Capoor, S. P. 1949. The movement of tobacco mosaic viruses and potato virus X through tomato plants. Annals of Applied Biology 36:307-319.

Cartwright, T. E., Ritchie, A. E., and Lauffer. M. A. 1956. The reaction of tobacco mosaic virus with formaldehyde. Virology 2:689-702.

Chamberlain, E. E. 1937. Tobacco Mosaic. New Zealand Journal of Science and Technology 19:209-226.

Chaplin, J. F. and Mann, T. J. 1978. Evaluation of tobacco mosaic resistance factor transferred from burley to flue-cured tobacco. Journal of Heredity 69:175-178.

Chatzivassiliou, E. K., Efthimiou, K., Drossos, E., Papadopoulou, A. Poimenidis, G. and Katis, N. I. 2004. A survey of tobacco viruses in tobacco crops and native flora in Greece. European Journal of Plant Pathology 110:1011-1023.

Citovsky, V. 1999. Tobacco mosaic virus: a pioneer of cell-to-cell movement. Philosophical Transactions Royal Society of London: Section B. 354:637-643.

Demski, J. W. 1981. Tobacco mosaic virus in seedborne in pimiento peppers. Plant Disease 65:723-724.

Diallo, I. and Mulchi, C. L. 1981. Influence of time of infection by TMV and TEV on agronomic, chemical and physical properties of tobacco cv. Md 609. Tobacco Science 25:1-5.

Erickson, F. L., Dinesh-Kumar, S. P., Holzberg, S., Ustach, C. V., Dutton, M., Handley, V., Corr, C., and Baker, B. J. 1999. Interactions between tobacco mosaic virus and the tobacco *N* gene. Philosophical Transactions of the Royal Society of London: Biological Sciences 354:653-658.

Fauquet, C. M., Mayo, M. A., Maniloff, J., Desselberger, U., and Ball, L. A. 2005. Virus Taxonomy. Eighth Report of the International Committee of the Taxonomy of Viruses. Elsevier Academic Press 1259 pp.

Gooding, G. V., Jr. 1975. Inactivation of tobacco mosaic virus on tomato seed with trisodium orthophosphate and sodium hypochlorite. Plant Disease Reporter 59:770-772.

Gooding, G. V., Jr. 1981. Control of tobacco mosaic virus on flue-cured tobacco by crop protection. Tobacco Science 40:30-31.

Gooding, G. V., Jr. 1986. Tobacco Mosaic Virus: infectivity and replication. p. 133-152. *In* The Plant Viruses: Volume 2. The rod-shaped plant viruses. Plenum Press, New York, USA.

Gooding, G. V., Jr. and Todd, F. A. 1976. Soil-borne tobacco mosaic virus as an inoculum source for flue-cured tobacco. Tobacco Science 20:133-135.

Gosalvez, B., Navarro, J. A., Lorca, A., Botella, F., Sanchez-Pina, M. A., and Pallas, V. 2003. Detection of melon necrotic spot virus in water samples and melon plants by molecular methods. Journal of Virological Methods 113:87-93.

Heinlein, M. 2002. The spread of Tobacco mosaic virus infection: insights into the cellular mechanism of RNA transport. Cellular and Molecular Life Sciences 59:58-82.

Holmes, F. O. 1938. Inheritance of resistance to tobacco mosaic disease in tobacco. Phytopathology 28:553-561.

Hull, R. 2002. *Matthew's Plant Virology*. Elsevier Academic Press London/CA 1001 pp.

Ishikawa, M., Meshi, T., Ohno, T. and Okada, Y. 1991. Specific cessation of minus-strand RNA accumulation at an early stage of tobacco mosaic virus infection. Journal of Virology 65:861-868.

Johnson, E. M. and Chapman, R. A. 1963. Chemical inactivation of tobacco mosaic virus in tomato roots. Plant Disease Reporter 47:389-391.

Koenig, R. 1986. Plant viruses in rivers and lakes. Advances in Virus Research 31:321-333.

Leisner, S. M. and Howell, S. H. 1993. Long-distance movement of viruses in plants. Trends in Microbiology 1:314-317.

Linger, L. R., Wolff, M. L., and Wernsman, E. A. 2000. Comparison of transgenic vs. interspecific sources of TMV resistance conferred by the N gene. Paper presented at the 39th Tobacco Workers' Conference, Williamsburg, VA. 10-13 Jan. 2000.

Lojkin, M. 1937. A study of ascorbic acid as an inactivating agent of tobacco mosaic virus. Contributions from Boyce Thompson Institute 8:445-465.

Lucas, W. L., Ding, B. and van der Schoot, C. 1993. Plasmodesmata and the supracellular nature of plants. New Phytologist 125:435-476.

Lusso, M., Davis, G., and Barker, H. 2002. The route of tobacco mosaic virus to Nicotiana tabacum seeds and evaluation of its seed transmission. Paper presented at the 40th Tobacco Workers Conference, Jan 14-17, Pinehurst, NC.

Miner, G. S., Gooding, G. V., and Moore, J. M. 1986. Prevention of mechanical transmission of tobacco mosaic virus in tobacco plant beds. Tobacco Science 30:25-29.

Niemyski, K. 1963. Inactivation of tobacco mosaic virus in tomato seed. Ada agrobot 14:233-244.

Nitzany, F. E. 1960. Transmission of mosaic virus through tomato seed and virus inactivation methods of seed extraction and seed treatments. Ktavim 2:59-61.

Noordam, D. 1973. Occurrence and prevention of undesiable infection (contamination). p.17-18. *In* Identificaiton of plant viruses: Methods and experiments. Centre for Agricultural Publishing and Documentation, Wageningen.

Nolla, J. S. B. and Roque, A. 1933. A variety of tobacco resistant to ordinary tobacco mosaic. Journal of the Puerto Rico Department of Agriculture 17:301-303.

Park, W. M., Lee, G. P., Ryu, K. H., and Park, K. W. 1999. Transmission of tobacco mosaic virus in recalculating hydroponic system. Scientia Horticulture 79:217-226.

Piazolla, P., Castellano, M. A. and Stradis, A. 1986. Presence of plant viruses in some rivers of southern Italy. Journal of Phytopathology 116:244-246.

Reed, T. D., Johnson, C. S., Semtner, P. J., and Wilkinson, C. A. 2004. 2005 Flue-cured tobacco production guide. Pub. 436-048 Virginia Agric. Exp. Stn., Blacksburg.

Reilly, J. J. 1979. Chemical control of tobacco mosaic virus. Tobacco Science 23:97-99.

Reilly, J. J., and Komm, D. A. 1983. Incidence of tobacco mosaic virus as affected by various concentrations of milk. Tobacco Science 27:62-63.

Samuel, G. 1934. The movement of tobacco mosaic virus within the plant. Annals of Applied Biology 21:90-111.

Santa Cruz, S. 1999. Perspective: phloem transport of viruses and macromolecules – what goes in must come out. Trends in Microbiology 7:237-241.

Scholthof, K-B. G. 2004. Tobacco mosaic virus: a model system for plant biology. Annual Review of Phytopathology 42:13-34.

Shew, H. D. and Lucas, G. B. 1991. Compendium of Tobacco Diseases. APS Press, USA.

Shinriki, N., Ishizaki, K., Yoshizaki, T., Miura, K., and Ueda, T. 1988. Mechanism of inactivation of tobacco mosaic virus with ozone. Water Resources 22:933-938.

Silber, G. and Burk, L. G. 1965. Infectivity of tobacco mosaic virus stored for fifty years in extracted 'unpreserved' plant juice. Nature 206:740-741.

Smith, K. M. 1972. A textbook of plant virus diseases. Academic Press.

Smith, P. R., Campbell, R. N. and Fry, P.R. 1969. Root discharge and soil survival of viruses. Phytopathology 59:1678-1687.

Taylor, R. H. 1962. The location of tobacco mosaic virus in tobacco seed. Australian Journal of Experimental Agriculture and Animal Husbandry 2:86-89.

Tosic, M. and Tosic, D. 1984. Occurrence of tobacco mosaic virus in water of the Danube and Sava rivers. Phytopathologische Zeitschrift 110:200-202.

Uppal, B. N. 1934. The movement of tobacco mosaic virus in leaves of Nicotiana sylvestris. Indian Journal of Agricultural Science 4:865-874.

Valentine, T. A., Roberts, I.M and Oparka, K.J. 2002. Inhibition of tobacco mosaic virus replication in lateral roots is dependant on an activated meristem-derived signal. Protoplasma 219:184-196.

Van Den Broeck, H., Vanachter, A. and Van Winckel, A. 1967. Inactivation of tobacco mosaic virus by chemical soil disinfectants. Agra-cultura, Louvain 15:120-126.

Waisel, Y., Eshel, A., and Kafkafi, U. 2002. *Plant Roots: The Hidden Half.* Marcel Dekker, Inc, NY. 1136 pp.

Wiggs, D. N. and Lucas, G. B. 1962. Inactivation of tobacco mosaic virus by volatile chemicals. Phytopathology 52:983-985.

Winckel, A. V. 1974. Effect of a fumigation on the biological degradation of tobacco mosaic virus in the soil. Agro-Ecosystems 1:131-138.

Wolf, S., Deom, C. M., Beachy, R. N. and Lucas, W. 1989. Movement protein of tobacco mosaic virus modifies plasmodesmatal size exclusion limit. Science 246:377-379.

Yarwood, C. E. 1960. Release and preservation of virus by roots. Phytopathology 50:111-114.

Zaitlin, M. 2000. Tobacco mosaic virus. AAB Descriptions of Plant Viruses No 370.

Zaitlin, M and Israel, H. W. 1975. Tobacco mosaic virus. AAB Descriptions of Plant Viruses No 151.

Zen, R., Teng, Y., Zhang, Q., Wu, D., Li, L., Xia, C., Wang, C., Mao, J., Xiao, Z., Ruan, D., Wang, J., Zhao, J. 2004. Comparison of disinfecting methods for TMV contamination in tobacco seedling floating trays and culture beds. (In Chinese.) Journal of Yunnan Agricultural University 19:692-695.

Chapter III

SEED TREATMENT OF FLUE-CURED TOBACCO SEED COLLECTED FROM TOBACCO MOSAIC VIRUS INFECTED PLANTS

Abstract

Tobacco mosaic virus (TMV) is localized in the nucellar layer of the tobacco seed coat and is seed transmitted at a low level. Seed sterilization could reduce or eliminate seedling infection by the virus and thereby eliminate an early season source of primary inoculum. The objective of this study was to determine effective chemical treatments that eliminate seed transmission of TMV without detrimental affects on germination. Growth chamber experiments were conducted in a randomized complete block design with three replications. Each test was conducted twice. Seed were collected from TMV infected and non-infected field grown 'K326' flue cured tobacco plants. Presence of TMV was assessed by DAS-ELISA and biological activity was verified by infectivity assays on *Nicotiana tabacum* cv. Xanthi NN. Treatment with formaldehyde severely reduced germination by 97%, while treatment with 10% bleach solution, 1% hydrochloric acid, 10% trisodium phosphate, 0.1% potassium permanganate, and 120 g/L non-fat dry milk reduced germination by 2% to 10% compared to the control. Percent germination decreased with increased treatment time of the seed. No chemical treatment eliminated TMV antigen in the seed coat of seed collected from a TMV infected plant based on ELISA, however, the virus was not biologically active based on infectivity assays. Hydrochloric acid seed treatment eliminated and reduced TMV in seedling groups to levels of 0% and 50% in experiments 1 and 2, respectively. Treatment with trisodium phosphate reduced presence of TMV to 12% and 22% in experiments 1 and 2, respectively. This research suggests a 1%

hydrochloric acid solution is an effective chemical treatment for seed stored approximately one year, but is not effective for eliminating TMV in newly collected seed.

Introduction

Tobacco (*Nicotiana tabacum* L.) is an economically important agricultural product. In the United States the annual crop value for tobacco in 2005 was slightly greater than $1 billion (NASS, 2006). Tobacco mosaic virus (TMV) is among several pathogens that can impact the production of tobacco. Estimates of the impact of TMV on tobacco production indicate reductions in yield could be from less than one percent to as much as 42% (Wolf and Moss, 1933; Johnson et al., 1983). Reducing or controlling the impact of TMV on tobacco yield could improve the profitability of tobacco production.

The age of the plant at the time of TMV infection relates directly to the reduction in yield and quality of tobacco. There is greater impact when tobacco plants are infected early in the growing season as compared to later in the season. Burley plants inoculated at the time of transplanting exhibit reductions in yield of approximately 30% and reduction in value of 40% (Valleau and Johnson, 1927). In contrast, plants inoculated at topping demonstrated no decrease in yield and a 25% reduction in value (Valleau and Johnson, 1927). Similar results were observed in flue-cured tobacco where a 31% reduction in yield and a 55% reduction in value occurred when plants were inoculated with TMV at transplanting (Wolf and Moss, 1933). Flue-cured tobacco yield decreased by 17% and value by 24% when infected at topping (Wolf and Moss, 1933). Decreases in cured leaf value are associated with poor color, reduced leaf size, thin texture, and/or spotting (Chamberlain, 1937).

TMV was not considered to be seed transmitted in tobacco for more than 100 years. Recent re-investigations have shown, however, that TMV is seed transmitted to 14-day-old flue-cured tobacco seedlings (C. A. Wilkinson, personal communication, 2007). TMV is also seed transmitted in tomato (*Lycopersicon esculentum* Mill.), chilli (*Capsicum annum* spp.), apple

(*Malus* P. Mill. spp.), pear (*Pyrus* L. spp.), and grape (*Vitis* L.) (Bennett, 1969; Gooding, 1975; Benoit and Maury, 1976; Hull 2002). Allard (1915) suggested that TMV was present in tobacco seed. Chamberlain (1937) reported possible seed transmission of TMV in tobacco seedlings. During a grow-out test of tobacco seed from mosaic infected plants, seven of 5,628 seedlings were found to be infected with TMV. Chamberlain (1937) was concerned that, even though precautions were taken, the infectious nature of the virus left doubt whether accidental inoculation caused the infection. Later research conducted by Taylor (1962) reported no seed transmission of TMV. Taylor (1962) observed virus in every replicate of seed coat samples in a dissection trial, but no biologically active virus was detected in seedlings grown from infected seed.

Benoit and Maury (1976) demonstrated that TMV is located within the nucellar layer of the infected tobacco seed coat. Although TMV is present within a tobacco seed coat, TMV could be seedborne and not seed transmitted in tobacco (Benoit and Maury, 1976). Some researchers have suggested that plant debris on the seed surface is the source of TMV resulting in seedling infection (Benoit, 1977; Stace-Smith and Hamilton, 1988). The need arises for a chemical treatment to inactivate TMV in the seed.

Zen et al. (2004) successfully disinfected the bottom and surface of culture beds and float trays contaminated with TMV using 0.1% potassium permanganate and 2% formalin. They also demonstrated that 100 x chloride treatment did not eliminate TMV infection of seedlings grown in contaminated float trays. Reilly and Komm (1983) showed that 120 g/L powdered milk treatment of seedlings prior to transplanting significantly reduced mechanical spread from workers hands.

Seed treatment methods have been successful in reducing or eliminating infectious TMV in tomato seeds (Alexander, 1960; Nitzany, 1960; Niemyski, 1963; Alekseev et al., 1975; Gooding, 1975). Tomato seed treated with Na_3PO_4 eliminated or reduced infectious TMV in tomato seedlings in most cases (Gooding, 1975). Hydrochloric acid (HCl) eliminated or reduced infection of seedlings (Alexander, 1960; Nitzany, 1960; Alekseev et al., 1975), whereas potassium permanganate ($KMnO_4$) only reduced the infection of seedlings (Niemyski, 1963). Gooding (1975) used a combination of trisodium phosphate (Na_3PO_4) and bleach (sodium hypochlorite, NaClO) to eliminate TMV infection in tomato seedlings without reducing seed germination.

Few studies of seed treatments for TMV in tobacco seeds have been conducted. Treating tobacco seed with trisodium phosphate did not reduce the number of infected seedlings or percent germination (Taylor, 1962). Because of the lack of research in this area, the objective of this research was to determine the effectiveness of chemical seed sterilization treatments on TMV stability. If the TMV particles, either in the nucellar layer of the seed coat or on the seed coat surface, could be denatured chemically, this would reduce or eliminate seed transmission of TMV in tobacco seedlings.

Materials and Methods

Growth chamber experiments were conducted at the Virginia Polytechnic Institute and State University Southern Piedmont Agricultural Research and Extension Center (SPAREC) near Blackstone, Virginia in 2006. The growth chamber used for the experiments was the Versatile Environmental Test Chamber (SANYO Scientific, Bensenville, IL). The growth chamber was

operated at 22°C, 65% relative humidity, and approximately 120 μmol/m^2/s photosynthetic photon flux density. The day length and night length was set at 12 h each.

Seed from TMV infected and noninfected plants were collected from field grown 'K326' flue-cured tobacco plants. Hereafter they are designated 'infected seed' and 'healthy seed,' respectively. Standard agronomic production practices for fertilization, transplanting, disease, weed, and insect control (Reed et al., 2005) were followed for plant production. Plants were mechanically inoculated with TMV in late June. TMV inoculum was prepared by grinding 0.5 g of TMV infected leaf tissue in 4.5 mL 1% potassium phosphate buffer at pH 7.0 and 1% Celite (Fisher Scientific Company, Pittsburgh, PA). Inoculum was diluted 1:100 with 1% potassium phosphate buffer at pH 7.0 and inoculations were conducted on a palm size leaf near the top of the plant using a foam tipped 12.7 cm polypropylene swab (Fisher Scientific Company). Seed heads were collected from both infected and non-infected plants during September of 2005 and 2006. Healthy and infected seed were shelled and cleaned using separate 600 μm (30 mesh) sieves designated for either seed type. All seed were stored in paper bags at room temperature.

Experiment 1 - Chemical by Exposure Time

Experiments to test for virus inactivation by chemical treatments for different periods of time were conducted in a randomized complete block design with three replications and the test was conducted twice. The treatment arrangement was a seven by three factorial. The seven chemical treatments were (i) 9.1% (v:v) ultra bleach, (ii) 2% (v:v) formaldehyde, (iii) 0.1% (w:v) potassium permanganate, (iv) 1% (v:v) hydrochloric acid, (v) 10% (w:v) trisodium phosphate, (vi) 120 g/L dry non-fat milk, and (vii) deionized water. Chemicals were obtained from Fisher Scientific Company (Pittsburgh, PA), except non-fat dry milk (Carnation, NESTLÉ, Glendale,

CA) and ultra bleach (Wal-Mart Stores, Inc, Bentonville, AK). All chemical treatments were dissolved or diluted in deionized water. Seed were treated for three time periods, 30, 60, and 120 min. All seed used in this experiment was collected in September of 2005, approximately 10 to 12 months prior to use. Seed from a non TMV infected K326 source was used as the control and treated only with deionized water for the same time periods listed above.

Experiment 2 - Selected Chemical Time Intervals

Experiments to test for virus inactivation by chemical treatments with selected periods of time were conducted in a randomized complete block design with three replications and the test was conducted twice. Ten treatments included were: (i) 9.1% (v:v) ultra bleach solution for 30 min, (ii) 18.2 % (v:v) ultra bleach solution for 10 min, (iii) 27.3% (v:v) ultra bleach solution for 10 min, (iv) 0.2% (v:v) hydrochloric acid for 10 min, (v) 0.5% (v:v) hydrochloric acid for 10 min, (vi) 1% (v:v) hydrochloric acid for 10 min, (vii) 1% (v:v) hydrochloric acid for 30 min, (viii) 10% (w:v) trisodium phosphate for 30 min, (ix) 15 % (w:v) trisodium phosphate for 10 min, and (x) 20% (w:v) trisodium phosphate for 10 min. The chemicals and exposure times were selected based on results from the chemical by exposure time test, especially effectiveness of the chemical treatment on reducing TMV transmission to seedlings and a minimal effect on germination. All seed used in this experiment were collected in September of 2006, approximately 2 weeks before testing. Seed collected from a non-infected source plant treated with deionized water for 30 min served as the control.

Seed Treatment and Seedling Technique

Lots of 50 K326 seed, from both infected and non-infected source plants, were counted, transferred into a 2 mL flat-top microcentrifuge tube (Fisher Scientific Company), and a volume of 1 mL of each chemical was added to its designated tube. The tubes were placed on their side and gently agitated on a Labquake Shaker/Rotisserie (Barnstead International, Dubuque, IA) at room temperature for a designated treatment time. Following each treatment time interval the chemical treatment solution was carefully decanted using an air-displacement pipette and seed were rinsed twice in situ with 1 mL deionized water. Seed were then rinsed out of the tubes with two 1mL deionized water rinses on to Whatman No. 4, 90 mm filter paper (Whatman, South Kearney, NJ) funnel where they were allowed to drain freely for 5 min. Seed were allowed to air dry, undisturbed over night in the filter paper funnel.

Seed were placed on 10.2 x 10.2 cm blue blotter paper (Hoffman Manufacturing Co., Albany, OR) in 10.5 x 10.5 x 3 cm rectangular containers (Hoffman Manufacturing Co.) (Figure 3-1). The blotter paper was supersaturated with doubly distilled water and acted as the growing medium for the seed. Tweezers and probes, which were flame sterilized between each treatment, were used to separate and space the seed.

Sample Collection and Assay Methods

Seedling and seed coat samples were collected from each box two weeks post seeding, and placed in separate 1.5 mL RNase-free microtubes (Kontes Glass, Vineland, NJ). Flame sterilized tweezers and probes were used to remove the samples from the boxes. Seedling samples were collected and each seedling sample was examined to verify that the seed coat was not attached. Two seedling samples, each weighing approximately 20 mg, were taken from each

box when possible. Typically, the average range for the number of seedlings included in a seedling sample was between 10 to 15 seedlings. All seed coats were collected after the seedling samples were collected from each box without regard to weight for testing. Seed, which had not germinated, were collected and included in the seed coat sample. Any seed or seedling with fungal contamination was not collected. The number of seedlings and seed coats per sample were recorded.

To maintain a 1:10 sample to buffer ratio, 200 µL of general extract buffer (GEB) (Agdia, Elkhart, IN) was added to each microcentrifuge tube. Seedling and seed coat samples were thoroughly macerated using Pellet Pestles (Kontes Glass) placed in hand operated, battery powered drills (Black and Decker, Towson, MD). The Pellet Pestle was changed between each tube to ensure against contamination between samples.

Agdia PathoScreen Kit double antibody sandwich enzyme-linked immunosorbent assay (DAS ELISA) test systems for the common strain of TMV (TMV-c) were utilized for all serological assays. A 100 µL of sample was loaded into a single well on the ELISA plate. Two positive and negative controls, also at a volume of 100 µL, were loaded onto each plate. The positive control was provided in the PathoScreen Kit and the negative control was GEB. All DAS ELISA plates were developed according to the instructions provided with the PathoScreen Kit.

Each plate was visually examined to record color development and measured with a MRX Revelation plate reader (DYNEX Technology, Chantilly, VA) at 405 nm following the recommended 45 min incubation. Wells containing TMV are considered positive if they are yellow and if the absorbance was twice that of the negative control. The intensity of the color is

relative to the concentration of virus in the sample; more intense yellow is a higher concentration of TMV, and less intense yellow is a lower concentration.

Infectivity assays were conducted using the remaining 100 µL of solution to demonstrate biological activity of the virus. *Nicotiana tabacum* cv. Xanthi NN was used as the assay plant due to its local lesion response to TMV infection. Infectivity assays were conducted under greenhouse conditions with a maximum and minimum temperature of 18 and 35°C, respectively. A clean cotton swab was dipped in Celite, in the remaining solution, and rubbed on a Xanthi leaf. The presence of local lesions was recorded 7 to 10 days after inoculation.

Analysis of variance was conducted using PROC GLM (SAS Institute, 1987). Data transformations were performed on percent data based on counts, including the percent positive TMV samples, using an arcsine transformation (Gomez and Gomez, 1984). Treatment means were separated using Duncan's multiple range test (Gomez and Gomez, 1984). An F-max test was conducted to determine if error variances between tests were homogeneous (Gomez and Gomez, 1984). The analysis of percent germination, number of seedlings or seed coats per sample, and weight of seedlings or seed coats per sample included the healthy control because these data should be consistent throughout, regardless of treatment. The healthy control was omitted from the analysis of percent TMV positive seedlings or seed coats and absorbance data, due to the fact that the healthy controls should always be negative for TMV and are thus, significantly different from the chemical treatments.

Results and Discussion

Experiment 1 - Chemical by Exposure Time

There were significant differences among chemicals for the percent germination, percent positive TMV seedling samples, weight of seedling sample, and number of seedlings per sample for each test (Tables A-1, A-2, A-4, and A-5). No significant differences were observed for seed coat weight, seed coat number, and percent positive seed coats in each test (Tables A-1, A-2, A-4, and A-5). There was no significance difference in exposure time for all attributes measured (Tables A-1, A-3, A-4, and A-6). Error variances between tests were homogeneous for each parameter measured based on an F-max test, therefore a combined analysis was conducted. Chemical by exposure and chemical by test interactions (Table 3-1) were a result of change in magnitude and not a change in rank, therefore only the main effects will be discussed.

Significant differences were observed among chemicals and exposure times for percent germination (Table 3-1). Formaldehyde reduced germination to 7% which was significantly lower than all other chemical treatments (Table 3-2). There was no significant difference for germination among the bleach, hydrochloric acid, potassium permanganate, milk, and deionized water infected control treatments. The trisodium phosphate treatment significantly reduced germination compared to bleach, potassium permanganate, and deionized water infected control. Germination of the deionized water healthy control was significantly higher compared to all treatments using seed produced on a TMV infected plant. A decline in germination was observed with increased exposure time of the seed sterilization treatments (Table 3-3). Germination was significantly higher at 30 min compared to 120 min of exposure.

Significant differences were observed among chemicals for TMV positive seedling samples (Table 3-2). The infected control treatment had 94% of seedling samples positive for TMV based on ELISA. An ELISA absorbance of about 0.3 to 0.4 was considered to be weakly positive, while an absorbance of greater than 0.4 was strongly positive (Table 3-2). Potassium

permanganate, milk, or bleach treatments, at 88%, 80%, and 76% TMV positives, respectively were not significantly different for the percentage of TMV positive seedling samples compared to the infected control treatment. Trisodium phosphate and hydrochloric acid significantly reduced TMV positive samples compared to all other treatments. The only chemical treatment that eliminated transmission of TMV to tobacco seedlings was hydrochloric acid (Table 3-2).

Significant differences among chemicals for the number of seedlings in a 20 mg sample were observed (Table 3-2). The healthy control had significantly more plants in a sample than all the other treatments. This suggests that the seedlings grown from seeds collected from a non-infected source plant are smaller. But, the increased number of seedlings could simply be due to the fact that the weight of the sample was slightly, but not statistically, higher than all of the other treatments (Table 3-2). There were significantly fewer plants per sample in the formaldehyde treatment compared to all other treatments. It is unclear why the seedlings seem to be larger, unless there is some chemical carryover on the seed coats which affects the seedlings treated with formaldehyde. There was no statistical difference observed among seedling sample weights with the average seedling sample weight being 20 mg (Table 3-2).

There was a significant difference in the number of seed coats collected from the chemical treatments (Table 3-2). Statistically, more seed coats were found in the formaldehyde treatment than in most of the other treatments except treatment with hydrochloric acid, trisodium phosphate, and the healthy control. Bleach and non-fat dry milk treatments had the lowest number of seed coats, but were statistically similar to potassium permanganate and the infected control. Visual location of the seed coats treated with bleach was more difficult to see and collect due to the bleaching of the coats (Figure 3-2). The milk treatment seemed to be more heavily contaminated with fungus covered seed or seed coats, which were not collected.

All seed coats collected from TMV infected source plants were found to be positive for TMV, except one set treated with hydrochloric acid (Table 3-2). Typically, the absorbance of the seed coats for the TMV source seed was 2.8 or higher. There was one incidence of a TMV positive sample in the seed from healthy source plants, and the origin of contamination is unknown.

There were no significant differences among exposure times for number of seed coats in a sample, seed coat sample weight, or number of TMV positive samples (Table 3-3). Thus, exposure time is not statistically significant for any of the parameters measured except percent germination.

Experiment 2 - Selected Chemical Time Intervals

There was a significant difference in percent germination among chemicals in the first selected chemical time interval test but not the second (Tables A-7 through A-10). No significant differences were observed for seedling or seed coat weight, seedling or seed coat number, and percent positive seed coats in either selected chemical time interval test (Tables A-7 through A-10). Error variances between tests were homogeneous for each parameter measured based on an F-max test, therefore a combined analysis was conducted.

A significant difference in percent germination among chemicals was observed (Table 3-4). Several treatments were statistically similar to the high germination of the control treatment at 88%. Those treatments include 30% bleach for 10 min, 0.2% hydrochloric acid for 10 min, 0.5% hydrochloric acid for 10 min, 15% trisodium phosphate for 10 min, and 20% trisodium phosphate for 10 min (Table 3-5). One percent hydrochloric acid treatment for 30 min was not significantly different from the 1% hydrochloric acid treatments for 10 min, but the 30 min

treatment was significantly lower than all the others in percent germination (Table 3-5). The 1% hydrochloric acid treatments exhibited the lowest germination rates among the chemical treatments. All other treatments, 10% bleach for 30 min, 20% bleach for 10 min, and 10% trisodium phosphate for 30 min, were intermediate and not statistically different from the treatments.

No statistical differences were observed for the number of seedlings in each sample or the weight of the seedlings in a sample (Table 3-5). Seedling weights also did not average over 19 mg for any treatment.

No chemical treatment was successful in eliminating TMV infection in seedlings in the selected chemical time interval tests (Table 3-5). Twenty percent trisodium phosphate reduced the percentage of positive groups of seedlings to 10%, while the 1% hydrochloric acid treatment for 30 min reduced positive seedling groups to only 50%. Seed treatment with 20% trisodium phosphate for 10 min is significantly similar to all trisodium phosphates treatments, 0.2% hydrochloric acid for 10 min, 1% hydrochloric acid for 30 min, 30% bleach for 10 min, and 10% bleach for 30 min, but statistically different from all others. The treatment of seed with 20% bleach for 10 min had the highest percentage of positive TMV seedlings, but this treatment is not significantly different from all other treatments except the 10% trisodium phosphate for 30 min. The lack of reduction of TMV in this test could be due to the age of the seed. Seed used in this test was collected approximately two weeks before they were used in the experiment. Studies have shown that the longer the seed are stored the less viable the virus is within the seed (Taylor, 1962; Benoit and Maury, 1976). Thus, the older the seed, the more likely the virus can be inactivated.

There was a statistical difference in the number of seed coats collected from each box (Table 3-5). The number of seed coats for the healthy control was significantly higher than treatments with bleach and 1% hydrochloric acid only. Seed treatment with 1% hydrochloric acid for either 30 or 10 min was statistically similar and lower than those for 0.2% hydrochloric acid, 0.5% hydrochloric acid, 15% trisodium phosphate, and the healthy control. The difference in the number of seed coats collected could be due to fungal contamination and subsequent noncollection.

There was a statistical difference in the weight of the seed coats collected from each box (Table 3-5). The seed coats from the 0.2% hydrochloric acid treatment for 10 min weighed significantly more than the seed coats collected from the 20% bleach for 10 min and the 1% hydrochloric acid for 30 min, which were statistically similar to each other. All other chemical treatments were intermediate and similar to each other.

All seed coats collected from TMV infected source plants were found to be positive for TMV (Table 3-5). Seed coats from seed treated with 1% hydrochloric acid had a reduced ELISA titer compared to all other treatments (Table 3-5). Absorbance readings for seed coat samples from TMV source seed were 3.2 or higher in all cases. There was no incidence of TMV positive samples in the healthy controls.

Conclusions

A total of 4,709 seedlings in 332 total samples and 9,495 seed coats in 210 total samples were assayed from all four tests. Of the 188 total TMV positive seedling samples, 2,374 total seedlings were represented within those samples. There were 8,372 seed coats assayed in 187

TMV positive seed coat samples in both seed sterilization tests. Among the healthy samples, there were 634 seedlings in 36 samples and 829 seed coats in 18 samples.

This study supports findings by Taylor (1962) and Benoit and Maury (1976) where TMV was detected in seed coats of seeds collected from TMV infected tobacco plants. Results also confirm that seed transmission of TMV can be detected in seedlings 14 days after seeding in groups of 12 to 15 seedlings. Not every group of seedlings grown from seed collected from a TMV infected source plant was found to be positive for TMV. This indicates that chemical seed treatments may provide a means of inactivating TMV in the seed coat and thereby reduce seed transmission of TMV in tobacco seedlings, but further research is required to optimize the chemical, chemical rate, and treatment time.

The stability of plant viruses in tobacco seeds may be due to the concentration and/or crystallization of the virus. Dehydration and crystallization of virus particles acts as a means for viral survival in desiccated seeds (Hull, 2002). Overcoming this survival strategy with chemical treatments would provide a realistic way to eliminate seed transmission of TMV in tobacco seed. Both 1% hydrochloric acid and 20% trisodium phosphate were effective in reducing seed transmission of TMV in these tests. The timing of the treatment and the chemical concentration still need to be refined, as does the influences of seed storage time on the efficiency of the treatment. Because the viability of the virus within a tobacco seed decreases with time (Taylor, 1962; Benoit and Maury, 1976), chemical seed treatments may need to be modified to account for seed age. This research suggests that a 1% hydrochloric acid solution is effective on seed stored for one year, but is not effective on fresh seed collected the same year as the study. Also, a 20% trisodium phosphate solution may provide inactivation on newer seed but the relative concentration to exposure time requires additional research.

Literature Cited

Alekseev, R. V., Shcherbinin, B. M., and Tokareva, N. N. 1975. Control of tobacco mosaic virus (TMV) in tomato seed crops. Ovoshchevodstva i Bakhchevodstva 3/4:82-87.

Alexander, L. J. 1960. Inactivation of TMV from tomato seed. Phytopathology 50:627.

Allard, H. A. 1915. Distribution of the virus of the mosaic disease in capsules, filaments, anthers and pistils of affected tobacco plants. Journal of Agricultural Research 5:251-256.

Bennett, C. W. 1969. Seed transmission of plant viruses. Advances in Virus Research 14:221-261.

Benoit, M. A. 1977. Does tobacco seed transmit tobacco mosaic seed? Planter 53: 197-201.

Benoit, M. A. and Maury, Y. 1976. The location of tobacco mosaic virus in tobacco seed. Planter 52:392-396.

Chamberlain, E. E. 1937. Tobacco Mosaic. New Zealand Journal of Science and Technology 19:209-226.

Gooding, G. V. 1975. Inactivation of tobacco mosaic virus on tomato seed with trisodium orthophosphate and sodium hypochlorite. Plant Disease Reporter 59:770-772.

Gomez, K. A. and Gomez, A. A. 1984. *Statistical Procedures for Agricultural Research.* John Wiley and Sons, Inc. New York, New York, United States. 680 pp.

Hull, R. 2002. *Matthew's Plant Virology*. Elsevier Academic Press London/CA 1001 pp.

Johnson, C. S., Main, C. E. and Gooding, G. V. 1983. Crop loss assessment for flue-cured tobacco cultivars infected with tobacco mosaic virus. Plant Disease 67:881-885.

National Agricultural Statistics Service (NASS). 2006. Crop values, 2005 summary. Pr2 (06). United States Department of Agriculture.

Niemyski, K. 1963. Inactivation of tobacco mosaic virus in tomato seed. Ada agrobot 14:233-244.

Nitzany, F. E. 1960. Transmission of mosaic virus through tomato seed and virus inactivation methods of seed extraction and seed treatments. Ktavim 2:59-61.

Reed, T. D., Johnson, C. S., Semtner, P. J., and Wilkinson, C. A. 2005. 2006 Flue-cured tobacco production guide. Pub. 436-048 Virginia Agric. Exp. Stn., Blacksburg.

Reilly, J. J., and Komm, D. A. 1983. Incidence of tobacco mosaic virus as affected by various concentrations of milk. Tobacco Science 27:62-63.

SAS Institute Inc. 1987. *SAS/STAT™ Guide for personal Computers Version 6 Edition.* Cary, NC: SAS Institute Inc., 1028 pp.

Stace-Smith, R. and Hamilton, R I. 1988. Inoculum thresholds of seed borne pathogens: viruses. Phytopathology 78:875-880.

Taylor, R. H. 1962. The location of tobacco mosaic virus in tobacco seed. Australian Journal of Experimental Agriculture and Animal Husbandry 2:86-89.

Valleau, W. D. and Johnson, E. M. 1927. The effect of a strain of tobacco mosaic on the yield and quality of burley tobacco. Phytopathology. 17:523-527.

Wolf, F. A. and Moss, E. G. 1933. Effect of flue-cured tobacco on yield and quality. Phytopathology 23:834-839.

Zen, R., Teng, Y., Zhang, Q., Wu, D., Li, L., Xia, C., Wang, C., Mao, J., Xiao, Z., Ruan, D., Wang, J., and Zhao, J. 2004. Comparison of disinfecting methods for TMV contamination in tobacco seedling floating trays and culture beds. (In Chinese.) Journal of Yunnan Agricultural University 19:692-695.

Table 3-1. Analysis of variance from two growth chamber tests evaluating effectiveness of chemicals and treatment time on germination and seed transmission of tobacco mosaic virus in K326 flue-cured tobacco seedlings and seed coats. [1]

Source of variation	df	Mean Square (MS) Germination	df	MS Number	MS Weight	df	MS TMV positive[2]
Seedlings[3]							
Test (T)	1	4,467**	1	152	0.000003	1	10,084
Test(Rep)	4	19	4	45	0.000082	4	3,690
Chemical (C)	7	8,370**	7	83**	0.000134	6	31,774**
Exposure (E)	2	414**	2	5	0.000011	2	167
C x E	14	144*	13	10	0.000009	11	1,080
C x T	7	467**	7	49**	0.000040**	6	509
E x T	2	2	2	7	0.000027	2	100
C x E x T	14	110	12	9	0.000008	10	1,103
Error	92	77	174	13	0.000014	143	786
Seed coats							
Test (T)	…	…	1	43	0.000293	1	63
Test(Rep)	…	…	4	18	0.000113	4	63
Chemical (C)	…	…	7	71**	0.000010	6	63
Exposure (E)	…	…	2	6	0.000001	2	63
C x E	…	…	14	25	0.000028*	12	63
C x T	…	…	7	16	0.000013	6	63
E x T	…	…	2	10	0.000007	2	63
C x E x T	…	…	14	8	0.000030*	12	63
Error	…	…	92	15	0.000014	80	63

*,** Significant at the 0.05 and 0.01 level of probability, respectively.

[1] Two tests were conducted in a growth chamber using a randomized complete block design with three replications in 2006.

[2] Healthy control data deleted prior to analysis.

[3] Seedlings and seed coats were separated 14 days after seeding and evaluated for TMV by ELISA. The seed were collected from field grown K326 infected with TMV in 2005. Healthy control seed were collected from healthy field grown K326 plants in 2005.

Table 3-2. Means from two growth chamber tests evaluating effectiveness of chemicals on germination and seed transmission of tobacco mosaic virus in K326 flue-cured tobacco seedlings and seed coats.[1]

Treatments[2]	Germination (%)	No. of units in each sample	Weight (mg)	No. positive total[3]	TMV positive (%)[4]	Average Absorbance
Seedlings						
9.1% ultra bleach	56 b[5]	15 b	21 a	22/29	76 ab	1.04
1% hydrochloric acid	53 bc	15 b	20 a	0/29	0 c	0.11
10% trisodium phosphate	48 c	15 b	20 a	3/26	12 c	0.24
0.1% potassium permanganate	55 b	13 b	20 a	31/35	88 a	0.90
120 g/L nonfat dry milk	52 bc	14 b	20 a	24/30	80 a	0.99
2% formaldehyde	7 d	9 c	20 a	4/7	57 b	0.26
Deionized water infected control	58 b	15 b	21 a	30/31	94 a	0.81
Deionized water healthy control	86 a	18 a	22 a	0/36	0	0.09
Seed coats						
9.1% ultra bleach	…	43 c	7 a	18/18	100 a	3.31
1% hydrochloric acid	…	46 ab	6 a	17/18	94 a	2.81
10% trisodium phosphate	…	46 ab	6 a	18/18	100 a	3.43
0.1% potassium permanganate	…	44 bc	5 a	18/18	100 a	3.44
120 g/L nonfat dry milk	…	43 c	5 a	18/18	100 a	3.48
2% formaldehyde	…	48 a	7 a	18/18	100 a	3.49
Deionized water infected control	…	44 bc	7 a	18/18	100 a	3.31
Deionized water healthy control	…	46 ab	6 a	2/18	11	0.32

[1] Two tests were conducted in a growth chamber using a randomized complete block design with three replications in 2006.
[2] Seedlings and seed coats were separated 14 days after seeding and evaluated for TMV by ELISA. The seed were collected from field grown K326 infected with TMV in 2005. Healthy control seed were collected from healthy field grown K326 plants in 2005.
[3] Sample N is the number of positive immunoassays over the total number of seedlings or seed coats immunoassays.
[4] Data presented are untransformed but Duncan's multiple range test is from transformed analysis.
[5] Means within a column followed by the same letter are not significantly different at the 0.05 probability level using Duncan's mean separation. The control treatment was excluded from the analysis of TMV positive.

Table 3-3. Means from two growth chamber tests evaluating effectiveness of treatment times on germination and seed transmission of tobacco mosaic virus in K326 flue-cured tobacco seedlings and seed coats.[1]

Exposure[2] (min)	Germination (%)	No. of units in each sample	Weight (mg)	No. positive / total[3]	TMV positive[4] (%)
Seedlings					
30	55 a[5]	15 a	20 a	44/71	62 a
60	52 ab	15 a	20 a	37/62	60 a
120	49 b	15 a	21 a	32/54	59 a
Seed coats					
30	…	45 a	8 a	48/48	100 a
60	…	45 a	6 a	48/48	100 a
120	…	45 a	6 a	47/48	98 a

[1] Two tests were conducted in a growth chamber using a randomized complete block design with three replications in 2006.
[2] Seedlings and seed coats were separated 14 days after seeding and evaluated for TMV by ELISA. The seed were collected from field grown K326 infected with TMV in 2005. Healthy control seed were collected from healthy field grown K326 plants in 2005.
[3] Sample N is the number of positive immunoassays over the total number of seedlings or seed coats immunoassays.
[4] Data presented are untransformed but Duncan's multiple range test is from transformed analysis.
[5] Means within a column followed by the same letter are not significantly different at the 0.05 probability level using Duncan's mean separation. The control treatment was excluded from the analysis of TMV positive.

Table 3-4. Analysis of variance from two growth chamber tests evaluating effectiveness of chemicals on germination and seed transmission of tobacco mosaic virus in K326 flue-cured tobacco seedlings and seed coats.[1]

Source of variation	df	Mean Square (MS) Germination	df	MS Number	MS Weight	MS TMV positive[2]
Seedlings[3]						
Test (T)	1	1,239**	1	411**	0.000581*	1,199
Test(Rep)	4	34	4	8	0.000061	1,815
Chemical (C)	10	237**	10	13	0.000019	3,076
C x T	10	138*	10	16	0.000030	1,154
Error	40	54	82	27	0.000026	1,913
Seed coats						
Test (T)	…	…	1	27	0.000272*	0
Test(Rep)	…	…	4	15	0.000015	0
Chemical (C)	…	…	10	31**	0.000004	0
C x T	…	…	10	8	0.000002	0
Error	…	…	40	9	0.000003	0

*,** Significant at the 0.05 and 0.01 level of probability, respectively.
[1] Two tests were conducted in a growth chamber using a randomized complete block design with three replications in 2006.
[2] Healthy control data deleted prior to analysis.
[3] Seedlings and seed coats were separated 14 days after seeding and evaluated for TMV by ELISA. The seed were collected from field grown K326 infected with TMV in 2006. Healthy control plants were collected from healthy field grown K326 plants in 2006.

Table 3-5. Means from two growth chamber tests evaluating effectiveness of chemicals on germination and seed transmission of tobacco mosaic virus in K326 flue-cured tobacco seedlings and seed coats.[1]

Treatments[2]	Germination (%)	No. of units in each sample	Weight (mg)	No. positive / total[3]	TMV positive[4] (%)	Average Absorbance
Seedlings						
10% bleach for 30 min	74 bc[5]	12 a	17 a	6/11	54 abc	0.52
20% bleach for 10 min	76 bc	14 a	17 a	7/9	78 a	0.91
30% bleach for 10 min	80 abc	13 a	19 a	6/11	54 abc	0.70
1% hydrochloric acid for 30 min	65 d	13 a	15 a	3/6	50 abc	0.37
0.2% hydrochloric acid for 10 min	83 ab	13 a	18 a	6/12	50 abc	0.38
0.5% hydrochloric acid for 10 min	81 abc	11 a	17 a	8/11	73 ab	0.77
1% hydrochloric acid for 10 min	73 dc	13 a	14 a	5/8	62 ab	0.38
10% trisodium phosphate for 30 min	77 bc	13 a	18 a	2/9	22 bc	0.25
15% trisodium phosphate for 10 min	82 abc	12 a	18 a	3/10	30 abc	0.28
20% trisodium phosphate for 10 min	82 abc	15 a	18 a	1/10	10 c	0.29
Deionized water healthy control for 30 min	88 a	13 a	19 a	0/12	0	0.12
Seed coats						
10% bleach for 30 min	…	44 bc	6.9 ab	6/6	100 a	3.43
20% bleach for 10 min	…	44 bc	6.2 b	6/6	100 a	3.43
30% bleach for 10 min	…	44 bc	6.2 ab	6/6	100 a	3.42
1% hydrochloric acid for 30 min	…	42 c	6.1 b	6/6	100 a	3.44
1% hydrochloric acid for 10 min	…	43 c	6.9 ab	6/6	100 a	3.44
0.2% hydrochloric acid for 10 min	…	48 ab	8.5 a	6/6	100 a	3.46
0.5% hydrochloric acid for 10 min	…	47 ab	7.9 ab	6/6	100 a	3.48
10% trisodium phosphate for 30 min	…	46 abc	6.7 ab	6/6	100 a	3.28
15% trisodium phosphate for 10 min	…	48 ab	7.5 ab	6/6	100 a	3.45
20% trisodium phosphate for 10 min	…	45 abc	6.3 ab	6/6	100 a	3.39
Deionized water healthy control for 30 min	…	49 a	6.7 ab	0/6	0	0.15

[1] Two tests were conducted in a growth chamber using a randomized complete block design with three replications in 2006.

[2] Seedlings and seed coats were separated 14 days after seeding and evaluated for TMV by ELISA. The seed were collected from field grown K326 infected with TMV in 2006. Healthy control seed were collected from healthy field grown K326 plants in 2006.
[3] Sample N is the number of positive immunoassays over the total number of seedlings or seed coats immunoassays.
[4] Data presented is untransformed but Duncan's multiple range test is from transformed analysis.
[5] Means within a column followed by the same letter are not significantly different at the 0.05 probability level using Duncan's mean separation. The control treatment was excluded from the analysis of TMV positive.

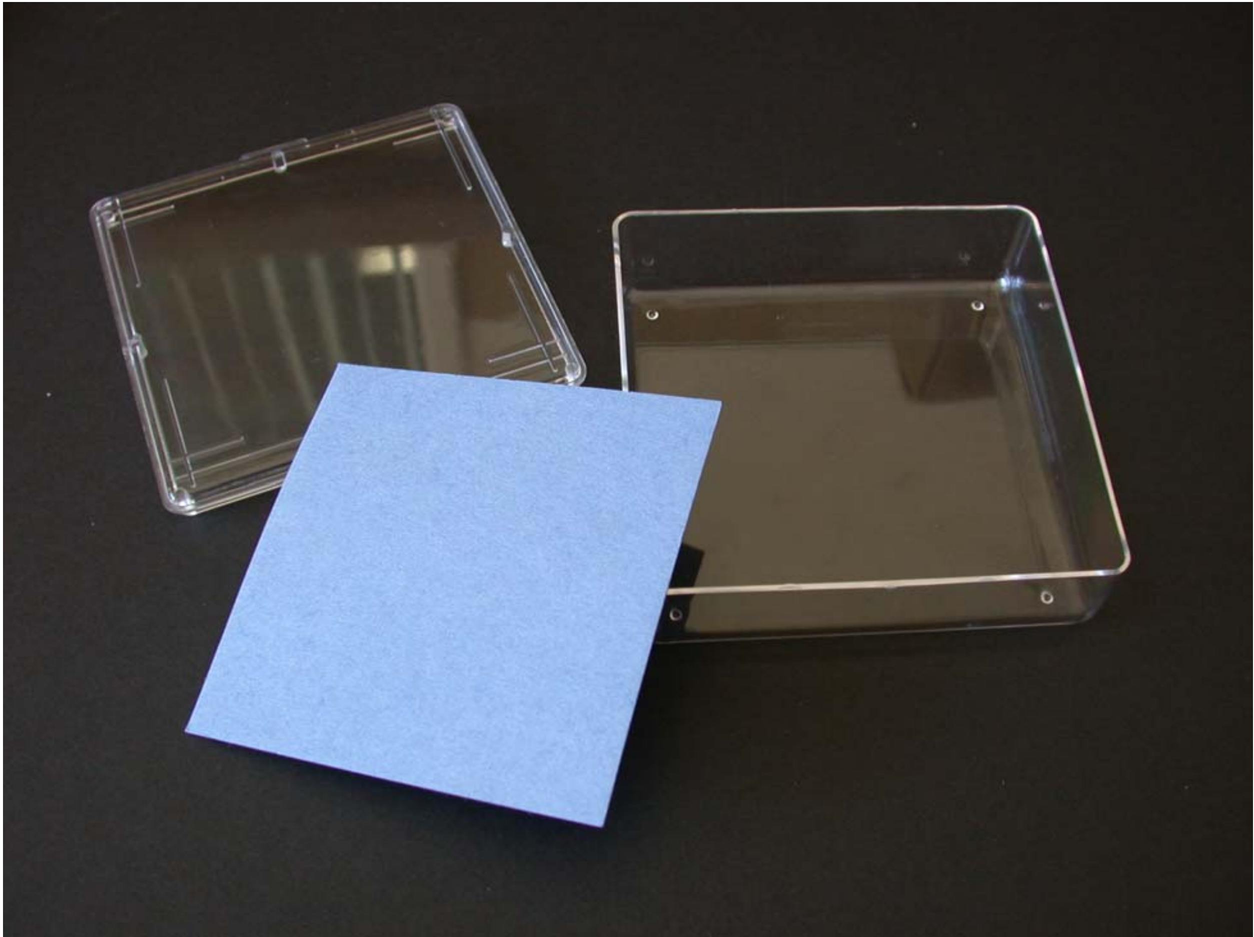

Figure 3-1. Germination box and blue blotter paper used in seed treatment tests.

Figure 3-2. Seed treated with 9.1% ultra bleach solution (left) and healthy control seed treated with deionized water (right) showing the color difference. Seed become bleached and difficult to detect after treatment with ultra bleach.

Chapter IV

EVALUATION OF TOBACCO MOSAIC VIRUS MOVEMENT IN GREENHOUSE FLOAT PRODUCTION OF K326 FLUE-CURED TOBACCO

Abstract

Seed transmission of tobacco mosaic virus in tobacco provides a source of primary inoculum very early in the production cycle. Tobacco seedlings are primarily produced in float beds in the United States. Evaluation of viral presence in the seedlings and nutrient solution will provide much needed information on spread of TMV in tobacco transplant production. The objectives of this study were to determine if TMV can be detected in plant tissue and in the nutrient solution in the float bed system. Greenhouse experiments were conducted in a randomized complete block design with three replications using 'K326' flue-cured tobacco. Seedlings were inoculated 21 DAS and sampled 12 dpi. Biologically active TMV was detected in 100% of leaf and root samples based on ELISA and infectivity assays. Based on polyethylene glycol (PEG) virus precipitation and biological assay, water samples collected from bays with seedlings inoculated with TMV always resulted in water contaminated with TMV. Additional greenhouse tests were conducted to compare seedlings grown from seed produced on a TMV infected plant and seed produced on a healthy plant. TMV was serologically and biologically detected in 2 of 1,045 seedlings sampled which were grown from seed from a TMV infected plant. Therefore, the seed transmission rate in these tests was 0.2%.

Introduction

Tobacco mosaic virus (TMV) has been an economically important pathogen under investigation for over 100 years. Viral spread can be very rapid due to mechanical transmission and many susceptible hosts. There is no chemical control for TMV; once the virus is in the plant, it cannot be inactivated in the living plant. The only resistance available after viral entry into the plant is genetic resistance. Actively employing sanitation and exclusion methods of the mosaic virus is very important in preventing movement into susceptible plants.

Tobacco transplant production in Virginia and much of the southeastern United States has shifted from seed bed to greenhouse production using hydroponic float bed systems (Clark, 2001). Expanded polystyrene trays, filled with soilless media and seeded, are floated on a nutrient solution. Capillary action carries water up through the soilless media to the seed (Reed, 1996) via a small opening in the bottom of the tray. Seedlings are grown closely together and are trimmed with an overhead clipper to provide manageable plugs for transplanting directly into prepared fields. Hundreds of thousands of plants can be grown in a single greenhouse. Although TMV has been an economically important disease for many years, infection of seedlings in float bed systems would lead to widespread field infections and severe losses (T. D. Reed, personal communication, 2007).

Mechanical spread can lead to viral infection of seedlings in greenhouse float or plant bed production. Potential sources of TMV inoculum can be contaminated equipment, workers hands, tobacco products, and seed transmission. Overhead clippers are likely to facilitate the spread of virus throughout the seedlings in the greenhouse. Allard (1915) suggested that TMV could be seed transmitted, but predicted that TMV was more likely seedborne. Others supported Allard's prediction (Beniot, 1977; Taylor, 1962; Chatzivassiliou et al., 2004), while few did not

(Chamberlain, 1937). The physical characteristics or phenotypes of plants suspected to be infected via seed transmission are unknown, but they are not recognized as showing typical mosaic symptoms. Such seedlings may be short, deformed, or appear normal and show no symptoms at all, due to an atypical viral-plant interaction mechanism in very young plants. Thus, clipping may or may not spread the mosaic virus.

Investigations have been conducted to examine the spread of plant viruses through water. Surface waters have been demonstrated to contain plant viruses (Tomlinson et al., 1982, 1983; Tomlinson and Faithfull, 1984; Tosic and Tosic, 1984; Koenig and Lesemann, 1985; Polák and Branisova, 1992; Fuchs et al., 1996; Horvath, et al., 1999). Tomato mosaic virus has even been detected in ancient glacial ice (Castello, 1999). The presence of TMV in surface waters was demonstrated by Tosic and Tosic (1984) and Koenig (1986). Park et al. (1999) studied the transmission of TMV in recirculating hydroponic systems using tobacco (*N. tabacum* cv. Samsun-nn and cv. Xanthi), tomato (*Lycopersicon esculentum* cv. Alchan), and hot pepper (*Capsicum annuum* cv. Dahong). Research demonstrated that TMV was easily transmitted through root-tip grafting of tobacco, tomato, and hot pepper but rarely through the nutrient solution (Park et al. 1999).

There has been limited research on the presence of TMV in tobacco float bays and its impact on greenhouse tobacco transplant production (Zen et al., 2004). Zen et al. (2004), in China, artificially contaminated float bed liners and expanded polystyrene trays with TMV in order to test chemical disinfection treatments. High disease incidences of TMV, 70% to 73.5%, were found in control treatments where water was used as a mock disinfectant (Zen et al., 2004). Artificial contamination of greenhouse float production systems leads to high, unfavorable rates of TMV infection in seedlings. This provides insight into what might happen when TMV enters

into the greenhouse float system. The objectives of this study were to detect and test viability of TMV in shoots, roots, and water in a greenhouse float production system.

Materials and Methods

Greenhouse experiments were conducted at the Virginia Polytechnic Institute and State University Southern Piedmont Agricultural Research and Extension Center (SPAREC) near Blackstone, Virginia in the October and November of 2006. The greenhouse experiments were conducted in a randomized complete block design with four replications. Each test was repeated three times. A shade cloth covered the glass greenhouse until mid October and temperatures were regulated between 18 to 35°C.

All ‘K326’ flue-cured tobacco seed used in these experiments were grown and collected at SPAREC. Standard agronomic production practices for fertilization, transplanting, disease, weed, and insect control (Reed at al., 2005) were followed for plant production. Plants were mechanically inoculated with TMV in late June, after layby cultivation. TMV inoculum was prepared by grinding 0.05 g of TMV infected leaf tissue in 1% potassium phosphate buffer at pH 7.0 and 1% Celite (Fisher Scientific Company, Pittsburg, PA). Inoculum was diluted 1:100 and inoculations were conducted on a palm size leaf near the top of the plant using foam tipped 12.7 polypropylene swabs (Fisher Scientific Company). Seed heads, from healthy and infected source plants, were collected during September 2006. Seed were shelled and cleaned using separate 600 μm (30 mesh) sieves designated for both seed types. All seeds were stored in paper bags at room temperature. Seed from a non TMV infected K326 source was used as the control and treated only with deionized water.

All trays were seeded using Clay's Seeder (Clay's Seeder Company, Carlisle, KY) (Figure 4-1). After seeding, the trays were moved to their bay containers, which were filled with water. Trays were floated on nutrient solution and the soilless media was allowed to moisten.

Evaluation of roots and water of infected seedlings

All seed used were collected from healthy parent plants. Treatments were seedlings mechanically inoculated with TMV and buffer-inoculated healthy seedlings. New 288-cell BeltWide expanded polystyrene hydroponic trays (BeltWide Inc., Tampa, FL) were cut into two pieces using a hand saw. Each half tray measured 11 by 12 cells wide. Each partial tray was hand filled with Carolina Choice Tobacco Mix (Carolina Soil Company, Kinston, NC). Healthy seed were sown into the trimmed trays and placed in the 40.5 by 50.6 by 7.9 cm plastic containers (Consolidated Plastics Company, Inc., Twinsburg, OH), herein called bay containers.

Seedlings were inoculated 21 DAS with either TMV or a buffer control. The buffer inoculation was used to standardize the wound defense mechanism across both treatments. The buffer inoculations were performed first using sodium phosphate buffer, pH 7.0 and 1% Celite. One finger was placed under the largest leaf, a glass rod was dipped into a 2 mL aliquot of buffer solution in a centrifuge tube, and the rod was pressed and pulled across about 1.5 cm length of the midrib and adjacent tissue. A new glass rod was used to inoculate the seedlings in each tray. Following the control inoculations, the inoculation with TMV was conducted using the same procedure. Each half tray was allotted 2 mL of TMV inoculum to infect all of the seedlings in the tray. Plants were not rinsed following inoculation.

Ten randomly selected plants were sampled 12 days post inoculation (dpi). Leaf and root tissue were sampled separately with 0.3 g samples for TMV detection. Roots were rinsed under

running water before sampling to remove soilless media from the roots. Also, approximately 50 mL of water was collected from each bay container for analysis.

Evaluation of TMV in seedlings via seed transmission

The two treatments include TMV seeds collected from an infected parent or a healthy parent plant. New 288-cell BeltWide expanded polystyrene hydroponic trays were cut in three pieces measuring 7 by 12 cells using a hand saw. Trays were hand filled with Carolina Choice Tobacco Mix (Carolina Soil Company, Kinston, NC), seeded, and floated in 45.7 by 30.4 by 8.9 cm bay containers (Rubbermaid Commercial Products, Inc., Winchester, VA).

Seedlings were allowed to grow until 28 DAS and had approximately five leaves before sampling began. There were three sampling dates, 28, 35, and 42 DAS. On the first and second sampling date, 0.3 g of leaf and the entire root system samples were collected from ten randomly selected plants. Roots were rinsed under running water to remove soilless media from the roots before sampling. Only 0.3 g leaf samples were taken from all of the remaining plants during the third sampling date. Water samples of 50 mL were collected 42 DAS.

Fertilization and management

Seven days after seeding the water in the bay containers was discarded and filled with nutrient solution at a rate of 100 ppm N. A stock tank was prepared using Ultrasol 20-10-20 water-soluble fertilizer (SQM North America, Atlanta, GA). Bay containers were filled with a fertilizer injector set at a rate of 1:100, such that 100 ppm N nutrient solution was being produced. The depth of nutrient solution in the bays was maintained at 4 cm. Bay containers

used in trials where seedlings were inoculated contained 8 L of nutrient solution and bay containers used in the seed transmission trial contained 3.7 L of nutrient solution.

Weekly treatments for blue mold (downy mildew, *Peronospora tabacina* D. B. Adam = *P. hyoscyami* de Bary f. sp. *tobacina*) were applied to the seedlings to prevent an outbreak of this fungus. Mancozeb (Dithane DF Rainshield, DOW AgroSciences LLC, Indianapolis, IN) was used at a rate of 1.06 g/L for blue mold. Applications of imidacloprid (Admire Pro, Bayer CropScience, Research Triangle PK, NC) for control of greenhouse whiteflies (*Trialeurodes vaporariorum*) were applied to the seedlings as needed. Imidacloprid was applied at a rate of 1.32 mL/L. When one or both chemicals were used they were mixed and poured into a hand sprayer and applied onto the leaves. Due to an infestation of shore fly (*Scatella stagnalis*) it was necessary to treat the bays to prevent seedling death. All bay water was treated with acephate (Orthene 97, Valent USA Corporation, Walnut Creek, CA) at a rate of 1.3 g/L. Bays were treated only once due to the efficiency of treatment.

Inoculum preparation

Tobacco mosaic virus inoculum was obtained from infected tissue from a greenhouse culture of the common strain of TMV, obtained from Dr. Sue Tolin at Virginia Polytechnic Institute and State University, Blacksburg, Virginia. The tissue was ground and the resulting sap was decanted. The original source of the virus was from a flue-cured tobacco plant growing at SPAREC. The sap was then diluted at a rate of 1:10 in sodium phosphate buffer, pH 7.0, to reach a final volume of 10 mL. Celite was added to the inoculum at a rate of 0.5%.

Detection of virus in tissue and water

Leaf and root tissue were placed in individual grinding bags (Agdia, Elkhart, IN) between the two mesh liners and stored at 2°C until processed. Prior to maceration, 3 mL of general extract buffer (GEB) (Agdia) was added to each bag. Leaf and root samples were thoroughly macerated using a hand operated tissue homogenizer (Agdia) with circular bearings.

All water samples were stored at 2°C to limit algae, fungal, and bacterial growth prior to laboratory analysis. Prior to filtration, samples were allowed to rest for 24 h to allow sedimentation of coarse particulate matter. Water samples were filtered through a Whatman No. 4, 90 mm filter paper (Whatman, South Kearney, NJ) funnel to remove debris, such as insect remains, algae, and soilless media. Water samples were allowed to filter gravitationally into 85 mL Oak Ridge centrifuge tubes (Thermo Fisher Scientific, Rochester, NY).

A polyethylene glycol (PEG) precipitation, adapted from Hebert (1963), was used to assay the water samples for the presence of TMV. Four percent PEG (w:v) and 0.3M NaCl (w:v) were added to 50 mL of bay water in each 85 mL centrifuge tube. Tubes were inverted gently by hand several times until the chemicals began to dissolve. Tubes were placed in a refrigerator at 2°C for 2 h. Tubes were then centrifuged in a Marathon multipurpose refrigerated centrifuge (Fisher Scientific Company, Pittsburg, PA) at 10,000 x g for 12 min at 20°C. The supernatant was poured off immediately and the pellet resuspended in 5 mL sodium phosphate buffer, pH 8.2. The PEG/NaCl precipitation and centrifugation were repeated and the resulting pellet was suspended in 0.5mL of sodium phosphate buffer, pH 8.2. The contents of each centrifuge tube was collected and assayed for TMV.

Following the preparation of the tissue and water samples, 100 µl of each sample was loaded per well into an enzyme-linked immunosorbent assay (ELISA) plate(s). The plate was

obtained from PathoScreen Kit (Agdia, Elkhart, IN) for the common strain of TMV (TMV-c). Two positive and negative controls were also loaded per plate. The positive control was provided in the PathoScreen Kit and the negative control was GEB (Agdia).

Plates were visually examined to record color development and measured by a MRX Revelation plate reader (DYNEX Technology, Chantilly, VA) at 405 nm. Positive TMV samples are characterized by yellow color development and absorbance greater than twice that of the negative control. Color intensity is relative to the concentration of virus in the sample; more intense yellow is a higher concentration of TMV and less intense yellow is less concentrated.

All ELISA results were followed up by infectivity assays using some of the remaining solution to demonstrate biological activity of the virus particles. *Nicotiana tabacum* cv. Xanthi NN was used as the assay plant due to its local lesion response to TMV infection. Infectivity assays were conducted under greenhouse conditions with a maximum and minimum temperature of 18 and 35°C, respectively. A clean cotton swab was dipped in Celite, in the remaining solution in the grinding bag, and rubbed on a Xanthi leaf. The presence of local lesions were recorded 7 to 10 dpi.

Analysis of variance was conducted using PROC GLM (SAS Institute, 1987). Data transformations were performed using arcsine transformation (Gomez and Gomez, 1984). Treatments means were separated using Duncan's multiple range test (Gomez and Gomez, 1984). An F-max test was conducted to determine if error variances between tests were homogeneous (Gomez and Gomez, 1984). Leaf and root samples were combined for statistical analysis due to the fact that root and leaf samples were always positive when collected from TMV inoculated seedlings and always negative when collected from a healthy source plant or buffer inoculated.

Results and Discussion

Evaluation of roots and water of infected seedlings

There were significant differences between treatments in all tests for percentage of TMV positive, absorbance, infectivity, and number of local lesions for plant tissue samples (Tables A-11 through A-16). Percentage of TMV positive and infectivity were also significantly different between treatments in all tests for nutrient solution samples. However, nutrient solution samples were significantly different between treatments for absorbance and local lesion number in only one test (Table A-11). Error variances among the three tests were homogeneous for each parameter measured based on an F-max test although there were a few exceptions. A combined analysis was conducted since the rank between treatments was consistent.

Significant differences were observed between treatments for the percentage positive TMV samples, absorbance, infectivity, and local lesion number for both plant tissues and the nutrient solution in the combined analysis (Table 4-1). Data from leaf and root samples were combined for statistical analysis since they were identical. Each of the 240 mechanically infected seedlings sampled was positive for TMV based on ELISA and infectivity assays (Table 4-2). Plant tissue samples positive for TMV had an average absorbance of 2.80, which is significantly higher than the absorbance of buffer inoculated samples measuring 0.14 (Table 4-2). The average number of local lesions for a positive tissue sample was 21 which was significantly higher than the buffer inoculated which had none (Table 4-2). All of the healthy plant tissue samples were negative for TMV. The nutrient solution from the TMV inoculated seedlings contained biologically active TMV and the buffer inoculated bays remained virus free

based on ELISA and local lesion infectivity results (Table 4-2). An average of four local lesions were counted from infectivity assays of positive nutrient solution samples.

Results of this research demonstrate that when 21-day-old flue-cured tobacco seedlings of K326 are mechanically inoculated with TMV, leaves and roots are infected with TMV 12 days after inoculation. TMV was also detected in the corresponding nutrient solution samples, which could provide a potential source of inoculum for passive plant-to-plant transmission of TMV (Table 4-2). Presence of virus in nutrient solution is supported by Yarwood (1960). TMV titer in the nutrient solution would need to be sufficiently high to contribute to plant-to plant transmission in greenhouse float production of tobacco seedlings.

Evaluation of TMV in seedlings via seed transmission

There were no significant differences observed between treatments for percentage of positive TMV samples, infectivity, and number of local lesions in all three sampling dates for all three tests (Tables A-17 through A-22). However, there was a significant difference between treatments for absorbance at 28 and 35 DAS (Tables A-17 through A-22). Error variances between the three tests were homogeneous for percentage of positive TMV samples, infectivity, and local lesion number based on an F-max test therefore but a combined analysis was conducted. A treatment by test interaction for absorbance at 28 DAS (Table 4-3) was a result of change in magnitude and not a change in rank, therefore only the main effects will be discussed.

No significant differences were observed for percentage of positive TMV samples, infectivity, and number of local lesions between treatments in all three tests at 28, 35, and 42 DAS (Table 4-3). There were two TMV positive plant tissue samples from the TMV infected seed treatment and one TMV positive plant tissue sample from the healthy treatment at 28 DAS

which resulted in TMV incidence levels of 0.8% to 0.4%, respectively (Table 4-4). Although this is not a statistically significant difference, the TMV source seed exhibit a higher disease incidence. The origin of contamination is unknown. All precautions were taken to keep the seedlings from being infected in the greenhouse. Watering was done carefully not to splash water from bay to bay. Gloves were worn and tools were flame sterilized when samples were taken. Some explanations for these results could include TMV source seed from previous tests were not cleaned out of the seeder efficiently or that plants were accidentally inoculated with TMV at some point during the study.

At 28 and 35 DAS there was a significant difference between treatments, seed grown from a healthy source plant and seed grown from an infected source plant, for absorbance (Table 4-3). The results from these two dates were contradictory. At 28 DAS the absorbance from an infected source plant was lower than a healthy source plant at 0.06 and 0.10, respectively (Table 4-4). At 35 DAS the absorbance from an infected source plant was higher than a healthy source plant at 0.09 and 0.08, respectively (Table 4-4).

For this seed transmission test 1,595 seedlings were sampled individually. Of those, 1,045 seedlings were from seed produced from an infected parent plant. All of those plants were tested for the presence of TMV using ELISA and infectivity and 2 were found positive for the virus using ELISA. There is a 0.2% incidence of infection by seed transmission in tobacco seedlings grown in a float bed production system. Of these, only three plants were small or distorted (Figure 4-2). The remainder of the infected seedlings were similar in size and color to the rest of the seedlings found to be healthy.

Conclusions

Seedlings produced in greenhouses with float bed production practices and are mechanically inoculated with TMV 21 DAS were demonstrated to have biologically active TMV in the leaves and roots based on ELISA and infectivity assays. Nutrient solution collected from those bays also contains biologically active viral particles. This study suggests that contamination of bay water could be an important reservoir for the spread of TMV in float bed production. This study also determined that the incidence of seed transmission of TMV in flue-cured tobacco using seed collected from a TMV infected plant is 0.2% in a greenhouse float production system. Given that the majority of the positive seedlings were visually no smaller than there noninfected counterparts, it is reasonable to suggest that these seedlings are overlooked and could provide the potential for spread of TMV throughout a greenhouse by the standard practice of clipping. Other research (Lusso, 2002; Park et al. 1999; Taylor, 1962) has determined that TMV is not seed transmitted because mosaic symptoms were not visible on seedlings produced from grow-out tests using seed produced on a TMV infected plant. Presence of TMV was determined using serological tests and biological activity of TMV was verified with infectivity assays in our tests. Results of this research lend credence to the claim of Chatzivassiliou et al. (2004) that TMV’s primary source of infection is through seed to seedling infection.

Literature Cited

Allard, H. A. 1915. Distribution of the virus of the mosaic disease in capsules, filaments, anthers and pistils of affected tobacco plants. Journal of Agricultural Research 5:251-256.

Benoit, M. A. 1977. Does tobacco seed transmit tobacco mosaic virus? Planter 53: 197-201.

Benoit, M. A. and Maury, Y. 1976. The location of tobacco mosaic virus in tobacco seed. Planter 52:392-396.

Castello, J. D., Rogers, S. O., Starmer, W. T., Catranis, C. M., Ma LiJun, Bachand, G. D., Zhao, Y., and Smith, J. E. 1999. Detection of tomato mosaic tobamovirus RNA in ancient glacial ice. Polar Biology 22:207-212.

Chamberlain, E. E. 1937. Tobacco Mosaic. New Zealand Journal of Science and Technology 19:209-226.

Chatzivassiliou, E. K., Efthimiou, K., Drossos, E., Papadopoulou, A. Poimenidis, G. and Katis, N. I. 2004. A survey of tobacco viruses in tobacco crops and native flora in Greece. European Journal of Plant Pathology 110:1011-1023.

Clark, J. J. 2001. Development of a greenhouse tobacco seedling performance index. M.S. thesis. Virginia Polytechnic Institute and State University, Blacksburg.

Fuchs, E., Schlufter, C. and Kegler, H. 1996. Occurrence of plant viruses in the northern sea. Archives of Phytopathology and Plant Protection 30:365-366.

Hebert, T. T. 1963. Precipitation of plant viruses by polyethylene glycol. Phytopathology 53:362.

Horváth, J., Pocsai, E., and Kazinczi, G. 1999. Plant virus contamination of natural waters in Hungary. Zbornik predavanj in referatov 4. Sloenskega Posvetovanja o Varstvu Rastlin v Portorožu od 3. do 4. Marca 1999. pp. 353-356.

Koenig, R. 1986. Plant viruses in rivers and lakes. Advances in Virus Research 31:321-333.

Koenig, R. and Lesemann, D. E. 1985. Plant viruses in German lakes: tombusviruses, a potexvirus and carnation mottle virus. Phytopathologische Zeitschrift 112:105-116.

Lusso, M., Davis, G., and Barker, H. 2002. The route of tobacco mosaic virus to Nicotiana tabacum seeds and evaluation of its seed transmission. Paper presented at the 40th Tobacco Workers Conference, Jan 14-17, Pinehurst, NC.

Park, W. M., Lee, G. P., Ryu, K. H., and Park, K. W. 1999. Transmission of tobacco mosaic virus in recalculating hydroponic system. Scientia Horticulture 79:217-226.

Polák, Z. and Branišová, H. 1992. Plant virus isolations from water of an irrigation ditch. Sbornik UVTIZ, Ochrana Rostlin 28:167-169.

Reed, D. W. 1996. A grower's guide to water, media, and nutrition for greenhouse crops. Ball Pub., Batavia, IL. 314 pp.

Reed, T. D., Johnson, C. S., Semtner, P. J., and Wilkinson, C. A. 2005. 2006 Flue-cured tobacco production guide. Pub. 436-048 Virginia Agric. Exp. Stn., Blacksburg.

Taylor, R. H. 1962. The location of tobacco mosaic virus in tobacco seed. Australian Journal of Experimental Agriculture and Animal Husbandry 2:86-89.

Tomlinson, J. A. and Faithfull, E. M. 1984. Studies of the occurrence of tomato bushy stunt virus in English rivers. Annals of Applied Biology 104:485-495.

Tomlinson, J. A., Faithfull, E. M. and Fraser, R. S. S. 1982. Plant viruses in river water. 33rd Annual Report for 1982, National Vegetable Research Station. pp. 81-82.

Tomlinson, J. A., Faithfull, E. M., Webb, M. J. W., Fraser, R. S. S. and Seeley, N. D. 1983. Chenopodium necrosis: a distinctive strain of tobacco necrosis virus isolated from river water. Annals of Applied Biology 102:135-147.

Tosic, M. and Tosic, D. 1984. Occurrence of tobacco mosaic virus in water of the Danube and Sava rivers. Phytopathologische Zeitschrift 110:200-202.

Yarwood, C. E. 1960. Release and preservation of virus by roots. Phytopathology 50:111-114.

Table 4-1. Analysis of variance for three greenhouse tests investigating distribution of tobacco mosaic virus (TMV) in plant tissue and the nutrient solution.[1]

Source of variation	df	Mean Square (MS) TMV positive[2]	MS Absorbance	MS Infectivity[2]	MS Local lesion no.
Plant tissues[3]					
Test	2	16	1.05	0	12,416**
Test(Rep)	9	16	0.66*	0	956**
Treatment[4]	1	945,709**	855.22**	953,640**	52,021**
Treatment x Test	2	16	0.66	0	12,416**
Error	465	16	0.32	0	182
Nutrient solution					
Test	2	0	1.74	0	4
Test(Rep)	9	0	0.41	0	3
Treatment	1	47,682**	12.02**	47,682**	81**
Treatment x Test	2	0	1.76	0	4
Error	9	0	0.42	0	3

*,** Significant at the 0.05 and 0.01 level of probability, respectively.

[1] Three tests were conducted in a greenhouse using a randomized complete block design with four replications in 2006. Plant tissue and nutrient solutions were collected 12 dpi, 28 DAS and evaluated for TMV by ELISA and infectivity assay on TMV resistant plants. The seed were collected from healthy, field grown K326 in 2006.

[2] Data presented are from arcsine transformed analysis.

[3] Plant tissues represent leaf and root samples combined.

[4] There were two inoculation treatments, which include inoculation with TMV and inoculation with buffer.

Table 4-2. Means from three greenhouse tests investigating distribution of tobacco mosaic virus (TMV) in plant tissue and the nutrient solution.[1]

Inoculation Treatment[2]	TMV positive[3] (%)	Absorbance (405 nm)	Infectivity[3] Assay	Local lesion no.
Plant tissues[4]				
TMV inoculated	100 a[5]	2.80 a	100 a	21 a
Buffer inoculated	0 b	0.14 b	0 b	0 b
Nutrient solution				
TMV inoculated	100 a	1.50 a	100 a	4 a
Buffer inoculated	0 b	0.09 b	0 b	0 b

[1] Tests were conducted in a greenhouse using a randomized complete block design with four replications in 2006. Plant tissue and nutrient solutions were collected 12 dpi, 28 DAS and evaluated for TMV by ELISA and infectivity assay on TMV resistant plants. The seed were collected from healthy, field grown K326 in 2006.
[2] There were two inoculation treatments, which include inoculation with TMV and inoculation with buffer.
[3] Data presented are untransformed but Duncan's multiple range test data are from arcsine transformed analysis.
[4] Plant tissues represent leaf and root samples combined.
[5] Means within a column followed by the same letter are not significantly different at the 0.05 probability level using Duncan's multiple range test.

Table 4-3. Analysis of variance for three greenhouse tests evaluating distribution of tobacco mosaic virus (TMV) via seed transmission.[1]

Source of variation	df	Mean Square (MS) TMV positive[2]	MS Absorbance	MS Infectivity[2]	MS Local lesion no.
28 DAS[3]					
Test	2	149**	29**	16	0
Test(Rep)	9	16	2	16	0
Treatment[4]	1	16	24**	16	0
Treatment x Test	2	16	9**	16	0
Error	465	50	0	16	0
35 DAS					
Test	2	0	73**	0	0
Test(Rep)	9	0	1	0	0
Treatment	1	0	14**	0	0
Treatment x Test	2	0	3	0	0
Error	465	0	0	0	0
42 DAS					
Test	2	0	7**	0	0
Test(Rep)	9	0	5**	0	0
Treatment	1	0	0	0	0
Treatment x Test	2	0	1	0	0
Error	610	0	0	0	0

*,** Significant at the 0.05 and 0.01 level of probability, respectively.
[1] Three tests were conducted in a greenhouse using a randomized complete block design with four replications in 2006.
[2] Data presented are from arcsine transformed analysis.
[3] Leaf samples were collected 28, 35, and 42 DAS and evaluated for TMV by ELISA and infectivity assay on TMV resistant plants. The seed were collected from healthy, field grown K326 in 2006.
[4] Treatments include seed collected from and infected source plant and seed collected from a healthy source plant.

Table 4-4. Means from three greenhouse tests evaluating distribution of tobacco mosaic virus (TMV) via seed transmission.[1]

Sample date[2]	TMV positive[3] (%)	Absorbance (405 nm)	Infectivity[3] Assay	Local lesion no.
28 DAS				
TMV source seed	0.8 a[4]	0.06 b	0 a	0 a
Healthy source seed	0.4 a	0.10 a	0.4 a	0 a
35 DAS				
TMV source seed	0 a	0.09 a	0 a	0 a
Healthy source seed	0 a	0.08 b	0 a	0 a
42 DAS				
TMV source seed	0 a	0.13 a	0 a	0 a
Healthy source seed	0 a	0.13 a	0 a	0 a

[1] Tests were conducted in a greenhouse using a randomized complete block design with four replications in 2006. The seed used were collected from healthy, field grown K326 in 2006.
[2] Leaf samples were collected 28, 35, and 42 DAS and evaluated for TMV by ELISA and infectivity assay on TMV resistant plants.
[3] Data presented are untransformed but Duncan's multiple range test data are from arcsine transformed analysis.
[4] Means within a column followed by the same letter are not significantly different at the 0.05 probability level using Duncan's multiple range test.

Figure 4-1. Clay's Seeder used to seed float trays with raw 'K326' flue-cured tobacco seed.

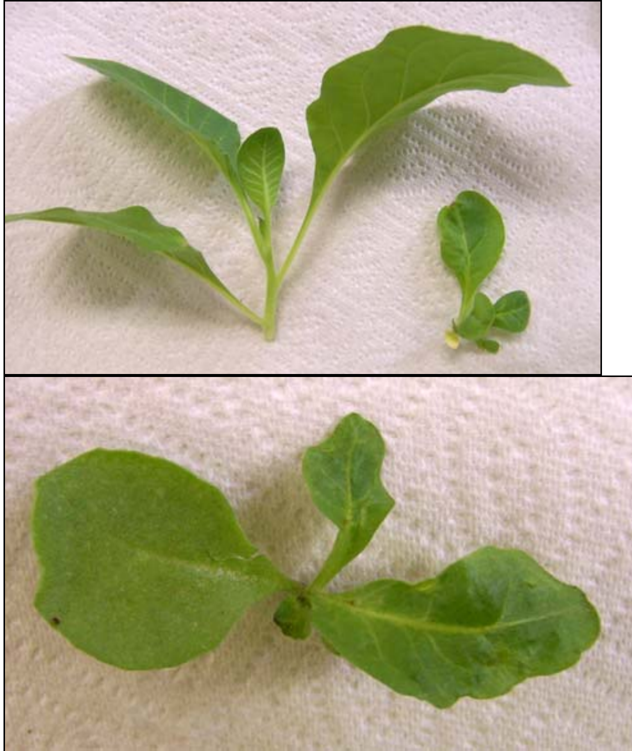

Figure 4-2. Comparison of various seedlings in the evaluation of tobacco mosaic virus in seedlings via seed transmission study. A normal seedling and small, deformed seedling collected from the same float tray (left) and a small deformed seedling found to be positive for TMV using ELISA (right).

Chapter V

SUMMARY

This study demonstrates TMV is present in all seed coat samples collected from infected source plants, even after chemical treatment. While most chemical treatments reduced infection of the seedlings, two treatments emerged as the most effective. They include hydrochloric acid and trisodium phosphate. Hydrochloric acid at a concentration of 1% eliminated infection in seedlings grown from seed stored for one year but not newly harvested seed at any concentration. Trisodium phosphate reduced, but did not eliminate, infection of seedlings at all concentrations. Neither chemical treatment significantly reduced germination. Reevaluation of chemical treatment times, chemical concentration, and seed storage time could provide a viable treatment for inactivation of TMV in tobacco seed. A combination of these two treatments should also be considered as they may work effectively together.

This study also found that when seedlings are mechanically inoculated with TMV, the root systems are invaded and infected with virus. Subsequently, nutrient solution in the float bed of infected seedlings is also contaminated with biologically active virus. This contaminated nutrient solution could provide a means for facilitating spread of the virus and should be avoided. The concentration of virus in the nutrient solution, or whether healthy seedlings floated in contaminated solution become infected, was not investigated in this study. Investigations of both aspects should be carried out. Calculating the concentration of the virus and determining the dilution end point where plants become infected from contaminated nutrient solution should also be investigated. Concentration of the virus and

dilution end point information could be used to determine infection thresholds under which healthy transplants could be produced in greenhouse float production.

This study demonstrated that seed treatments were effective in reducing, but not eliminating seed transmission of TMV. This study also demonstrated that TMV is seed transmitted at a rate of 0.2% of germinated seed. Such a rate is relatively high considering the potential number of seedlings present in a typical greenhouse, 0.5 to 1 million, and considering management practices such as clipping that can readily spread the virus from originally infected seedlings. The two seedlings that tested positive for TMV were similar in appearance to the seedlings found to be healthy. In addition, the presence of biologically viable virus particles in the bay water was demonstrated and thus the potential for spread to other seedlings in greenhouse transplant production. The occurrence of seed transmission of TMV places great importance on producing virus free tobacco seed.

Appendix

TABLES

Table A-1. Analysis of variance from the first of two growth chamber tests evaluating effectiveness of chemicals and treatment time on germination and seed transmission of tobacco mosaic virus (TMV) in flue-cured tobacco seedlings and seed coats.[1]

Source of variation	df	Mean Square (MS) Germination	df	MS Number	MS Weight	df	MS TMV positive[2]
Seedlings[3]							
Rep	2	7	2	53**	0.000070**	2	872
Chemical (C)	7	4,880**	7	88**	0.000145**	6	16,826**
Exposure (E)	2	181	2	2	0.000009	2	27
C x E	14	100	13	5	0.000009	11	854
Error	46	74	76	7	0.000010	61	938
Seed coats							
Rep	…	…	2	14	0.000221**	2	0
Chemical (C)	…	…	7	26	0.000022	6	0
Exposure (E)	…	…	2	8	0.000007	2	0
C x E	…	…	14	17	0.000061**	12	0
Error	…	…	46	13	0.000023	40	0

*,** Significant at the 0.05 and 0.01 level of probability, respectively.

[1] The test was conducted in a growth chamber using a randomized complete block design with three replications in 2006.

[2] Healthy control data deleted prior to analysis.

[3] Seedlings and seed coats were separated 14 days after seeding and evaluated for TMV by ELISA. The seed were collected from field grown K326 infected with TMV in 2005. Healthy control seed were collected from healthy field grown K326 plants in 2005.

Table A-2. Means from the first of two growth chamber tests evaluating effectiveness of chemicals on germination and seed transmission of tobacco mosaic virus (TMV) in flue-cured tobacco seedlings and seed coats.[1]

Treatments	Mean germination (%)	Mean no. in sample	Mean Weight (mg)	No. positive / total[2]	TMV positive (%)[3]
Seedlings[4]					
10% bleach solution	51 b[5]	12 c	22 a	13/15	87 a
1% hydrochloric acid	43 bc	14 bc	21 ab	0/12	0 b
10% trisodium phosphate	38 b	15 ab	23 a	2/9	22 b
0.1% potassium permanganate	52 b	13 bc	20 ab	16/17	94 a
120 g/L nonfat dry milk	43 bc	14 b	19 b	11/12	92 a
2% formaldehyde	5 d	6 d	5 c	3/4	75 a
deionized water infected control	48 b	14 b	21 ab	13/14	93 a
deionized water healthy control	90 a	18 a	22 ab	0/18	0
Seed coats					
10% bleach solution	…	44 b	10 a	9/9	100 a
1% hydrochloric acid	…	43 ab	7 a	9/9	100 a
10% trisodium phosphate	…	46 ab	7 a	9/9	100 a
0.1% potassium permanganate	…	47 ab	7 a	9/9	100 a
120 g/L nonfat dry milk	…	44 b	5 a	9/9	100 a
2% formaldehyde	…	48 a	9 a	9/9	100 a
deionized water infected control	…	43 b	8 a	9/9	100 a
deionized water healthy control	…	46 ab	7 a	1/9	11

[1] The test was conducted in a growth chamber using a randomized complete block design with three replications in 2006.
[2] No. positive/total is the ratio of positive immunoassays to the total number of seedlings or seed coats in the sample immunoassays.
[3] Data presented are untransformed but Duncan's multiple range test is from transformed analysis.
[4] Seedlings and seed coats were separated 14 days after seeding and evaluated for TMV by ELISA. The seed were collected from field grown K326 infected with TMV in 2005. Healthy control seed were collected from healthy field grown K326 plants in 2005.
[5] Means within a column followed by the same letter are not significantly different at the 0.05 probability level using Duncan's multiple range test. The control treatment was excluded from the analysis of TMV positive.

Table A-3. Means from the first of two growth chamber tests evaluating effectiveness of treatment time on germination and seed transmission of tobacco mosaic virus (TMV) in flue-cured tobacco seedlings and seed coats.[1]

Exposure (min)	Germination (%)	No. in sample	Weight (mg)	No. positive/total[2]	TMV positive[3] (%)
Seedlings[4]					
30	49 a[5]	14 a	20 a	23/33	70 a
60	46 ab	14 a	20 a	19/29	70 a
120	44 b	14 a	21 a	16/25	69 a
Seed coats					
30	…	45 a	8 a	21/21	100 a
60	…	46 a	8 a	21/21	100 a
120	…	46 a	7 a	21/21	100 a

[1] The test was conducted in a growth chamber using a randomized complete block design with three replications in 2006.
[2] No. positive/total is the ratio of positive immunoassays to the total number of seedlings or seed coats in the sample immunoassays.
[3] Data presented are untransformed but Duncan's multiple range test data are from transformed analysis.
[4] Seedlings and seed coats were separated 14 days after seeding and evaluated for TMV by ELISA. The seed were collected from field grown K326 infected with TMV in 2005. Healthy control seed were collected from healthy field grown K326 plants in 2005.
[5] Means within a column followed by the same letter are not significantly different at the 0.05 probability level using Duncan's multiple range test. The control treatment was excluded from the analysis of TMV positive.

Table A-4. Analysis of variance from the second of two growth chamber tests evaluating effectiveness of chemicals and treatment time on germination and seed transmission of tobacco mosaic virus (TMV) in flue-cured tobacco seedlings and seed coats.[1]

Source of variation	df	Mean Square (MS) Germination	df	MS Number	MS Weight	df	MS TMV positive[2]
Seedlings[3]							
Rep	2	30	2	38	0.000101**	2	7,604
Chemical (C)	7	3,957**	7	49*	0.000050*	6	23,250**
Exposure (E)	2	235	2	5	0.000032	2	448
C x E	14	155*	12	13	0.000007	10	1,769
Error	46	80	98	18	0.000018	83	1,017
Seed coats							
Rep	…	…	2	23	0.000004	2	159
Chemical (C)	…	…	7	61	0.000002	6	159
Exposure (E)	…	…	2	9	0.000002	2	159
C x E	…	…	14	16	0.000001	12	159
Error	…	…	46	17	0.000002	40	159

*,** Significant at the 0.05 and 0.01 level of probability, respectively.

[1] The test was conducted in a growth chamber using a randomized complete block design with three replications in 2006.

[2] Healthy control data deleted prior to analysis.

[3] Seedlings and seed coats were separated 14 days after seeding and evaluated for TMV by ELISA. The seed were collected from field grown K326 infected with TMV in 2005. Healthy control seed were collected from healthy field grown K326 plants in 2005.

Table A-5. Means from the second of two growth chamber tests evaluating effectiveness of chemicals on germination and seed transmission of tobacco mosaic virus (TMV) in flue-cured tobacco seedlings and seed coats.[1]

Treatments	Mean germination (%)	Mean no. in sample	Mean Weight (mg)	No. positive / total[2]	TMV positive (%)[3]
Seedlings[4]					
10% bleach solution	61 bc	18 a	20 a	9/14	64 b
1% hydrochloric acid	63 bc	16 ab	18 a	0/17	0 c
10% trisodium phosphate	58 c	15 ab	18 a	1/17	6 d
0.1% potassium permanganate	57 c	14 b	20 a	15/18	83 a
120 g/L Carnation nonfat dry milk	61 bc	14 b	20 a	13/18	72 a
2% formaldehyde	10 b	14 ab	13 b	1/3	33 b
deionized water infected control	68 b	15 ab	22 a	16/17	94 a
deionized water healthy control	82 a	18 ab	22 a	0/18	0
Seed coats					
10% bleach solution	…	41 c	5 a	9/9	100 a
1% hydrochloric acid	…	46 ab	4 a	8/9	89 a
10% trisodium phosphate	…	46 ab	6 a	9/9	100 a
0.1% potassium permanganate	…	42 bc	5 a	9/9	100 a
120 g/L Carnation nonfat dry milk	…	42 bc	6 a	9/9	100 a
2% formaldehyde	…	49 a	5 a	9/9	100 a
deionized water infected control	…	44 bc	6 a	9/9	100 a
deionized water healthy control	…	46 ab	7 a	1/9	11

[1] The test was conducted in a growth chamber using a randomized complete block design with three replications in 2006.
[2] No. positive/total is the ratio of positive immunoassays to the total number of seedlings or seed coats in the sample immunoassays.
[3] Data presented are untransformed but Duncan's multiple range test data are from transformed analysis.
[4] Seedlings and seed coats were separated 14 days after seeding and evaluated for TMV by ELISA. The seed were collected from field grown K326 infected with TMV in 2005. Healthy control seed were collected from healthy field grown K326 plants in 2005.
[5] Means within a column followed by the same letter are not significantly different at the 0.05 probability level using Duncan's multiple range test. The control treatment was excluded from the analysis of TMV positive.

Table A-6. Means from the second of two growth chamber tests evaluating effectiveness of treatment time on germination and seed transmission of tobacco mosaic virus (TMV) in flue-cured tobacco seedlings and seed coats.[1]

Exposure (min)	Germination (%)	No. in sample	Weight (mg)	No. positive / total[2]	TMV positive[3] (%)
Seedlings[4]					
30	61 a[5]	16 a	19 a	21/38	55 a
60	57 ab	15 a	20 ab	18/33	51 a
120	54 b	15 a	21 a	18/31	52 a
Seed coats					
30	…	44 a	4 a	21/21	100 a
60	…	45 a	4 a	21/21	100 a
120	…	44 a	5 a	20/21	95 a

[1] The test was conducted in a growth chamber using a randomized complete block design with three replications in 2006.
[2] No. positive/total is the ratio of positive immunoassays to the total number of seedlings or seed coats in the sample immunoassays.
[3] Data presented are untransformed but Duncan's multiple range test data are from transformed analysis.
[4] Seedlings and seed coats were separated 14 days after seeding and evaluated for TMV by ELISA. The seed were collected from field grown K326 infected with TMV in 2005. Healthy control seed were collected from healthy field grown K326 plants in 2005.
[5] Means within a column followed by the same letter are not significantly different at the 0.05 probability level using Duncan's multiple range test. The control treatment was excluded from the analysis of TMV positive.

Table A-7. Analysis of variance from the first of two growth chamber tests evaluating effectiveness of selected chemicals and treatment time combinations on germination and seed transmission of tobacco mosaic virus (TMV) in flue-cured tobacco seedlings and seed coats.[1]

Source of variation	df	Mean Square (MS) Germination	df	MS Number	MS Weight	df	MS TMV positive[2]
Seedlings[3]							
Rep	2	61	2	5	0.000121	2	2,879
Chemical (C)	10	313**	10	4	0.000034	9	4,043
Error	20	37	47	7	0.000016	42	2,112
Seed coats							
Rep	…	…	2	28	0.000025*	2	0
Chemical (C)	…	…	10	23	0.000003	9	0
Error	…	…	20	10	0.000004	18	0

*,** Significant at the 0.05 and 0.01 level of probability, respectively.

[1] The test was conducted in a growth chamber using a randomized complete block design with three replications in 2006.

[2] Healthy control data deleted prior to analysis.

[3] Seedlings and seed coats were separated 14 days after seeding and evaluated for TMV by ELISA. The seed were collected from field grown K326 infected with TMV in 2006. Healthy control plants were collected from healthy field grown K326 plants in 2006.

Table A-8. Means from the first of two growth chamber tests evaluating effectiveness of selected chemicals and treatment time combinations on germination and seed transmission of tobacco mosaic virus (TMV) in flue-cured tobacco seedlings and seed coats.[1]

Treatments	Germination (%)	No. in sample	Weight (mg)	No. positive / total[2]	TMV positive[3] (%)
Seedlings[4]					
10% bleach for 30 min	65 de	9 a	18 abc	3/6	50 ab
20% bleach for 10 min	68 cd	10 a	17 bc	3/5	60 ab
30% bleach for 10 min	77 bcd	12 a	23 ab	3/6	50 ab
1% hydrochloric acid for 30 min	55 e	12 a	16 c	2/3	67 ab
0.2% hydrochloric acid for 10 min	78 bc	11 a	22 ab	2/6	33 ab
0.5% hydrochloric acid for 10 min	84 ab	11 a	20 abc	1/6	83 a
1% hydrochloric acid for 10 min	67 cd	12 a	16 c	3/4	75 a
10% trisodium phosphate for 30 min	73 bcd	11 a	19 abc	1/6	17 ab
15% trisodium phosphate for 10 min	81 b	11 a	20 abc	1/6	17 ab
20% trisodium phosphate for 10 min	73 bcd	12 a	18 abc	0/6	0 b
Deionized water healthy control for 30 min	93 a	12 a	23 a	0/6	0
Seed coats					
10% bleach for 30 min	…	44 abc	10 a	3/3	100 a
20% bleach for 10 min	…	44 abc	8 a	3/3	100 a
30% bleach for 10 min	…	46 abc	9 a	3/3	100 a
1% hydrochloric acid for 30 min	…	40 c	8 a	3/3	100 a
1% hydrochloric acid for 10 min	…	48 a	10 a	3/3	100 a
0.2% hydrochloric acid for 10 min	…	47 ab	10 a	3/3	100 a
0.5% hydrochloric acid for 10 min	…	41 bc	7 a	3/3	100 a
10% trisodium phosphate for 30 min	…	45 abc	8 a	3/3	100 a
15% trisodium phosphate for 10 min	…	47 ab	10 a	3/3	100 a
20% trisodium phosphate for 10 min	…	44abc	9 a	3/3	100 a
Deionized water healthy control for 30 min	…	49 a	8 a	0/3	0

[1] The test was conducted in a growth chamber using a randomized complete block design with three replications in 2006.
[2] No. positive/total is the ratio of positive immunoassays to the total number of seedlings or seed coats in the samples immunoassays.

[3] Data presented are untransformed but Duncan's multiple range test data are from transformed analysis.
[4] Seedlings and seed coats were separated 14 days after seeding and evaluated for TMV by ELISA. The seed were collected from field grown K326 infected with TMV in 2006. Healthy control seed were collected from healthy field grown K326 plants in 2006.
[5] Means within a column followed by the same letter are not significantly different at the 0.05 probability level using Duncan's mean separation. The control treatment was excluded from the analysis of TMV positive.

Table A-9. Analysis of variance from the second of two growth chamber tests evaluating effectiveness of selected chemicals and treatment time combinations on germination and seed transmission of tobacco mosaic virus (TMV) in flue-cured tobacco seedlings and seed coats.[1]

Source of variation	df	Mean Square (MS) Germination	df	MS Number	MS Weight	df	MS TMV positive[2]
Seedlings[3]							
Rep	2	7	2	11	0.000002	2	1,683
Chemical (C)	10	63	10	22	0.000015	9	1,674
Error	20	70	35	53	0.000040	31	2,799
Seed coats							
Rep	…	…	2	2	0.000004	2	0
Chemical (C)	…	…	10	16	0.000003	9	0
Error	…	…	20	9	0.000002	18	0

*,** Significant at the 0.05 and 0.01 level of probability, respectively.

[1] The test was conducted in a growth chamber using a randomized complete block design with three replications in 2006.

[2] Healthy control data deleted prior to analysis.

[3] Seedlings and seed coats were separated 14 days after seeding and evaluated for TMV by ELISA. The seed were collected from field grown K326 infected with TMV in 2006. Healthy control plants were collected from healthy field grown K326 plants in 2006.

Table A-10. Means from the second of two growth chamber tests evaluating effectiveness of selected chemicals and treatment time combinations on germination and seed transmission of tobacco mosaic virus (TMV) in flue-cured tobacco seedlings and seed coats.[1]

Treatments	Germination (%)	No. in sample	Weight (mg)	No. positive / total[2]	TMV positive[3] (%)
Seedlings[4]					
10% bleach for 30 min	83 ab	15 a	14 a	3/5	60 a
20% bleach for 10 min	83 ab	19 a	17 a	4/4	100 a
30% bleach for 10 min	83 ab	14 a	14 a	3/5	60 a
1% hydrochloric acid for 30 min	74 b	15 a	14 a	1/3	33 a
0.2% hydrochloric acid for 10 min	88 ab	15 a	13 a	2/4	67 a
0.5% hydrochloric acid for 10 min	79 ab	12 a	13 a	3/5	60 a
1% hydrochloric acid for 10 min	79 ab	13 a	11 a	4/6	50 a
10% trisodium phosphate for 30 min	81 ab	17 a	14 a	1/3	33 a
15% trisodium phosphate for 10 min	83 ab	14 a	15 a	2/4	50 a
20% trisodium phosphate for 10 min	91 a	18 a	19 a	1/4	25 a
Deionized water healthy control for 30 min	83 ab	15 a	15 a	0/5	0
Seed coats					
10% bleach for 30 min	…	44 ab	4 b	3/3	100 a
20% bleach for 10 min	…	44 ab	4 ab	3/3	100 a
30% bleach for 10 min	…	42 b	3 b	3/3	100 a
1% hydrochloric acid for 30 min	…	45 ab	5 ab	3/3	100 a
1% hydrochloric acid for 10 min	…	48 ab	7 a	3/3	100 a
0.2% hydrochloric acid for 10 min	…	48 ab	6 ab	3/3	100 a
0.5% hydrochloric acid for 10 min	…	45 ab	5 ab	3/3	100 a
10% trisodium phosphate for 30 min	…	48 ab	5 ab	3/3	100 a
15% trisodium phosphate for 10 min	…	49 a	5 ab	3/3	100 a
20% trisodium phosphate for 10 min	…	46 ab	4 b	3/3	100 a
Deionized water healthy control for 30 min	…	49 a	6 ab	0/3	0

[1] The test was conducted in a growth chamber using a randomized complete block design with three replications in 2006.
[2] No. positive/total is the ratio of positive immunoassays to the total number of seedlings or seed coats in the samples immunoassays.

[3] Data presented is untransformed but Duncan's multiple range test data are from transformed analysis.
[4] Seedlings and seed coats were separated 14 days after seeding and evaluated for TMV by ELISA. The seed were collected from field grown K326 infected with TMV in 2006. Healthy control seed were collected from healthy field grown K326 plants in 2006.
[5] Means within a column followed by the same letter are not significantly different at the 0.05 probability level using Duncan's multiple range test. The control treatment was excluded from the analysis of TMV positive.

Table A-11. Analysis of variance for the first of three greenhouse tests investigating distribution of tobacco mosaic virus (TMV) in plant tissue and the nutrient solution.[1]

Source of variation	df	Mean Square (MS) TMV positive[2]	MS Absorbance	MS Infectivity[2]	MS Local lesion no.
Plant tissues[3]					
Rep	3	50	1*	0	771**
Treatment[4]	1	309,983**	294**	317,880**	14,138**
Error	155	50	0.3	0	96
Nutrient solution					
Rep	3	0	1	0	2
Treatment[4]	1	15,894**	26*	15,894**	36**
Error	3	0	0.4	0	2

*,** Significant at the 0.05 and 0.01 level of probability, respectively.

[1] Three tests were conducted in a greenhouse using a randomized complete block design with four replications in 2006. Plant tissue and nutrient solutions were collected 12 dpi, 28 DAS and evaluated for TMV by ELISA and infectivity assay on TMV resistant plants. The seed were collected from healthy, field grown K326 in 2006.

[2] Data presented are from arcsine transformed analysis.

[3] Plant tissues represent leaf and root samples combined.

[4] There were two inoculation treatments, which include inoculation with TMV and inoculation with buffer.

Table A-12. Means for the first of three greenhouse tests investigating distribution of tobacco mosaic virus (TMV) in plant tissue and the nutrient solution.[1]

Inoculation Treatment[2]	TMV positive[3] (%)	Absorbance (405 nm)	Infectivity[3] Assay	Local lesion no.
Plant tissues[4]				
TMV inoculated	100 a[5]	2.80 a	100 a	19 a
Buffer inoculated	0 b	0.14 b	0 b	0 b
Nutrient solution				
TMV inoculated	100 a	2.39 a	100 a	4 a
Buffer inoculated	0 b	0.08 b	0 b	0 b

[1] Tests were conducted in a greenhouse using a randomized complete block design with four replications in 2006. Plant tissue and nutrient solutions were collected 12 dpi, 28 DAS and evaluated for TMV by ELISA and infectivity assay on TMV resistant plants. The seed were collected from healthy, field grown K326 in 2006.
[2] There were two inoculation treatments, which include inoculation with TMV and inoculation with buffer.
[3] Data presented are untransformed but Duncan's multiple range test data are from arcsine transformed analysis.
[4] Plant tissues represent leaf and root samples combined.
[5] Means within a column followed by the same letter are not significantly different at the 0.05 probability level using Duncan's multiple range test.

Table A-13. Analysis of variance for the second of three greenhouse tests investigating distribution of tobacco mosaic virus (TMV) in plant tissue and the nutrient solution.[1]

Source of variation	df	Mean Square (MS) TMV positive[2]	MS Absorbance	MS Infectivity[2]	MS Local lesion no.
Plant tissues[3]					
Rep	3	50	0.4*	0	2,079**
Treatment[4]	1	309,983**	307**	317,880**	61,976**
Error	155	50	0.3	96	442
Nutrient solution					
Rep	3	0	1	0	7
Treatment[4]	1	15,894**	4	15,894**	45
Error	3	0	1	0	7

*,** Significant at the 0.05 and 0.01 level of probability, respectively.

[1] Three tests were conducted in a greenhouse using a randomized complete block design with four replications in 2006. Plant tissue and nutrient solutions were collected 12 dpi, 28 DAS and evaluated for TMV by ELISA and infectivity assay on TMV resistant plants. The seed were collected from healthy, field grown K326 in 2006.

[2] Data presented are from arcsine transformed analysis.

[3] Plant tissues represent leaf and root samples combined.

[4] There were two inoculation treatments, which include inoculation with TMV and inoculation with buffer.

Table A-14. Means for the second of three greenhouse tests investigating distribution of tobacco mosaic virus (TMV) in plant tissue and the nutrient solution.[1]

Inoculation Treatment[2]	TMV positive[3] (%)	Absorbance (405 nm)	Infectivity[3] Assay	Local lesion no.
Plant tissues[4]				
TMV inoculated	100 a[5]	2.92 a	100 a	39 a
Buffer inoculated	0 b	0.15 b	0 b	0 b
Nutrient solution				
TMV inoculated	100 a	1.60 a	100 a	5 a
Buffer inoculated	0 b	0.09 b	0 b	0 b

[1] Tests were conducted in a greenhouse using a randomized complete block design with four replications in 2006. Plant tissue and nutrient solutions were collected 12 dpi, 28 DAS and evaluated for TMV by ELISA and infectivity assay on TMV resistant plants. The seed were collected from healthy, field grown K326 in 2006.
[2] There were two inoculation treatments, which include inoculation with TMV and inoculation with buffer.
[3] Data presented are untransformed but Duncan's multiple range test data are from arcsine transformed analysis.
[4] Plant tissues represent leaf and root samples combined.
[5] Means within a column followed by the same letter are not significantly different at the 0.05 probability level using Duncan's multiple range test.

Table A-15. Analysis of variance for the third of three greenhouse tests investigating distribution of tobacco mosaic virus (TMV) in plant tissue and the nutrient solution.[1]

Source of variation	df	Mean Square (MS) TMV positive[2]	MS Absorbance	MS Infectivity[2]	MS Local lesion no.
Plant tissues[3]					
Rep	3	0	0.5*	0	18**
Treatment[4]	1	317,880**	255**	317,880**	740**
Error	155	0	0.3	0	9
Nutrient solution					
Rep	3	0	0.04	0	0.3
Treatment[4]	1	15,894**	0.37	15,894**	8*
Error	3	0	0.05	0	0.3

*,** Significant at the 0.05 and 0.01 level of probability, respectively.
[1] Three tests were conducted in a greenhouse using a randomized complete block design with four replications in 2006. Plant tissue and nutrient solutions were collected 12 dpi, 28 DAS and evaluated for TMV by ELISA and infectivity assay on TMV resistant plants. The seed were collected from healthy, field grown K326 in 2006.
[2] Data presented are from arcsine transformed analysis.
[3] Plant tissues represent leaf and root samples combined.
[4] There were two inoculation treatments, which include inoculation with TMV and inoculation with buffer.

Table A-16. Means for the third of three greenhouse tests investigating distribution of tobacco mosaic virus (TMV) in plant tissue and the nutrient solution.[1]

Inoculation Treatment[2]	TMV positive[3] (%)	Absorbance (405 nm)	Infectivity[3] Assay	Local lesion no.
Plant tissues[4]				
TMV inoculated	100 a[5]	2.64 a	100 a	4 a
Buffer inoculated	0 b	0.12 b	0 b	0 b
Nutrient solution				
TMV inoculated	100 a	0.52 a	100 a	2 a
Buffer inoculated	0 b	0.09 b	0 b	0 b

[1] Tests were conducted in a greenhouse using a randomized complete block design with four replications in 2006. Plant tissue and nutrient solutions were collected 12 dpi, 28 DAS and evaluated for TMV by ELISA and infectivity assay on TMV resistant plants. The seed were collected from healthy, field grown K326 in 2006.
[2] There were two inoculation treatments, which include inoculation with TMV and inoculation with buffer.
[3] Data presented are untransformed but Duncan's multiple range test data are from arcsine transformed analysis.
[4] Plant tissues represent leaf and root samples combined.
[5] Means within a column followed by the same letter are not significantly different at the 0.05 probability level using Duncan's multiple range test.

Table A-17. Analysis of variance for the first of three greenhouse tests evaluating distribution of tobacco mosaic virus (TMV) via seed transmission.[1]

Source of variation	df	Mean Square (MS) TMV positive[2]	MS Absorbance	MS Infectivity[2]	MS Local lesion no.
28 DAS[3]					
Rep[4]	3	0	0.0027*	0	0
Treatment	1	0	0.1875**	0	0
Error	155	0	0.0009	0	0
35 DAS					
Rep[4]	3	0	0.0004	0	0
Treatment	1	0	0.0022	0	0
Error	155	0	0.0008	0	0
42 DAS					
Rep[4]	3	0	0.0035**	0	0
Treatment	1	0	0.0001	0	0
Error	215	0	0.0001	0	0

*,** Significant at the 0.05 and 0.01 level of probability, respectively.

[1] Three tests were conducted in a greenhouse using a randomized complete block design with four replications in 2006.

[2] Data presented are from arcsine transformed analysis.

[3] Leaf samples were collected 28, 35, and 42 DAS and evaluated for TMV by ELISA and infectivity assay on TMV resistant plants. The seed were collected from healthy, field grown K326 in 2006.

[4] Treatments include seed collected from and infected source plant and seed collected from a healthy source plant.

Table A-18. Means for the first of three greenhouse tests evaluating distribution of tobacco mosaic virus (TMV) via seed transmission.[1]

Sample date[2]	TMV positive[3] (%)	Absorbance (405 nm)	Infectivity[3] Assay	Local lesion no.
28 DAS				
TMV source seed	0 a	0.029 b	0 a	0 a
Healthy source seed	0 a	0.098 a	0 a	0 a
35 DAS				
TMV source seed	0 a	0.103 a	0 a	0 a
Healthy source seed	0 a	0.095 a	0 a	0 a
42 DAS				
TMV source seed	0 a	0.154 a	0 a	0 a
Healthy source seed	0 a	0.153 a	0 a	0 a

[1] Tests were conducted in a greenhouse using a randomized complete block design with four replications in 2006. The seed used were collected from healthy, field grown K326 in 2006.
[2] Leaf samples were collected 28, 35, and 42 DAS and evaluated for TMV by ELISA and infectivity assay on TMV resistant plants.
[3] Data presented are untransformed but Duncan's multiple range test data are from arcsine transformed analysis.
[4] Means within a column followed by the same letter are not significantly different at the 0.05 probability level using Duncan's multiple range test.

Table A-19. Analysis of variance for the second of three greenhouse tests evaluating distribution of tobacco mosaic virus (TMV) via seed transmission.[1]

Source of variation	df	Mean Square (MS) TMV positive[2]	MS Absorbance	MS Infectivity[2]	MS Local lesion no.
28 DAS[3]					
Rep[4]	3	50	0.02248	50	0
Treatment	1	50	0.00332	50	0
Error	155	150	0.02034	150	0
35 DAS					
Rep[4]	3	0	0.00004	0	0
Treatment	1	0	0.00004	0	0
Error	155	0	0.00007	0	0
42 DAS					
Rep[4]	3	0	0.01411**	0	0
Treatment	1	0	0.00190	0	0
Error	203	0	0.00312	0	0

*,** Significant at the 0.05 and 0.01 level of probability, respectively.

[1] Three tests were conducted in a greenhouse using a randomized complete block design with four replications in 2006.

[2] Data presented are from arcsine transformed analysis.

[3] Leaf samples were collected 28, 35, and 42 DAS and evaluated for TMV by ELISA and infectivity assay on TMV resistant plants. The seed were collected from healthy, field grown K326 in 2006.

[4] Treatments include seed collected from and infected source plant and seed collected from a healthy source plant.

Table A-20. Means for the second of three greenhouse tests evaluating distribution of tobacco mosaic virus (TMV) via seed transmission.[1]

Sample date[2]	TMV positive[3] (%)	Absorbance (405 nm)	Infectivity[3] Assay	Local lesion no.
28 DAS				
TMV source seed	2 a	0.141 a	0.005a	0 a
Healthy source seed	1 a	0.132 a	1.255 a	0 a
35 DAS				
TMV source seed	0 a	0.071 a	0 a	0 a
Healthy source seed	0 a	0.070 a	0 a	0 a
42 DAS				
TMV source seed	0 a	0.128 a	0 a	0 a
Healthy source seed	0 a	0.138 a	0 a	0 a

[1] Tests were conducted in a greenhouse using a randomized complete block design with four replications in 2006. The seed used were collected from healthy, field grown K326 in 2006.
[2] Leaf samples were collected 28, 35, and 42 DAS and evaluated for TMV by ELISA and infectivity assay on TMV resistant plants.
[3] Data presented are untransformed but Duncan's multiple range test data are from arcsine transformed analysis.
[4] Means within a column followed by the same letter are not significantly different at the 0.05 probability level using Duncan's multiple range test.

Table A-21. Analysis of variance for the third of three greenhouse tests evaluating distribution of tobacco mosaic virus (TMV) via seed transmission.[1]

Source of variation	df	Mean Square (MS) TMV positive[2]	MS Absorbance	MS Infectivity[2]	MS Local lesion no.
28 DAS[3]					
Rep[4]	3	0	0.0158**	0	0
Treatment	1	0	0.1503**	0	0
Error	3	0	0.0032	0	0
35 DAS					
Rep[4]	3	0	0.0010**	0	0
Treatment	1	0	0.0043**	0	0
Error	155	0	0.0001	0	0
42 DAS					
Rep[4]	3	0	0.0019**	0	0
Treatment	1	0	0.0015*	0	0
Error	192	0	0.0002	0	0

*,** Significant at the 0.05 and 0.01 level of probability, respectively.

[1] Three tests were conducted in a greenhouse using a randomized complete block design with four replications in 2006.

[2] Data presented are from arcsine transformed analysis.

[3] Leaf samples were collected 28, 35, and 42 DAS and evaluated for TMV by ELISA and infectivity assay on TMV resistant plants. The seed were collected from healthy, field grown K326 in 2006.

[4] Treatments include seed collected from and infected source plant and seed collected from a healthy source plant.

Table A-22. Means for the third of three greenhouse tests evaluating distribution of tobacco mosaic virus (TMV) via seed transmission.[1]

Sample date[2]	TMV positive[3] (%)	Absorbance (405 nm)	Infectivity[3] Assay	Local lesion no.
28 DAS				
TMV source seed	0 a	0.072 a	0 a	0 a
Healthy source seed	0 a	0.011 b	0 a	0 a
35 DAS				
TMV source seed	0 a	0.096 a	0 a	0 a
Healthy source seed	0 a	0.085 a	0 a	0 a
42 DAS				
TMV source seed	0 a	0.108 a	0 a	0 a
Healthy source seed	0 a	0.099 b	0 a	0 a

[1] Tests were conducted in a greenhouse using a randomized complete block design with four replications in 2006. The seed used were collected from healthy, field grown K326 in 2006.
[2] Leaf samples were collected 28, 35, and 42 DAS and evaluated for TMV by ELISA and infectivity assay on TMV resistant plants.
[3] Data presented are untransformed but Duncan's multiple range test data are from arcsine transformed analysis.
[4] Means within a column followed by the same letter are not significantly different at the 0.05 probability level using Duncan's multiple range test.

Vita

Rachel Ellen Leonard Loveday was born January 18, 1981 to Jimmie H. and Linda K. Leonard in Farmville, Virginia. She attended Nottoway County Public Schools and graduated from Nottoway County High School in 1999. She enrolled at Southside Virginia Community College, John H. Daniel Campus, as a dual-enrolled high school student in 1997 and received an Associate of Art and Science in May of 2001. She transferred to Longwood University in 2001 and graduated with a Bachelor of Science in Biology in May of 2003. She enrolled in the graduate school of Virginia Polytechnic Institute and State University to pursue a Master of Science in Crop and Soil Environmental Science under the direction of Dr. Carol Wilkinson at the Southern Piedmont Agriculture Research and Extension Center, located in Blackstone, Virginia and Dr. Sue Tolin at Virginia Polytechnic Institute and State University in Blacksburg, Virginia.