Tracking *Tobacco Mosaic Virus* Infection from Infected Seeds to Seedlings Confirms Seed Transmission in Tobacco (*Nicotiana tabacum* L.)

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Abstract

The *Tobacco mosaic virus* (TMV) is a positive sense single stranded RNA virus and is found across the world. TMV can impact the overall yield and quality of the crop resulting in an economic loss. Plants that are infected with TMV show a variety of symptoms such as mosaic pattern, mottling, necrotic lesions and stunted growth. Historically, TMV has caused controversy on whether this economically significant virus is seedborne or seed transmitted. The objective of this study is to track TMV infection from infected seeds to seedlings to determine the percentage of seed transmission. This experiment used three pods from three different TMV infected cultivar K 326 flue-cured tobacco plants. Seeds from each pod were germinated in a growth chamber for approximately ten days. Samples were separated into seed coat, root and leaves after germination. Total RNA was extracted from each part and synthesized into cDNA for analysis. A quantitative real-time PCR (RT-qPCR) assay was used to determine TMV concentration of each sample. Endpoint RT-PCR was used to determine a conservative threshold value from the RT-qPCR results. These results demonstrated that TMV influenced percent germination with a range from 94% to 50%. Seed coats had a significantly higher virus titer concentration ($P < 0.05$) when compared to the roots and leaves. Statistical analysis revealed highly significant ($P < 0.0001$) differences among pods for virus titer and there is a highly significant plant by pod interaction ($P < 0.0001$). Endpoint RT-PCR confirmed TMV infection in leaves, roots and seed coats. Percent infection in leaves ranged from 2% to 24% and percent infection for roots ranged from 8% to 40%. Results demonstrate that TMV is seed transmitted in flue-cured tobacco.

Tracking *Tobacco Mosaic Virus* Infection from Infected Seeds to Seedlings Confirms Seed Transmission in Tobacco (*Nicotiana tabacum* L.)

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General Audience Abstract

The *Tobacco mosaic virus* (TMV) is an RNA virus that occurs globally in areas where tobacco is grown. TMV is a tobamovirus and infects over 350 plant species. TMV can reduce the yield and quality of the crop which will result in an economic loss for the grower. Plants that are infected with TMV show a variety of symptoms such as mosaic pattern, necrotic lesions, and stunted growth, and there are no effective ways to eradicate the virus. There has been controversy on whether to categorize TMV as a seedborne virus or a seed-transmitted virus because the location of the virus within a seed is unknown. This study examined seeds from three pods grown on three different TMV-infected flue-cured tobacco plants of cultivar K 326 to track TMV infection from infected seeds to seedlings. Seeds from each pod were germinated in a growth chamber for ten days and samples were separated into leaves, root and seed coat. Each sample had total RNA extracted and synthesized into cDNA for analysis. A quantitative real-time PCR (RT-qPCR) assay was used to determine TMV concentration of each sample since this technology can detect small amounts of virus. Endpoint RT-PCR was used to conservatively determine an infection threshold value from the RT-qPCR results. Percent germination of TMV infected seeds ranged from 94% to 50%. Seed coats had a significantly higher virus titer ($P < 0.05$) when compared to the roots and leaves in each pod. Statistical analysis showed ($P < 0.0001$) differences among pods for virus titer and there is a highly significant plant by pod interaction ($P < 0.0001$). Endpoint RT-PCR confirmed TMV infection in leaves, roots and seed coats. Percent infection in

leaves ranged from 2% to 24% and percent infection for roots ranged from 8% to 40%.

Therefore, results show that TMV is seed-transmitted in flue-cured tobacco.

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Chapter 1: Literature Review

Introduction

Tobacco mosaic virus (TMV) was the first virus discovered over a century ago. TMV is structurally a simple virus and has been a paradigm for numerous studies over the years (Heinlein 2002). The TMV particle has a rigid rod-shaped structure and TMV's genetic material is a single, positive-sense strand of RNA. TMV is a tobamovirus that can infect over 350 species and tobacco is the main plant infected by this virus (Scholthof 2004). TMV does not require vectors to transmit the virus, TMV is dependent on mechanical transmission by physical contact between damaged plant cells and a contaminated surface (Heinlein 2002). Primary infection of TMV occurs when the plant is infected from a source of inoculum such as workers or from overwintering of the virus. TMV spreads from primary infected plants to other plants in the field and this is known as secondary spread.

Yield and quality of tobacco is negatively impacted by TMV and results in an economic loss. Plants infected with TMV earlier in the growing season have a greater negative effect. This virus is of economic importance in both field and greenhouse production (Losenge et al. 2012). TMV will affect the quality of a cured leaf by making the leaf thinner and less durable; which reduces the average price for the crop (Williams-Woodward 2000). TMV has been difficult to control and eradicate due to the virus being highly stable. There are a variety of preventative measures producers can use to minimize transmission and resistance to TMV has been incorporated into many cultivars, especially in burley tobacco.

Past research used serological assays such as ELISA to detect TMV, but these technologies were not sensitive enough to detect small amounts of virus in small samples. This lack of sensitivity led to controversy on whether to classify TMV as a seedborne or seed-

transmitted virus because there is an inconsistency in the literature on the location of the virus in the seed and if it infects the seedlings. Therefore, a more sensitive technique to detect TMV in a sample with limited amount of RNA is needed. The objective of this study is to establish the rate of transmission for TMV from a seed to a seedling by using RT-qPCR.

TMV Structure and Assembly

The *Tobacco mosaic virus* (TMV) is a positive-sense rod-shaped virus and is a helical array of identical protein subunits embedded in a single molecule of RNA (Klug 1999). The virus particle measures 300 nm x 18 nm with an axial radius of 2 nm. TMV contains a genomic single strand RNA that is 6,400 bases long encapsidated in a helical coat of 2,160 subunits of coat protein (Namba and Stubbs 1986; Heinlein 2002). RNA is capped at the 5' end and will fold into a histidine-accepting transfer RNA structure at the 3' end (Heinlein 2002). TMV encodes four functional proteins that will result in successful infection. The 126- and 183- kDa proteins are known as the replicase proteins and enable viral replication from the RNA genome (Liu and Nelson 2013). There is a 30-kDa movement protein (MP) that allows the virus to move from cell to cell. The last protein is a 17.5-kDa coat protein (CP) that will express and encapsidate the single strand genome and allow it to be systemically transmitted (Liu and Nelson 2013).

Infection, Replication and Movement

TMV is mechanically transmitted by physical contact of plant tissue with contaminated surfaces or infected plant tissue (Heinlein 2002). TMV can enter the plant through openings in the cell wall and plasma membrane, or through pinocytosis after mechanical wounds (Liu and Nelson 2013). Once TMV enters the cell the CP is disassembled within seconds by cotranslational disassembly. Replicase proteins are immediately translated, initiating replication of the viral genome. The virus uses its parental genome to synthesize a negative strand copy of the viral RNA that will serve as a template for synthesis of progeny full length positive strands (Heinlein 2002; Liu and Nelson 2013). The capsid releases TMV RNA at the site of viral RNA (vRNA) granule formation that are associated with the endoplasmic reticulum (ER). ER is the site for virus replication complex (VRC) where VRC is formed in the cortical vertices or

perinuclear regions of the ER. VRCs are known to have vRNA, MP, replication proteins and host proteins (Liu and Nelson 2013). A membrane protein holds together the replication proteins and the ER. VRCs can move intercellularly from replication sites to the plasmodesmata (PD). MP associates with PD and increases the size exclusion limit to initiate intercellular transfer of the RNA genome. vRNA is transported to release the vRNA for translation in the next cell. The virus can move within a cell to establish an infection site, multiply and move to the next cell (Liu and Nelson 2013).

Symptoms

The main symptom of TMV is a two-toned discoloration of the tobacco leaf consisting of light and dark green areas and is referred to as a mosaic pattern (Gooding 1986). TMV can reduce the production of chlorophyll which results in the discoloration of the leaf. Other symptoms such as leaf distortion, necrotic local lesions, and stunted growth may also occur (Scholthof 2004). Symptoms will vary for each plant and are dependent on age, environmental conditions, and type of plant. TMV will reduce crop yield and quality of the leaf due to discoloration and distortion. Valleau and Johnson (1927) reported a 60% reduction in crop value caused by early mosaic infection. McMurtrey (1929) showed that TMV caused an average yield reduction of 30% to 35% when crops were infected earlier in the growth of the plant and the crop's gross value decreased by 50%. Wolf and Moss (1933) observed a higher yield loss (31%) when plants were inoculated with TMV at transplanting compared to being inoculated at topping (17%). Chaplin (1964) observed similar results and reported a yield loss of 20% when plants were infected during transplanting whereas plants infected after topping did not impact yield. In 2000, Georgia reported a 4.5% reduction in crop value due to TMV that was equivalent to $5.42 million dollars in damage (Williams-Woodward 2000). Most of the losses in Georgia were

associated with specific cultivars of plants grown in contaminated greenhouses. TMV is an economically significant virus that impacts growers.

Primary and Secondary Infection

TMV is mechanically transmitted through mechanical wounds or micro-abrasions on the host plant tissue that allows the virus to enter injured cells (Liu and Nelson 2013; Mink 1993; and Scholthof 2004). Both primary infection and secondary infection occurs with TMV. Primary infection of TMV is the initial spread of the virus from the first site of infection. Primary infection usually impacts <1% of the plants in a field. Subsequent spread of the virus to healthy plants is known as secondary spread (Gooding 1986). When plants in a field (<1%) are infected, especially in the early growth stages, TMV can significantly negatively impact all other plants in the field. TMV related field epidemics are a result of a primary infected plant spreading the virus in field production. That is why it is crucial for farmers to know the source of inoculum for primary infection.

Primary infection can occur from a variety of sources of inoculum. The main source of primary infection is from contaminated hands, tools or equipment coming into contact with a healthy plant (Henn 2016). Contaminated workers are the main source of primary infection, and Losenge et al. (2012) found that TMV transmission from contaminated clothing material such as latex, cotton or high-density polyethylene fiber can result in TMV infection of 35% to 57% if contaminated. Another source of primary infection is overwintering of TMV in infected plant material remaining in the soil, on contaminated seeds, and even in tobacco products. TMV remains infectious in the soil up to 20 months after removal of infected plant material (Sastry 2013). TMV can reside in weeds in a field and be a source of inoculum for the next crop year (Gooding 1986). Mechanical transmission from a contaminated surface to a healthy host is the

main cause of transmission during field epidemics. Secondary spread can occur when the leaves from an infected plant rub against leaves from a healthy plant causing micro-abrasions to the surface for the virus to enter. Equipment and tools could also cause secondary spread by spreading contaminated sap from a primary infected plant across a field.

Seed Transmission

Seed transmitted viruses are classified into two categories based on where the virus is located within the seed and how the virus infects the seedling. The location of a virus in a seed could influence the transmissibility of that virus, especially if the virus is in the embryo (de Assis Filho and Sherwood 2000). A virus present in the seed but not transmitted to the seedling is known as a seedborne virus. Most seedborne viruses are located on or within the seed coat. A virus that is found in the embryo and infects the seedling is known as a seed-transmitted virus (de Assis Filho and Sherwood 2000). There has been controversy on whether to classify TMV as a seedborne or seed-transmitted virus because the exact location of the virus in a seed is unknown. Tobacco seeds are egg-shaped with a prominent raphe along the side with a projecting hilum (Avery 1933). They are dark brown in color with a reticulated surface. Tobacco seeds are extremely small and average 0.75 mm long, 0.53 mm wide and 0.47 mm thick. The outer portion of the seed is a protective layer known as the seed coat. Underneath the seed coat is the endosperm which is made of three to five layers of cells. The embryo is housed within the endosperm (Avery 1933). Seed will germinate in six to eight days after planting. During germination the primary root applies pressure to the seed coat and breaks through (Avery 1933).

Taylor (1962) attempted to locate the virus in the seed but was unable to due to difficulty separating the endosperm from the embryo. He found a 2.8% TMV infection in the seed coat and approximately a 10% infection for the endosperm and embryo. He reported that the endosperm

has the potential to carry TMV, but the embryo is not infected and only seed-transmitted viruses are able to infect and survive in the embryo (Taylor 1962). Gooding (1969) classified TMV as a seedborne virus since there were no signs of mosaic symptoms on the seedlings even though he was able to recover active virus from the seed. Benoit and Maury (1976) used a fluorescent antibody technique to show that TMV was found in the nucellar layer of the seed coat of an infected tobacco seed but there was no evidence of the virus in the embryo. They reported that TMV is present only in the seed coat with no evidence of TMV being present in the endosperm contrary to what was reported by Taylor (1962). They concluded that seed transmission depends on virus-host interactions, not just location of the virus in a seed (Benoit and Maury 1976). Mink (1993) reported TMV as being the only virus that does not infect the embryo but is classified as a seed-transmitted virus since the virus on the surface of the seed can infect the seedling. This is a rare occurrence and it is believed that since TMV is a stable virus TMV can remain viable on the seed surface and seedling infection occurs through mechanical transmission (Johansen et al. 1994). Seed transmission is crucial for the survival of the virus from season to season (Ali and Kobayashi 2010). Even low transmission rates of a virus can be vital for virus perpetuation, overwintering, and dissemination (Ali and Kobayashi 2010).

Resistance

Plants can limit the infection of pathogenic microbes by induction of localized cell death or hypersensitive response (HR) at the site of infection (Marathe et al. 2002). This response is dependent on the virus's avirulence (Avr) gene being recognized by the plant's resistance (R) gene. In tobacco, the R gene has been cloned to confer resistance to the *Tobacco mosaic virus* and is known as the N gene (Marathe et al. 2002). Holmes (1938) showed a single dominant gene in *Nicotiana glutinosa* L. controlled the necrotic response to TMV infection and referred to

it as the N gene for necrotic-type response to TMV. *Nicotiana glutinosa* plants that were infected with TMV and contained the N gene induced HR within 48 h at the site of infection and limited the spread of the virus to the necrotic spot (Holmes 1934). When Clausen and Goodspeed (1925) created a *N. glutinosa-tabacum* hybrid, *N. digluta*, Holmes (1938) crossed this new species with *N. tabacum* to develop a resistant line with the N gene. Valleau (1952) was able to use *N. digluta* to develop burley and dark fired-cured resistant tobacco cultivars. Legg et al. (1979) confirmed that the N gene had been successfully transferred to burley tobacco and the N gene did not impact yield or quality. There has been difficulty developing a commercially viable TMV-resistant inbred flue-cured tobacco cultivar because there have been reductions in yield and quality when the N gene is incorporated into flue-cured tobacco. Chaplin and Mann (1978) tested the TMV resistance derived from the Burley 21 cultivars which has the *N. glutinosa* form of resistance and reported reduced yield and off quality of TMV resistant plants. TMV resistant plants had a significantly reduced grade index due to the discoloration, woody texture in the leaves and thickness of a cured leaf (Chaplin and Mann 1978). Lewis et al. (2007) reported linkage drag effects contributed to effects on yield in TMV-resistant flue-cured tobacco due to the presence of unfavorable genes of *N. glutinosa* origin linked to *N. tabacum* (Lewis et al. 2007).

Another form of resistance involves the expression of coat protein genes known as coat protein-mediated resistance (CP-MR) (Beachy et al. 1990). CP-MR is the resistance caused by the expression of a virus CP gene in a transgenic plant and accumulation of the CP confers resistance to infection of the virus that the CP was derived from. Abel et al. (1986) used transgenic *Nicotiana tabacum* cv., Xanthi, that expressed TMV CP gene and found that seedlings infected with TMV had delayed symptom development and 10% to 60% of the transgenic plants

failed to develop any symptoms. Their results showed that plants can be genetically transformed for viral resistance. Register and Beachy (1988) reported late disease symptom development of TMV infected transgenic tobacco plants that expressed the TMV CP. This protection was observed in leaf mesophyll protoplasts, but these protoplasts were resistant to lower TMV concentrations. They reported little protection against TMV infection when TMV RNA was used as an inoculum (Register and Beachy 1988).

Although the N gene provides TMV resistance with little to no negative impacts on yield or quality of burley and some flue-cured hybrid varieties, the N gene has not been successfully implemented into inbred lines of flue-cured tobacco. Also, CP-MR results showed that there is limited protection against TMV infection based on the source of inoculum. Therefore, tobacco producers have to implement the use of resistant cultivars and control measures to reduce TMV infection.

Control

The *Tobacco mosaic virus* has been difficult to control and eradicate due to the virus being highly stable. Steam has been used to inactivate TMV in roots at high temperatures (90-95°C) but this treatment would be difficult to apply in a field setting (Broadbent et al. 1965). Inosine monophosphate dehydrogenase (IMPDH) chemicals have shown permeation of the chemical through the plant cell, but this treatment is economically impractical (Luvisi et al. 2017). The use of the antiviral agent ningnanmycin showed curative percentages of 30% to 60% in TMV infected leaves but these results were based on lab tests using the half leaf method (Ouyang et al. 2008). Therefore, a chemical approach to eradicating TMV is not an effective control measure based on current chemical treatments (Luvisi et al. 2017).

There are preventative measures farmers can take to avoid TMV infection in the field. Sanitation is a major contributor to preventing the spread of a virus (Henn 2016). One sanitation method is workers washing hands thoroughly between fields and greenhouses. Another method is washing equipment with a diluted (10%) bleach (sodium hypochlorite) solution to inactivate any virus that has contaminated the surface (Henn 2016). Lastly, worker's clothes can be cleaned with non-fat powdered milk and two concentrations of detergent to prevent TMV transmission (Losenge et al. 2012). Milk has also been used to wash workers hands during transplanting to avoid contamination with TMV.

Another preventative measure for TMV is keeping the field free of plants that may have the virus (Henn 2016). Primary infection occurs when the pathogen infects a plant for the first time. This usually happens once during a growing season. Whereas, secondary spread occurs after primary infection and results in rapid distribution of the virus in the field. This can happen multiple times in a growing season. The most effective way to prevent secondary spread is to prevent primary infection in a field by eliminating any source of inoculum (Gooding 1969). It is crucial for a grower to remove plants that exhibit symptoms of TMV to avoid secondary spread. Plants such as tobacco and various weeds can serve as a host for TMV and transmit TMV across a field. Contamination of a field with TMV can be extremely difficult to manage due to the ability of a plant to infect others (Gulser et al. 2008). Crop rotation of plants that are not a host for TMV in alternate years can reduce the amount of virus in the soil (Gulser et al. 2008). The virus can remain infectious in dead, dried tissue for years. It is essential that roots and stalks are removed from an infected field to prevent the virus from overwintering.

There are TMV resistant cultivars of tobacco. These cultivars would be beneficial to farmers who have high losses due to TMV. N gene resistance was successfully transferred to

burley tobacco with no deleterious agronomic effects. Resistant varieties can be used to break the cycle of virus carry-over (Scholthof 2004).

TMV Detection

Visual observation of TMV symptoms is a traditional way of detecting the virus in a field. This requires the ability to identify plants that exhibit a mosaic pattern or necrotic lesions on the leaves. However, young seedlings do not always display mosaic symptoms early in the growing cycle. It would be difficult to monitor an outbreak of TMV when not all plants are showing symptoms of the virus.

Serology-based assays such as ImmunoStrips (Agdia, Elkhart, IN) or enzyme-linked immunosorbent assays (ELISA) have been used to detect TMV. ImmunoStrips are an easy and rapid method to confirm TMV in the field. The test works by recognizing antigens that are specific to TMV. Typically, one red line means negative for TMV and two red lines means positive. These diagnostic tests are great for an onsite test for TMV, but if a plant has a low virus titer, this method may show a negative result. Also, this method can only give a qualitative result and there is no way to quantify the amount of virus in a sample.

ELISA has been the more dominant serological assay for detecting TMV in previous research. ELISA works by using antigen-antibody interactions that are specific to TMV. The ELISA will result in a colored end product that correlates to the amount of virus in the sample. ELISA data can be used to quantitatively calculate the concentrations of the specific antigen in samples. ELISA is used to determine infection of a seed, but the ELISA cannot differentiate seedborne from seed-transmitted viruses (de Assis Filho and Sherwood 2000). Yang et al. (2012) demonstrated sensitivity of ELISA by testing for a detection limit of TMV in serially diluted infected leaf sap from $1:10^1$ to $1:10^6$ dilutions, and results showed ELISA detected TMV up to

1:10^3 dilution. This result showed that ELISA lacked the sensitivity to detect low TMV concentrations. de Assis Filho and Sherwood (2000) used PAS-ELISA to assay individual seeds from TMV infected *Arabidopsis thaliana* L. and results indicated that the seed was healthy. They also performed a grow-out test with infected seed and results indicated that none of the 1,606 seedlings tested positive for the virus. They concluded TMV is not seed transmitted since the virus did not invade the embryo (de Assis Filho and Sherwood 2000). In contrast, Wilkinson et al. (2006, unpublished data) observed in a growth chamber test that bulked tobacco seed coat samples from infected 'K 326' flue-cured tobacco assayed for TMV by ELISA had 71% to 100% positive results for TMV. In addition, tobacco seedlings from the growth chamber tests had positive results that ranged from 2% to 77% for TMV (Wilkinson et al. 2006). It was documented that similar results were found in greenhouse tests but had lower transmission rates overall (Wilkinson et al. 2006). Sevik and Kose-Tohumeu (2011) showed that tomato seedlings grown from TMV infected seeds had a transmission rate of 23.5% by ELISA (Sevik and Kose-Tohumeu 2011). These results indicate that ELISA may not be sensitive enough to detect TMV in an individual sample, therefore, a more sensitive detection method is needed to determine transmission of TMV from a seed. Another limitation to this technique is the amount of time the ELISA takes to conduct the test.

Nucleic acid-based analysis such as end-point RT-PCR and reverse transcriptase real time PCR quantitative analysis (RT-qPCR) are the most sensitive techniques for detecting small amounts of nucleic acids and can be used to quantify gene expression (Fraga et al. 2014). These techniques use RNA samples that have been extracted and synthesized into cDNA through reverse transcription. Endpoint RT-PCR measures the amount of DNA at the end of PCR amplification. Quantification is determined by gel electrophoresis, ethidium bromide

visualization of the DNA product and measuring of band intensity (Fraga et al. 2014). Kumar et al. (2011) tested this method to determine the detection limits in TMV infected pepper and tomato leaves. Samples were diluted up to a 6-fold dilution and results showed bands up to 6-fold dilution of total RNA extracted from TMV infected leaves. Similar results were demonstrated by Yang et al. (2012), who showed that for a series of dilutions of $1{:}10^1$ to $1{:}10^6$ of TMV infected leaf sap there was a positive detection of TMV in a plant leaf down to the $1{:}10^6$ dilution. Similar to TMV, there was limited evidence that the *Cucumber mosaic virus* (CMV) was seedborne and transmitted through pepper seed. Ali and Kobayashi (2010) germinated 200 seeds for 7 to 8 d and found a 29.5% of transmission for infected seedling and 59.5% rate of transmission for infected seed coat by RT-PCR. In another test, there were 108 seeds germinated from infected plants and sampled four weeks post germination; none of the plants showed symptoms of CMV but 12% tested positive for CMV by RT-PCR. This study confirmed CMV was seedborne and is transmitted from seed to seedling (Ali and Kobayashi 2010). This technique is semi-quantitative because the amount of virus present in a sample is determined by the intensity of a band. This procedure does not give an exact value for virus concentration. Also, endpoint RT-PCR requires an additional series of post-PCR procedures for data collection that is not required for RT-qPCR such as gel electrophoresis and staining before the data can be collected. These steps require additional hours for data collection.

RT-qPCR also uses RNA extracted from an infected sample which is synthesized into cDNA. This technique uses fluorescence-based technology to measure the amount of amplicon produced during each cycle (Fraga et al. 2014). Results are measured in real time (as it's being produced) by labeling it with a fluorescent dye during amplification. RT-qPCR compares the relative concentration of TMV since it is testing the TMV RNA against the tobacco 18*S* rRNA.

The 18*S* is one of the only host RNAs at the same concentration level as the TMV RNA. RT-qPCR measures the cycle threshold (Ct) by measuring when the cycle quantification crosses the threshold line. This line is the level of detection when a reaction reaches a fluorescent intensity above background levels. The Ct shows how many cycles the sample went through to detect a real signal. The main advantages to this technique are time because there are no post PCR procedures and higher sensitivity of the fluorescent dyes detecting amplification.

Objectives and Significance

TMV has historically not been considered seed-transmitted. There is an inconsistency in the literature on whether a TMV infected seed produces an infected seedling. A large portion of the literature is reliant on grow out tests for visual symptoms or serological assays that require a larger amount of viral RNA to be in a sample. Seed transmission of TMV was demonstrated previously by Wilkinson et al. (2006) but the rate of transmission from a seed to seedling could not be determined because samples were pooled for analysis by ELISA. The objective for this study is to establish the rate of transmission for TMV from seed to seedling. Previous research used technology that was not sensitive enough to detect low levels of viral RNA or only looked for visual symptoms on the plant, therefore this research could clarify the rate of transmission. A plant can be infected with TMV and not show symptoms and there has been difficultly linking TMV transmission from the seed to the seedling. It is hypothesized that the virus will transfer from the seed to the seedling. Real-time qPCR (RT-qPCR) will be more sensitive in detecting the virus and can provide a quantitative result.

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Chapter: 2

Tracking *Tobacco Mosaic Virus* Infection from Infected Seeds to Seedlings Confirms Seed Transmission in Tobacco (*Nicotiana tabacum* L.)

Abstract

The *Tobacco mosaic virus* (TMV) is a rod-shaped virus with a positive sense single stranded RNA genome and is found in all areas that grow tobacco. TMV is an economically significant virus because TMV can impact the yield and quality of tobacco. Historically, there has been controversy on whether this virus is categorized as a seed-transmitted virus. The goal of this study is to track TMV infection from infected seeds to seedlings to demonstrate seed transmission. Seeds from three pods from three different TMV infected cultivar K 326 flue-cured tobacco plants were evaluated in this experiment. Seeds were germinated in a growth chamber and separated into seed coat, root and leaves. Total RNA was extracted from each part and synthesized into cDNA for analysis. This is the first report using quantitative real-time PCR (RT-qPCR) assay to determine TMV concentration of each sample. In order to validate results from RT-qPCR, endpoint RT-PCR was used to determine a conservative infection threshold value. Germination percent from TMV infected seeds ranged from 50% to 94%. RT-qPCR showed a consistent virus titer distribution pattern across all plants and pods. Seed coats had a significantly higher virus titer concentration ($P < 0.05$) when compared to the roots and leaves in each pod. Statistical analysis showed highly significant ($P < 0.0001$) differences among pods for virus titer and there is a highly significant plant by pod interaction ($P < 0.0001$). Endpoint RT-PCR confirmed TMV infection in leaves, roots and seed coats. Percent infection in leaves ranged from 2% to 24% and percent infection for roots ranged from 8% to 40%. Therefore, since virus was detected in leaves and roots of 10-day-old seedlings, TMV is seed transmitted in flue-cured tobacco.

Introduction

The *Tobacco mosaic virus* is a single stranded, positive-sense RNA virus and is an extremely hardy virus (Heinlein 2002). TMV is mechanically transmitted by the virus entering through open wounds on the host plant (Liu and Nelson 2013). The plant will show symptoms such as mosaic pattern, necrotic local lesions, and stunted growth (Gooding 1986). This is an economically significant virus because TMV can reduce the quality and yield which results in a decrease in average price.

TMV has been difficult to eradicate due to the stability of the virus. There are multiple ways to reduce transmission of TMV such as sanitation, removal of infected plants, crop rotation, and resistant cultivars. There have been several serological assays that test for TMV used in previous research such as ImmunoStrips and ELISA, but these assays are limited to the amount of virus present in a sample. Nucleic acid-base analysis such as endpoint RT-PCR and RT-qPCR are more sensitive technologies and can detect low amounts of RNA. RT-qPCR quantifies the virus concentration in a given sample, even with low amounts of RNA (Fraga et al. 2014). RT-qPCR has also been more effective in detecting TMV in the plant material. There is a gap in the literature on quantifying the virus concentration of an infected plant due to the lack of sensitivity in technology. Recent advancements in technology can provide a better understanding of TMV by producing accurate data on virus concentration.

Past research used serological assays such as ELISA for TMV detection but due to sensitivity limitations, this technique could not detect samples with a small amount of virus. Due to this lack of sensitivity, there has been controversy on whether TMV is classified as a seedborne or seed-transmitted virus because there is an inconsistency in the literature on the location of the virus in the seed and if TMV infects the seedlings. Therefore, a more sensitive

technique to detect TMV in a sample with limited amount of RNA is needed. The objective of this study is to establish that rate of transmission for TMV from a seed to a seedling by using RT-qPCR.

Materials and Methods

Plant Inoculation and Seed Collection

This study was conducted at the Virginia Tech Southern Piedmont Agricultural Research and Extension Center (SPAREC) in Blackstone, VA. K 326 cultivar flue-cured tobacco was grown in a geographically isolated field at SPAREC in 2017. Plants were grown using standard agronomic practices for fertilization, transplanting, disease and insect control (Reed et al. 2019). Test plants were inoculated after layby in June using a sponge brush (Fisher Scientific, Pittsburgh, PA) dipped in TMV inoculum and rubbed against a palm sized leaf. Inoculum was prepared by grinding infected tissue in TMV buffer (1% Celite (diatomaceous earth); Fisher Scientific) and 1% potassium phosphate dibasic buffer (Fisher Scientific) and creating an inoculum with 1:100 dilution. The pH of this solution was adjusted to 7.4 by adding concentrated HCl. The TMV inoculum was made by using a mortar and pestle to grind two grams of infected leaf tissue in 20 mL of TMV buffer. The sap was filtered through a cheesecloth to create a 1:10 dilution. A volume of 0.5 mL filtered sap was added to 4.5 mL of TMV buffer to create the 1:100 dilution of TMV inoculum.

Mature seed pods were collected from infected plants at the end of the season in October and stored at room temperature. In this experiment, three different plants of the cultivar K 326 infected with TMV were used and three pods per plant were tested. Seed collected from each pod were separated by sieves (Gilson, Middleton, WI) to be categorized as small, medium or large

seed. Seeds that fell through the 0.600 mm sieve was classified as large, 0.500 mm sieve was classified as medium and 0.425 mm sieve was classified as small.

Seed Germination

The effect of TMV infection on seed germination was evaluated by germinating 100 seeds for pod A, and 50 seeds for pod B and C, in a growth chamber (Sanyo Scientific, Wood Dale, IL) with 16-h light, 8-h dark cycles at 22°C and 70% relative humidity for 10 d. To serve as a control, 50 seeds from healthy K 326 flue-cured cultivar tobacco were also germinated using the same conditions as the TMV-infected seeds (Reed et al. 2019). According to the 2019 Flue-cured Tobacco Guide, maximum germination is seven to eight days when exposed to ideal temperatures of 22°C (Brown et al. 2019). Seeds were placed on deionized water-dampened blotter paper (Fisher Scientific) in a petri dish (Fisher Scientific) and tweezers (Fisher Scientific) were flame sterilized between each seed to minimize contamination. The seed was considered to have germinated if cotyledons (leaves) and primary root were observed. Germinated seeds were counted, dissected and assayed for virus by RT-qPCR and endpoint RT-PCR. The seed coat (SC), leaves (L), and root (R) were separated and each part collected in separate 1.5 mL microcentrifuge tubes (USA Scientific, Ocala, FL). Tweezers and scalpel (PFM Medical, Kölm, Germany) were flame sterilized between each cut to minimize contamination. Plant samples were stored in the freezer at -80°C.

Detection of TMV from infected samples

Total RNA was extracted from the leaves and root using TRIzol (Life Technologies, Carlsbad, CA) with a Direct-zol™ RNA MiniPrep Plus Kit (Zymo Research, Irvine, CA) following manufacturer's instructions. The total RNA extraction of the seed coat was done using TRIzol with a Direct-zol™ RNA MicroPrep Kit (Zymo Research) following the manufacturer's

instructions. Two different kits were used due to the lower amount of RNA in the seed coat in comparison to the root and leaf tissue. The RNA extracted into the labeled RNase free tube was stored in a freezer at -20°C.

Complementary DNA (cDNA) synthesis protocol was developed by Dr. Tim Sit (personal communication) of North Carolina State University. The reaction mixture in a total volume of 12 µL contained 6 µL of dH_2O, 1 µL 10 mM dNTPs (New England Biolabs, Ipswich, MA), 2 µL 60 µM random hexamer primers (Thermo Fisher, Waltham, MA), and 3 µL RNA sample into a 0.5 mL microcentrifuge tube (USA Scientific). Microcentrifuge tubes were vortexed and incubated for five minutes on a Isotemp dry heat block (Fisher Scientific) at 70°C to denature RNA. Tubes are placed immediately in ice for three to four minutes to anneal primers. Samples are taken off ice and 4 µL of 5X ProtoScript buffer (New England Biolabs), 2 µL of 0.1 M dithiothreitol (New England Biolabs), 1 µL of Murine RNase inhibitor (40 U/µL; New England Biolabs), and 1 µL Protoscript II Reverse Transcriptase (New England Biolabs) was added into the tube and vortexed. Samples were incubated for 5 min at room temperature followed by 45 min at 42°C in the Isotemp dry heat block. Once incubation is done, samples were incubated for five minutes at 80°C to denature reverse transcriptase (RT). After incubation, samples were vortexed and stored in a freezer at -20°C.

The reverse transcriptase qPCR (RT-qPCR) protocol was also developed by Dr. Tim Sit (personal communication) of North Carolina State University. Data were collected using a Thermo Fisher QuantStudio 3 real-time PCR machine (Thermo Fisher). The samples were run in triplicate for TMV 126 primer (TMV primer set; Eurofin Genomics, Louisville, KY) and 18*S* primer (internal rRNA control primer set; Eurofin Genomics) on a 96-well optical PCR plate (ThermoFisher) sealed with optical film (Applied Biosystems, Foster City, CA). Dr. Tim Sit

from NC State University developed primers used in this protocol. TMV primer set: forward, 5'-CCCCTACACCAGTCTCCATCA-3'; reverse, 5'-CGAACAGGTGTGCCTTGACA-3'. Internal rRNA control primer set: forward, 5'-CGTCCCTGCCCTTTGTACAC-3'; reverse, 5'-CGAACACTTCACCGGATCATT-3'. The TMV primer reaction mixture for each sample has 8.4 µL dH_2O, 0.3 µL TMV forward primer (10 pmoles/µL), 0.3 µL TMV reverse primer (10 pmoles/µL), and 10 µL Fast SYBR Green Master Mix (AppliedBiosystems by ThermoFisher) for a total of 19 µL TMV primer set. The 18*S* primer reaction mixture for each sample has 8.4 µL dH_2O, 0.3 µL 18*S* forward primer (10 pmoles/µL), 0.3 µL 18*S* reverse primer (10 pmoles/µL), and 10 µL Fast SYBR Green Master Mix for a total of 19 µL 18*S* primer set. Primer mixtures were aliquoted into plates, 19 µL of each primer set was aliquoted in triplicate for each sample (19 µL per well, three wells per sample). Samples were run in triplicate and 1 µL of cDNA sample was added to each well. Each plate had 1 µL TMV positive cDNA sample to serve as a positive control and 1 µL dH_2O sample to serve as the negative control. Plates were processed through a QuantStudio 3 Real Time PCR system. RT-qPCR conditions were as follows: 1 cycle 95°C for 20 sec, 40 cycles 95°C for 1 sec, 40 cycles 60°C for 20 sec, 1 cycle 95°C for 15 sec, 1 cycle 60°C for 1 min, and 1 cycle 95°C for 15 sec.

To validate the results from RT-qPCR and to determine a conservative infection threshold value, endpoint RT-PCR was performed in a Bio-Rad C1000 Touch Thermal Cycler (Bio-Rad, Hercules, CA). The RT-PCR mixture in a total volume of 25 µL consisted of the following: 9.5 µL dH_2O, 1 µL 5' forward primer (10 pmoles/µL; TobUni2; Eurofin Genomics, Louisville, KY), 1 µL 3' reverse primer (10 pmoles/µL; TobUni1; Eurofin Genomics; Letschert et al. 2002), 12.5 µL One *Taq* 2X Master Mix (New England Biolabs) and 1 µL cDNA (same cDNA used for RT-qPCR). RT-PCR mixture was run in a Bio-Rad Thermal Cycler under the

following conditions for routine TMV amplification: 94°C for 5 min, 94°C for 1 min, 60°C for 45 sec, 68°C for 1 min for 40 cycles, 68°C for 5 min, and 12°C for ∞. PCR fragments were visualized by horizontal gel electrophoresis (Bio-Rad) in a 1X TAE buffer (Promega Corporation, Madison, WI) on a 1% agarose gel (Promega Corporation). Template mixture for each sample consisted of the following: 2 µL dH_2O, 3 µL PCR reaction mixture and 1 µL 6X loading dye (New England Biolabs). DNA ladder (New England Biolabs) used to estimate the size of fragments consisted of the following: 4 µL dH_2O, 1 µL 100bp DNA ladder and 1 µL 6X loading dye. Proper negative (water blanks) and positive (cDNA from a TMV positive sample) controls were used for electrophoresis. Samples were electrophoresed at 90V for 45 min and stained with 2 µg/mL ethidium bromide staining (Sigma Aldrich, St. Louis, MO) followed by destaining in dH_2O for 20 to 30 minutes. Gels were examined in a Gel Doc-It system (UVP, Upland, CA) and samples were considered positive for TMV if a well-defined band appeared.

Data Analysis

Quantitative data from RT-qPCR analysis were transferred into QuantStudio Design and Analysis Software v1.4 (ThermoFisher) from QuantStudio 3 PCR machine. Mean TMV Ct values and mean 18*S* Ct values were transferred into Microsoft Office 365 ProPlus Excel for *Windows* (Microsoft, Redmond, WA). A series of calculations developed by Dr. Tim Sit (personal communication) were done to calculate the virus titer concentration in each sample: First, the normalized 18*S* was calculated (virus primer$^{18S\ Ct}$) and then normalized TMV was calculated with the following equation:

(normalized 18*S*) / (virus primer$^{\text{mean TMV Ct}}$)

Finally, the virus titer was calculated by using the Log_{10}(normalized TMV). Percent germination of all components were calculated in Microsoft Office 365 ProPlus Excel by dividing the number

of samples germinated by the total number of samples sown. The percent infection for TMV was calculated using the Ct threshold values from endpoint RT-PCR. Any samples below the designated Ct value were considered positive and percent infected was calculated by the number of positive samples in each component divided by the total number of samples for each component. Graphs were created in GraphPad Prism 7.04 for Windows (GraphPad Prism, San Diego, CA). GraphPad Prism 7.04 was used for statistical analysis of virus titer means in each pod by an analysis of variance ANOVA and Tukey's multiple comparisons of the log_{10} of normalized data. SAS 9.4 for Windows (SAS Institute Inc., Cary, NC) was used for statistical analysis of plant components across plants and pods by ANOVA and Tukey's multiple comparisons of the log_{10} of normalized data.

Results

Seed Germination

The percentage of seeds that germinated from TMV-infected plants was scored after 10 days. Healthy K 326 flue-cured cultivar seed was germinated to use as a control and had a germination of 92% (Table 1). Germination percentage varied across plants and pods; plant 1 pod B had the highest germination of 94% and plant 3 pod C had the lowest germination of 50%. All plants and pods expect for plant 1 pod B had a germination percentage less than the control (<92%).

Detection of TMV from infected samples

TMV Ct values were calculated for all samples using the QuantStudio Design and Analysis Software to show which cycle the viral RNA was detected. The lower the Ct value, the more viral RNA is present. TMV Ct values had a consistent distribution across all plants and pods (Figure 1). The seed coats had lower TMV Ct values when compared to leaves and roots for

all plants and pods. Standard deviation bars showed that seed coats had the most variation of the plant components in all plants and pods. This indicated that there was more viral RNA in the seed coats than the leaves and roots. Ct values for leaves and roots were similar in all pods.

A range of TMV Ct values from 15 to 35 were used to establish a conservative threshold for TMV positive samples. All samples that displayed a band were considered positive for TMV. When testing the range of TMV Ct values by endpoint RT-PCR, results showed positive bands in all three components (Figure 2). These results validated that there was virus present in the leaves, root and seed coat. According to endpoint RT-PCR results, the maximum TMV Ct value detected for positive leaf sample was 29.596 (Figure 2). A sample with a Ct value of 30.576 was negative, therefore, samples above 30 were considered negative. Roots had a maximum mean TMV Ct value of 30.729 to detect a positive sample (Figure 2). A sample with a Ct value of 31.494 was negative, therefore any Ct value over 31 was considered negative. Seed coats had a maximum mean TMV Ct value of 31.851 for positive samples (Figure 2). A sample with a Ct value of 33.251 was negative, therefore any samples above 33 were considered negative.

Virus titer for each sample was calculated using the Ct values reported in the QuantStudio Design and Analysis Software. Virus titer is the log10 of the normalized virus. These values represent the virus concentration in a sample. RT-qPCR data showed a similar virus titer distribution across all samples (Figure 3). The seed coats had higher virus titer values when compared to leaves and roots for all plants and pods. This indicated that there was a higher virus concentration in the seed coats than the leaves and roots. Virus titer for leaves and roots were similar in all pods. Standard deviation bars showed that seed coats had the most variation of the plant components (Figure 3).

There were no significant differences among plants for virus titer in leaves, roots, or seed coats (Table 2). In contrast, highly significant differences ($P < 0.0001$) were observed among pods for virus titer in all three plant components. There were also highly significant ($P < 0.0001$) plant*pod interactions for virus titer in all three plant components therefore data for individual pods will be presented (Table 2).

Highly significant ($P < 0.001$) differences among roots, leaves, and seed coats within each pod were observed for virus titer (Table 3).

Within each pod there was a statistically significant ($P < 0.05$) difference among plant components for virus titer as determined by one-way ANOVA (Table 4). Seed coat has a significantly higher viral titer than leaves and roots in all pods ($P < 0.05$). Roots had a significantly higher ($P < 0.05$) virus titer than leaves in four pods (1A, 1C, 2C and 3A). There was no significant difference in viral titer between leaves and roots for the other five pods (Table 4).

Using the RT-qPCR results and the endpoint RT-PCR results, the percent incidence was calculated using TMV Ct cutoff values for each component (leaves Ct < 30, root Ct < 31, and seed coat Ct < 33). All samples that were below the cutoff TMV Ct value were considered infected. Leaves had a percent infection range from 2% in plant 3 pod B, to 24% in plant 1 pod B (Table 5). Roots had a percent infection range of 8% in plant 2 pod A and plant 2 pod C to 40% in plant 1 pod B. Seed coats had a percent infection range of 30% in plant 2 pod C to 100% in plant 2 pod B (Table 5). These results indicate that TMV was successfully transmitted to the seedling. Therefore, there is evidence that TMV is seed-transmitted in flue-cured tobacco.

Discussion

Seeds disperse viruses globally because most seed transmitted viruses remain viable within the seed for years. Virus transmitted from the seed to the seedling and the virus' efficiency may result in an epidemic. Therefore, it is crucial to understand which viruses are seed-transmitted and can potentially infect the seedling. There has been controversy whether TMV can be transmitted via seed, and if so, should TMV be categorized as seedborne or seed-transmitted because previous research has only been able to find the virus on the seed coat, but this did not always lead to an infected seedling.

Biological assays and serological detections can be time-consuming processes and lead to ambiguous results. Serological assays such as ELISA have previously been used to detect TMV in bulk samples, but ELISA was not sensitive enough to test on seed or seedlings with limited amount of virus. RT-qPCR is being recognized as a new way to quantify virus in a sample. Here, we report for the first time the use of RT-qPCR as a diagnostic tool to detect TMV in individual flue-cured tobacco seed and seedlings. In comparison with ELISA to quantify virus, RT-qPCR has a higher sensitivity and can detect virus even when limited amounts of RNA is available. RT-qPCR data demonstrated the root and leaf tissue from germinated seed were infected with TMV therefore demonstrating seed transmission of TMV in flue-cured tobacco. Leaves from infected seedlings had a percent infection of 2% to 24% depending on the plant and pod, and roots had a percent infection of 8% to 40% (Table 5). Chamberlain (1937) documented that 0.12% of tobacco seedlings grown from infected plants were positive for TMV. Although this number is low, it is still significant since the 0.12% of infected seedlings could serve as primary infection. Wilkinson et al. (2006; unpublished data) reported 2% to 71% pooled seedling samples were TMV positive when TMV infected K 326 flue-cured tobacco seed were grown in growth

chamber tests. When compared to other plants that TMV can impact, the percent infection rates differ. de Assis Filho and Sherwood (2000) used ELISA for detection of TMV in *Arabidopsis thaliana* L. which showed that 0% of seedlings from TMV infected seed were positive for TMV. In contrast, Demski (1981) reported that 28% of roots and 3% of shoots from infected *N. glutinosa* was considered positive by a bioassay. Sevik and Kose-Tohumeu (2011) observed 23.5% of tomato seedlings grown from infected seeds tested positive by ELISA. There is evidence that TMV could infect seedlings from a variety of plants such as tobacco, tomato and pepper. It is crucial to understand how this virus infected the seedling and where TMV might reside in the seed.

Taylor (1962) reported virus located in the seed coat and endosperm but there was not virus located in the embryo. He classified TMV as seedborne since there were no signs of mosaic symptoms on seedlings. Whereas, Benoit and Maury (1976) reported virus only being in the nucellar layer of the seed coat and no evidence in the endosperm or embryo. de Assis Filho and Sherwood (2000) found that TMV was only detected in the seed coat of a TMV infected *Arabidopsis thaliana* L. seed. Kumar et al. (2011) used multiplex RT-PCR to detect TMV in 9 out of 18 pepper and tomato seed samples from TMV infected plants, but the location of the virus within the seeds was not determined.

In contrast, to previous studies reporting TMV is not detected in the embryo, M. Ellis (2019) dissected seed from TMV infected flue-cured plants and pods (same plants and pods used in this study), and found virus in the embryo, endosperm and seed coat for all pods and plants (Table 6). These results indicated that TMV is seed-transmitted since there is evidence of virus in all three components of a seed and all components in a seedling (Table 5 and Table 6). These

results indicate that infection of the leaves and root may be a result from the embryo being infected rather than the seed coat.

Results showed that the number of TMV infected components varied among seed pods. There were significant differences among pods for virus titer in leaves, roots, and seed coat (Table 2). Seed coats had a significantly higher mean viral titer when compared to the leaves and roots for all plants (Table 4). This variation could be from the timing of infection. Seedlings were incubated for 10 d due to fungal contamination beyond that time point. If seeds had been surface sterilized, there could have been an extended germination period but it would be unknown how the surface sterilization affects TMV. An extended germination time may have led to a greater percentage of the leaves and roots showing presence of TMV as the infection would have more time to proceed and increase viral titer. The virus can move across tissues because the inoculated leaf cells are symplastically connected by the plasmodesmata. TMV can move from infected areas to new areas that will develop into maternal derived seed coat. The seed coats are developed from maternal tissue and this explains why TMV was most commonly found in the seed coats in previous research and this study. During the later stages of development, the endosperm becomes impermeable and would be expected to block the movement of viruses (Yim and Bradford 1998). Although this study did not examine the permeability of the endosperm, Argerich and Bradford (1989) showed that mature tomato seeds lacked a permeable endosperm by failed electroconductivity tests to measure leachate from aged seeds. A similar loss of endosperm permeability in the later stages of tobacco seed development would prevent apoplastic movement of TMV into the embryo and passively protect the embryo from infection. Transcellular movement of TMV could not happen in senescing tissue after development is

complete and the seeds are dry. Therefore, there is a limited amount of time tobacco embryos would be susceptible to TMV infection.

Conclusion

In conclusion, a new method was used to track TMV from a seed to seedling. Germination percentages varied among plants and pods. There was a similar virus concentration gradient across plants where seed coats had the highest concentration of virus followed by roots then leaves. Seed coats had a significantly higher viral titer across all pods. Presence of TMV was validated in each component and a conservative cutoff value was established to determine percent transmission, demonstrating that the virus was transmitted to the roots and leaves. The roots had a higher percent transmission because the roots are the initial site of infection since roots are the first cells to emerge from the embryo and would have the longest amount of time to harbor multiplying virus. TMV was detected in seedling components and seed components, therefore, TMV can be classified as a seed-transmitted virus.

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Table 1. Percent germination of seed collected from field grown K 326 flue-cured tobacco seed infected with *Tobacco mosaic virus* (TMV).[1]

Plant	Pod A (%)	Pod B (%)	Pod C (%)
1	87	94	57
2	85	84	85
3	70	83	50

[1]Number of seed evaluated was 100, 50 and 50 from pods A, B, and C, respectively. Seeds were germinated in a growth chamber with 16-h light, 8-h dark cycles at 22°C and 70% relative humidity for 10 days.

Table 2. Analysis of variance of *Tobacco mosaic virus* (TMV) titer for each plant component from growth chamber tests.[1]

Component	Sources of Variation	df	*p*-value
Leaves	Plant	2	0.5996
	Pod	2	<0.0001
	Plant*Pod	4	<0.0001
	Error	591	
	Total	599	
Root	Plant	2	0.6157
	Pod	2	0.0012
	Plant*Pod	4	0.0019
	Error	591	
	Total	599	
Seed Coat	Plant	2	0.5928
	Pod	2	<0.0001
	Plant*Pod	4	<0.0001
	Error	591	
	Total	599	

[1]Plant components were separated 10 d after seeding and evaluated for TMV by RT-qPCR. Seeds were collected from field grown K 326 infected with TMV in 2017.

Table 3. Analysis of variance from comparing Log_{10} values of each plant component from each individual pod of each seed collected from a *Tobacco mosaic virus* (TMV) infected plant.[1]

Plant	Pod	Sources of Variation	df	*p*-value
1	A	Treatment	2	<0.001
		Error	297	
		Total	299	
	B	Treatment	2	<0.001
		Error	147	
		Total	149	
	C	Treatment	2	<0.001
		Error	147	
		Total	149	
2	A	Treatment	2	<0.001
		Error	297	
		Total	299	
	B	Treatment	2	<0.001
		Error	147	
		Total	149	
	C	Treatment	2	<0.001
		Error	147	
		Total	149	
3	A	Treatment	2	<0.001
		Error	297	
		Total	299	
	B	Treatment	2	<0.001
		Residual	147	
		Total	149	
	C	Treatment	2	<0.001
		Residual	147	
		Total	149	

[1]Plant components were separated 10 d after seeding and evaluated for TMV by RT-qPCR. Seeds were collected from field grown K 326 infected with TMV in 2017. Treatments are the root, leaves, and seed coat from each germinated seed. Pod A n = 300 and pods B and C n = 150.

Table 4. *Tobacco mosaic virus* (TMV) titer (log_{10} of normalized virus) means for each plant component after germination in a growth chamber.[1]

Plant	Pod	Leaves	Root	Seed Coat
1	A	-4.218 c [2]	-3.749 b	-0.051 a
	B	-3.957 b	-3.759 b	-0.297 a
	C	-4.768 c	-4.015 b	-0.843 a
2	A	-3.939 b	-3.521 b	-0.217 a
	B	-3.419 b	-3.924 b	1.771 a
	C	-5.624 c	-4.330 b	-1.896 a
3	A	-4.387 c	-3.506 b	-0.135 a
	B	-4.597 b	-4.333 b	0.631 a
	C	-4.059 b	-3.502 b	-1.047 a

[1]Seed were collected from field grown K 326 infected with TMV in 2017.
[2]Means within a row followed by the same letter are not significantly different at the 0.05 probability level using the Tukey test.

Table 5. Percent *Tobacco mosaic virus* (TMV) infection of plant components of K 326 flue-cured tobacco.[1]

Plant	Pod	Leaves (%)	Root (%)	Seed Coat (%)
1	A	16	14	99
1	B	24	40	70
1	C	10	20	86
2	A	11	8	86
2	B	20	26	100
2	C	4	8	30
3	A	8	12	79
3	B	2	32	94
3	C	4	16	84

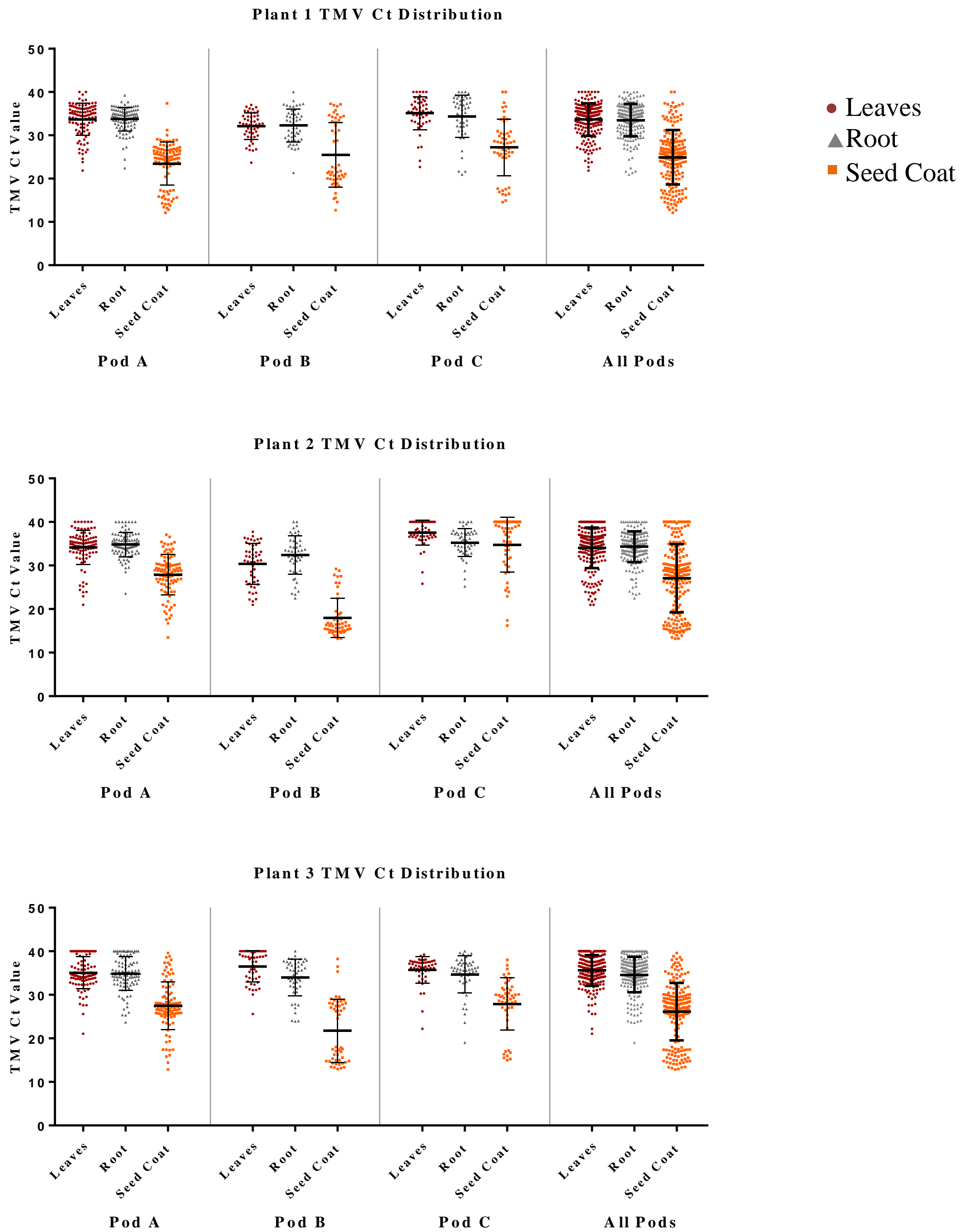
[1]Based on conservative TMV Ct cutoff values for leaves Ct < 30, root Ct < 31, and seed coat Ct < 33. All samples that had a TMV Ct value lower than the cutoff associated with each component was considered a positive sample. Pod A was based on 100 samples, pod B was based on 50 samples and pod C was based on 50 samples.

Table 6. Percent infection of *Tobacco mosaic virus* (TMV) from data within a flue-cured tobacco seed.[1]

Plant	Pod	Embryo (%)	Endosperm (%)	Seed Coat (%)
1	A	35	78	79
1	B	82	96	98
1	C	94	100	100
2	A	23	71	68
2	B	88	100	100
2	C	14	54	52
3	A	24	87	83
3	B	54	66	94
3	C	20	40	56

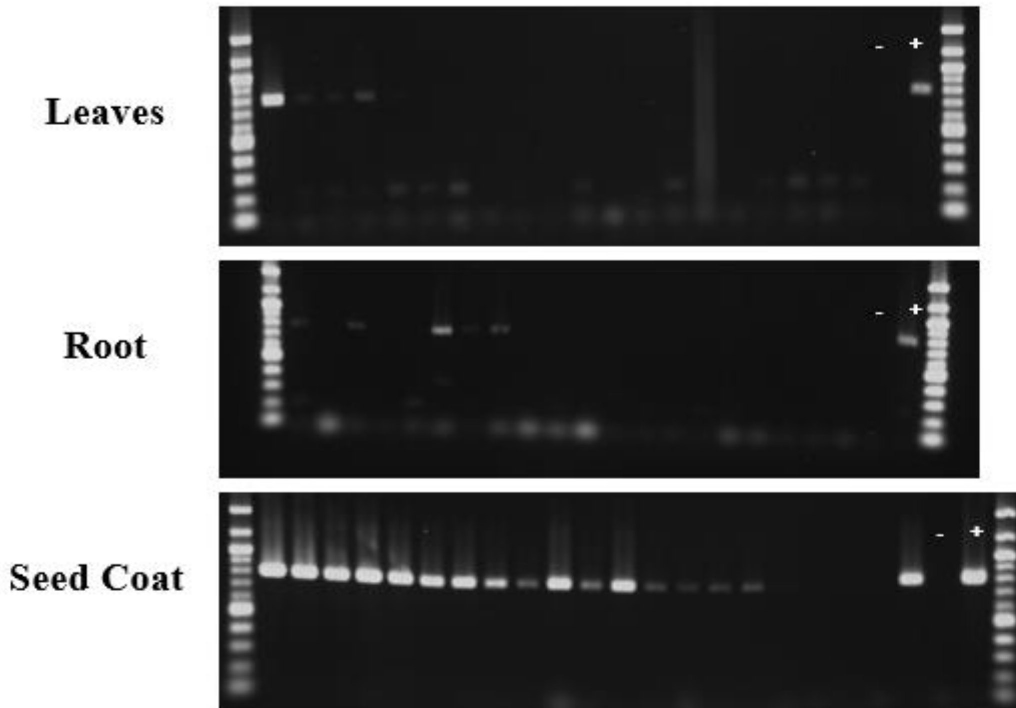
[1]Data was collected by Madeleine Ellis (2019) of Virginia Tech (personal communication) and she used the same three plants and the same three seed pods used in this thesis. Based on a conservative TMV Ct cutoff value of embryo Ct < 31, endosperm Ct < 32, and seed coat Ct < 30. All samples that had a TMV Ct value lower than the cutoff associated with each component was considered a positive sample. Pod 1 was based on 100 samples, pod 2 was based on 50 samples and pod 3 was based on 50 samples.

Figure 1. *Tobacco mosaic virus* (TMV) cycle threshold (Ct) value distribution of infected samples generated by RT-qPCR.[1]



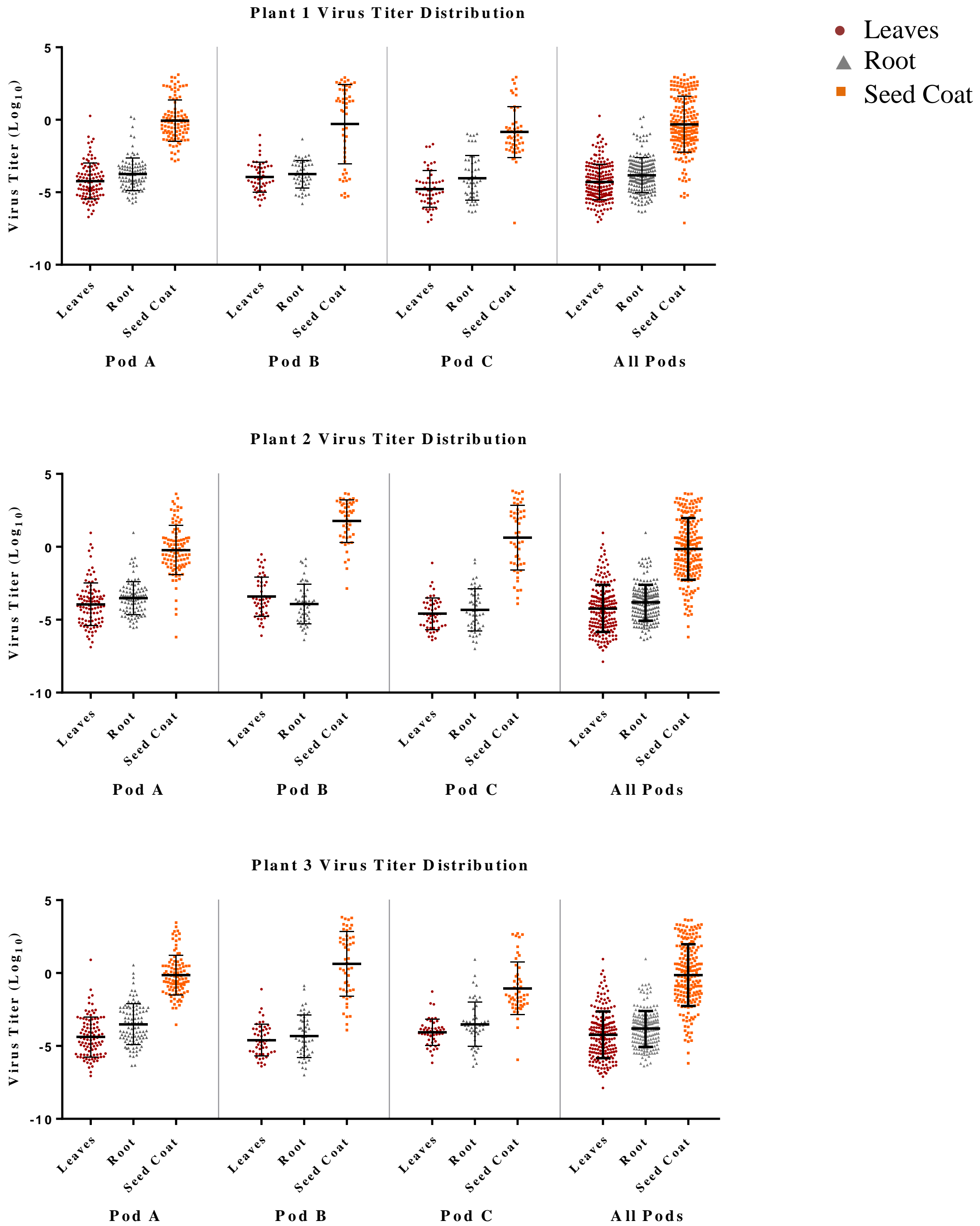
[1]For each component there are 100 samples for pod A, 50 samples for pod B and 50 samples for pod C. Center bars represent means and error bars represent the standard deviation of each population.

Figure 2. Endpoint RT-PCR was used to determine a conservative *Tobacco mosaic virus* (TMV) Ct value based on RT-qPCR results. A band indicates a positive sample.[1]



[1]20 samples per component from plant 8 pod A were analyzed with endpoint RT-PCR that had a TMV Ct range of 15 to 35. Any sample that showed a band was considered positive for TMV. TMV Ct cutoff values were established as leaves Ct < 30, root Ct < 31, and seed coat Ct < 33 based on endpoint RT-PCR results. Each set has a DNA ladder (first and last lanes), positive control (second lane from the right) and a negative control (third lane from the right).

Figure 3. *Tobacco mosaic virus* (TMV) virus titer (log10 of normalized virus) distribution of infected samples generated by RT-qPCR.[1]



[1]For each component there are 100 samples for pod A, 50 samples for pod B and 50 samples for pod C. Center bars represent means and error bars represent the standard deviation of each population.