THE SEXUALLY–TRANSMITTED WESTERN AUSTRALIAN WILD–PLANT VIRUS YELLOW TAILFLOWER MILD MOTTLE VIRUS:

DOES IT POSE A THREAT TO GLOBAL FOOD SECURITY?

Thesis presented by

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Declaration

I declare that this thesis is my own account of my research and contains as its main content, work which has not previously been submitted for a degree at any tertiary education institution.

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Frontispiece: A plant of *Anthocersis illicifolia* at Cervantes, Western Australia. Photo Steve Wylie.

Abstract

Yellow tailflower mild mottle virus is a species in the internationally-distributed genus *Tobamovirus*, other species of which are some of the most damaging plant viruses known. Yellow tailflower mild mottle virus (YTMMV) is the first tobamovirus described only from Australia and only from native plants. Because of the bad reputation of related tobamoviruses such as tobacco mosaic virus and cucumber green mottle mosaic virus as destroyers of valuable crops, we studied YTMMV to understand aspects of its biology and to assess its potential to spillover from the indigenous flora and threaten crops on national and international stages. Unlike many damaging plant viruses, tobamoviruses are not transmitted host-to-host by vectors such as aphids. Thus, understanding how YTMMV is transmitted between host plants is key to understanding aspects of its epidemiology. A further aim of our work was to assess the damage we might expect to see in some susceptible crops should YTMMV spillover.

Sexual transmission - seed: After manual transmission, YTMMV readily infected the solanaceous species and varieties we tested, which were *Solanum lycopersicum* (tomato), two cultivars of *Capsicum annuum* (bell pepper, chili), *Nicotiana tabacum* and two accessions of *Nicotiana benthamiana* (tobacco species). Genotypes of *C. annuum* and *N. benthamiana* quickly died after becoming infected while others displayed milder symptoms, and some produced flowers and set viable seed. We grew the seed and tested for presence of YTMMV in the seedlings. Rates of transmission of the virus *via* seed to the next generation were high—from 3% in *N. tabacum* 'Wisconsin 38' to 57.5% in *N. benthamiana* accession MtA-6. In some other tobamoviruses, the virus adheres to the external surface of the testa and infects the seedling as it germinates. We surface-sterilised seeds and tested the resulting seedlings for infection. Rates of

transmission remained the same, providing evidence that seedlings were infected by virus particles located within the testa.

YTMMV was transmitted via seed produced from an infected mother plant. Surface-sterilisation of seed cannot be used to control YTMMV.

Sexual transmission - pollen: Pollen was transferred from the stamens of virus-infected 'father' *N. tabacum* 'Wisconsin 38' plants to the stigmas of emasculated flowers of uninfected 'mother' plants. The virus was transmitted in pollen. A surprising finding was that virus-infected pollen transmitted YTMMV both vertically and horizontally with the rate below 1%, sometimes in the same pollination event. Vertical transmission occurred when the pollen tube delivered the virus directly to the developing ovule and seeds became infected. Rarely, the mother plant also became systemically-infected after pollination, demonstrating horizontal transmission. Thus, both vertical and horizontal virus transmissions occur at pollination. Subsequent seed produced by horizontally-infected mother plant was virus-infected, demonstrating vertical transmission of virus sourced from both the male gamete (pollen) and the female gamete (ovule). Pollinators such as bees may act as agents of virus transmission as they carry infected pollen from flower to flower, but they are not classed as viral vectors *per se* because there is no direct molecular interaction between the virus and the pollinator.

YTMMV is transmitted vertically and hortizontally via pollen.

Transmission *via* direct contact: Direct leaf-to-leaf contact transmission is well described for other tobamoviruses, and it occurs for YTMMV. Transmission *via* roots is less well-described although root contact between plants occurs in close-planted agricultural and horticultural crops. We grew virus-infected and virus-free plants of two species side-by-side in pots. Above-ground

contact was prevented but root contact underground was encouraged. Transmission between the plants occurred in both species at 83% and 50% for *N. benthamiana* and *C. annuum* 'Jalapeno', respectively. When seedlings were planted in soil containing decaying root materials of virus-infected plants, transmission was not detected. When uninfected scions were grafted onto rootstocks of infected plants, and vice verse, transmission occurred in both directions.

Root contact between live plants growing close together is a direct route of YTMMV transmission.

Potential yield losses: *S. lycopersicum* 'Tigerella', *C. annuum* 'Californian Wonder' and *C. annuum* 'Jalapeno' plants were infected at a young seedling stage, a mid-development stage, and immediately pre-flowering stage. In general, early infection induced more severe symptoms and sometimes death of the plant compared with later infection. In some plants, notably *S. lycopersicum* and one variety of *C. annuum*, late infection induced very few symptoms and fruit/seed was produced. When infection occurred in young *C. annuum* 'Californian Wonder' plants, fruit was damaged and yield reduced, but in mature-infected plants, average weight and length of fruits was similar to virus-free plants. *C. annuum* 'Jalapeno' plants reacted with hypersensitivity to YTMMV infection and rapidly died. In contrast, *Solanum lycopersicum* 'Tigerella' plants were mildly affected, irrespective of the age of virus infection, and yield and quality were not significantly reduced.

YTMMV infection caused minimal symptoms and yield loss in the C. annuum 'Californian Wonder' *and S. lycopersicum* 'Tigerella' *varieties tested, which made it difficult to detect by symptom expression alone.*

Conclusions: YTMMV is probably primarily a pollen-transmitted and seed-transmitted virus in wild systems. YTMMV is able to perpetuate itself through host generations *via* seed from both infected and uninfected mother plants that have been fertilised by YTMMV-infected pollen sourced from another plant of the same species. Our study showed that if infected *S. lycopersicum* 'Tigerella' and *C. annuum* 'Californian Wonder' plants, and probably other varieties of these species, were exported as fruit or seed from Western Australia, they could carry YTMMV to other places because symptoms are not clearly visible and YTMMV is transmitted in their seeds. Insects that carry infected pollen are potential agents of transmission. Thus, we consider that spillover of YTMMV to commercial solanaceous crops in the regions where YTMMV is endemic is likely, and spread to new areas, including internationally, *via* trade in infected plants and seed is possible. This should be confirmed by surveys, and antibodies to the YTMMV coat protein should developed for this purpose. Whether YTMMV has breached Australia's international borders is unknown, and the risk posed by YTMMV to the global food supply is less certain. More work is needed to assess effects of infection on a wider range of commercial solanaceous species and varieties. Of particular urgency is the need to assess YTMMV risk in potato. A significant proportion of the WA potato crop is exported as 'seed' and ware potatoes.

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Abbreviations

cDNA	Complementary DNA
CGMMV	Cucumber green mottle mosaic virus
CP	Coat protein
cm	Centimetre
DNA	Deoxyribonucleic acid
dpg	Days post-germination
dpi	Days post-inoculation
dps	Days post-sowing
EtOH	Ethanol
g	Gram
GPS	Global Positioning System
H	Height of plant
h	Hour
ICTV	International Committee on Taxonomy of Viruses
kDa	Kilodalton
Lf	Length of fruit
MP	Movement protein
mL	Millilitre

N/A	Not applicable
NOB	Number of branches (on a plant)
NOF	Number of fruits (on a plant)
nt	Nucleotide
ObPV	Obuda pepper virus
ORF	Open Reading Frame
PCR	Polymerase Chain Reaction
PMMoV	Pepper mild mottle virus
PSbMV	Pea seed-borne mosaic virus
RNA	Ribonucleic acid
RA-4	Research accession 4
RT-PCR	Reverse transcription PCR
SARS-CoV	Severe acute respiratory syndrome coronavirus
TMGMV	Tobacco mild green mosaic virus
TMV	Tobacco mosaic virus
ToMMV	Tomato mottle mosaic virus
ToMV	Tomato mosaic virus
ToBRFV	Tomato brown rugose fruit virus
TSAMV	Tropical soda apple mosaic virus

WA	Western Australia
Wf	Weight of fruit
YTMMV	Yellow tailflower mild mottle virus
ZGMMV	Zucchini green mottle mosaic virus

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to my life. Being their mother and being a daughter of my parents are the most fortunate experiences in my life.

Chapter 1: Introduction, Literature Review, Aims

1.1. Introduction

Viruses are tiny (nanometre scale) acellular parasites that consist of one or more fragments of nucleic acid, often enclosed in a protein or lipoprotein capsid, encoding one or more genes. As we have seen with the spillover in late 2019 of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), probably from a wild animal in China, to infect and kill millions of humans all over the world, viruses have the potential to cause enormous damage. Most, if not all, viruses that infect species that we have bred and domesticated for our purposes evolved in wild species before infecting domesticated species. This process is known as spillover or emergence, and it has undoubtedly happened many times over the evolutionary history of life on Earth. Given that there may be billions of virus species present on Earth (Domingo, 2020), spillovers appear to be relatively rare events because most viruses are constrained to one or a limited number of host species. Successful spillover depends on the simultaneous or near-simultaneous occurrence of several factors, and the small statistical likelihood of them co-occurring in space and time goes a long way to explaining the relative rarity of spillover events (Elena *et al.*, 2014).

In this study, one plant virus, yellow tailflower mild mottle virus (YTMMV), was studied. The species *Yellow tailflower mild mottle virus* belongs to an ancient group of plant-infecting virus species classified in genus *Tobamovirus* (from Tobacco mosaic virus). These viruses have probably evolved with their hosts for over at least 110 million years (Gibbs *et al.*, 2015). Even though tobamoviruses have no known vectors to transmit them, several of them cause serious economic damage to crops. YTMMV is the first member of this internationally-distributed group to be identified only from Australia's indigenous flora. Besides one isolated case where YTMMV spilled over to capsicum plants grown commercially in the Carnarvon region of Western Australia, this virus does not appear to have spilled over into other domesticated

solanaceous crops. Given the serious crop losses and costs of biosecurity measures to contain other internationally-distributed tobamoviruses, here we will attempt to gather information on means of transmission and potential to damage some domesticated solanaceous plant species. Almost all recorded research on other tobamoviruses has occurred "after the horse has bolted"—they have already emerged and become pathogens of domesticated species—whereas in this case, as far as we know, YTMMV remains principally within the wild flora of Australia. Previous research in our group showed YTMMV was capable of infecting a wild range of exotic-to-Australia solanaceous plants, and so there is evidence that YTMMV may be in a pre-spillover stage. Our research aims to identify the possible mechanism(s) of spillover of YTMMV, and to understand the potential costs of such an event.

1.2. Literature review

1.2.1. Viruses

Viruses are acellular but are considered to be biological entities because they possess genomes and are able to adapt/evolve to infect particular hosts (Van Regenmortel *et al.*, 2013). Because they are subject to evolution like all other biological entities, they have recently been classified into the same taxonomic categories—from realm to species—as all other forms of life (Walker et al, 2020). Some viruses have DNA genomes, but unlike other forms of life, many viral genomes are based on RNA, tobamoviruses being one group with RNA genomes that resemble messenger RNAs (mRNAs). Even more unusual, the genomes of a subset of RNA-based virus are in the negative-sense—complementary to the mRNA sense. The genomes of viruses may or may not be enclosed in protein capsids with or without lipid membranes. Viruses cannot replicate alone and must infect cells and use components (enzymes, membranes) of the host cell (animals, plants, bacteria) to make copies of themselves (Hegde *et al.*, 2009). Thus, they are obligate parasites.

According to the International Committee on Taxonomy of Viruses (ICTV), there were 9110 virus species belonging to 189 families from 59 orders officially recognised in 2021 (https://talk.ictvonline.org). This number undoubtedly underrepresents the extent of virus diversity. Because they are obligate parasites, viruses have intimate links with all cellular life, and may play important roles in ecosystem function.

1.2.1.1. Spillover

Pathogen spillover (also known as emergence) is a term that describes the infection of a new host species from a natural reservoir population (Power & Mitchell, 2004). Spillover is more likely to

occur within species of the same genus, but it may also occur between members of a family or order.

For a virus spillover to occur, several barriers must be overcome:

1. Encounter: The original source of the virus and the potential new host must come into close proximity and exposure must occur.

2. Vector: For some viruses, but not tobamoviruses, a vector is required to transmit the virus between the original host and the new host.

3. Cellular contact: The virus must interact with cellular receptors on the surface of the cell in the new host.

4. Nuclear/cytoplasmic entry, replication, systemic spread: the virus's genome must be imported into the cell of the new host and replicate there. Some virus genomes must be imported into the nucleus of the cell.

5. Overcoming host immunity: During replication, the host may mount an immune response against the virus. The virus must overcome these.

6. Virion release/systemic spread within the host: The virus is released from the cell to infect other cells.

7. New host to new host infection: The virus is transmitted to another individual of the new species.

There may be a requirement for several genetic changes to occur within the viral genome to achieve all or most of the above steps.

The only reported spillover of YTMMV from the indigenous flora to an exotic species was recorded by Wylie & Li, 2017, in this case two plants of *Capsicum annuum* collected from a commercial crop located near the town of Carnarvon, Western Australia. The vegetable farm is located adjacent to natural vegetation where wild solanaceous plants occur, including species of *Solanum*, *Physalis*, *Nicotiana* and *Anthocercis*. It seems likely that the original source of the virus was the indigenous solanaceous plants growing nearby, although this was not tested. It remains to be seen whether YTMMV has emerged as a pathogen in solanaceous crops or in exotic weeds of the same family.

1.2.1.2. Tobamoviruses

Tobamoviruses have coevolved with angiosperms for about 110 million years ago (Gibbs *et al.*, 2015). They are characterized by a typical rod-shaped particle morphology with a diameter of 18 nm, 300-310 nm in length (Adams *et al.*, 2017). The single-stranded RNA (+ssRNA) sense genome of 6.2 to 6.4 kb encoding four Open Reading Frames (ORF). ORF1 and ORF2 are separated by a leaky stop codon and encode non-structural proteins that have a mass of 126 and 183 kDa, respectively. ORF3 on the large sub-genomic RNA encodes the non-structural movement protein (MP) of 30 kDa. ORF4 on the small sub-genomic RNA encodes coat protetin (CP) subunits of 17-18 kDa (Luria, 2017).

Tobamoviruses infect plants of the Solanaceae, Brasscicaceae, Cactaceae, Apocynaceae, Cucurbitaceae, Malvaceae, Leguminosae, Passifloraceae, and Orchidaceae. The viruses are subdivided based on host range and phylogeny (Dorokhov *et al.*, 2018). According to the ICTV,

in 2020, genus *Tobamovirus* comprised 37 ratified species (https://talk.ictvonline.org/ictv-reports/ictv_online_report/positive-sense-rna-viruses/w/virgaviridae/672/genus-tobamovirus online meeting, October 2020). Based on genome organization and phylogenetic clustering, tobamoviruses are classified into three subgroups (Lartey *et al.*, 1996). One includes most of the viruses with rosid primary hosts (blue branches in Fig. 1.1), and the other two have asterid and caryophyllid hosts (pink and green, respectively in Fig. 1.1) (Gibbs *et al.*, 2015).

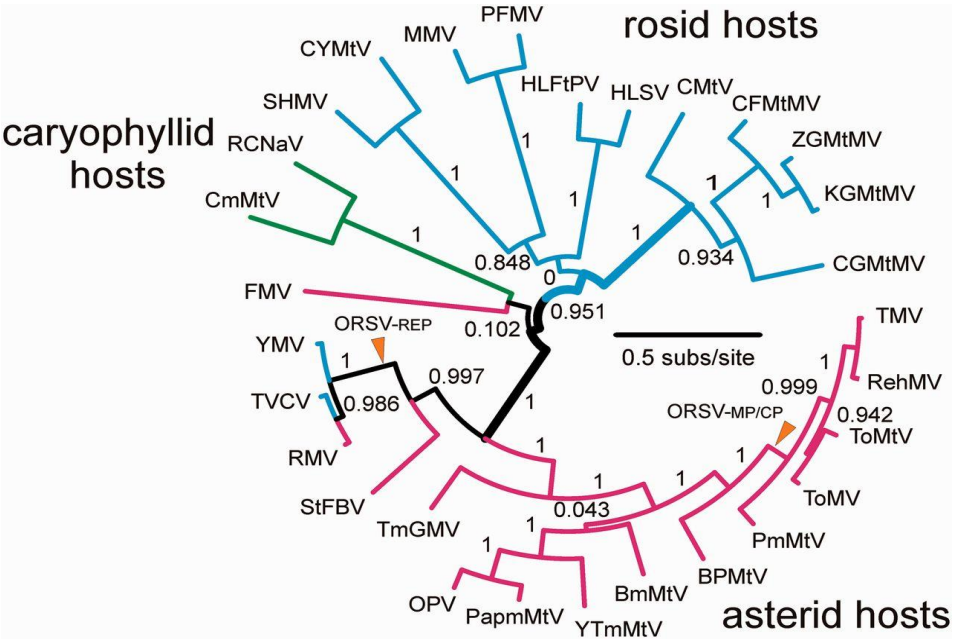


Figure 1.1 The phylogeny of twenty-nine tobamoviruses calculated from 100 percent consensus concatenated amino acid sequences of their three main proteins; replicase, MP and CPs (Gibbs *et al.*, 2015)

Tobamoviruses are among the most studied plant viruses and are used as models for the study of virus evolution (Fraile & García-Arenal, 2018). Several tobamovirus species are considered very damaging pathogens of some crops, especially those infecting members of the Solanaceae and Cucurbitaceae (Dombrovsky & Smith, 2017). Tobacco mosaic virus (TMV) is a major viral pathogen across 200 hosts from 30 families, especially in solanaceous plants, including tobacco (*Nicotiana tabacum*) and food crops like tomato (Iftikhar *et al.*, 2015). Currently, yield losses for tobacco due to TMV are estimated at only 1% because the N gene from the South American tobacco species *N. glutinosa* was introgressed and provides resistance to TMV in most commercial varieties of *N. tabacum* (Lewis *et al.*, 2005). In contrast, yield loss in bell pepper caused by TMV have been reported to be as high as 90% (Chitra *et al.*, 2002). Furthermore, poor

fruit quality may reduce the value of the crop on the commercial fresh market. Chewing insects such as grasshoppers and caterpillars do occasionally transmit the virus on their mouthparts (Parizipour & Shahriari, 2020; Mphuthi, 2017). They are not considered important vectors. Virus-contaminated soils may play a role in transmission of TMV (Yang *et al.*, 2012).

Tomato mosaic virus (ToMV) was reported for the first time on tomato in 1909 in the US and was the most persistent virus in terms of its ability to survive outside plant cells and in dead tissues (Broadbent, 1976). ToMV is the most troublesome viral disease of tomatoes, distorting leaves and fruit and stunting of growth. Besides that, this virus also infects pepper, potato, apple, pear, cherry, and numerous weeds, including pigweed and lamb's quarters. ToMV is transmitted on the surface of seeds on tomato (external contamination).

Tomato mottle mosaic virus (ToMMV) was first described in 2013 infecting tomato in Mexico. This new species caused rapid necrosis on the upper leaves of tomato seedlings or mosaic patterns and deformation on leaves on mature plants (Li *et al.*, 2013). After the first report, this virus was quickly detected in the United States (Webster *et al.*, 2014; Fillmer *et al.*, 2015), Spain (Ambrós *et al.*, 2016), Israel (Turina *et al.*, 2016), and China (Li *et al.*, 2014), indicating that it had been spreading for some time. It was also detected recently by Australia on capsicum seeds for sowing exported from the Netherlands (https://www.agriculture.gov.au/import/goods/plant-products/seeds-for-sowing/emergency-measures-tommv-qa#to-which-host-species-do-the-emergency-measures-apply). The close phylogenetic relationship between this new viral species to ToMV and TMV has been demonstrated (Li *et al.*, 2017). To respond to the emerging risk of ToMMV, Australia has implemented emergency measures for tomato and capsicum seed. ToMV and ToMMV are both considered major viral threats to tomato production (Nagai *et al.*, 2019).

Cucumber green mottle mosaic virus (CGMMV) was discovered in England in 1935 by Ainsworth (as described in Hollings, 1975). The most problematic of cucurbit-infecting tobamoviruses, CGMMV is currently considered a significant threat to the production of cucumber, melon, watermelon, gherkin, and pumpkin (Dombrovsky, 2017). The typical symptoms of CGMMV include mosaic patterning of infected leaves and fruit distortions (Komuro, 1971). The occurrence of CGMMV has been reported in Europe (e.g., Denmark, Finland, Germany, Greece, Holland, Norway, Russia, and Spain), United States, Asia (e.g., China, India, Indonesia, Japan, Korea, Pakistan, Thailand), Australia and the Middle East (Iran, Israel, and Saudi Arabia) (Moradi & Jafarpour, 2011; Zhang *et al.*, 2009; Yoon *et al.*, 2008; Zhou *et al.*, 2008; Ali *et al.*, 2004; Varveri *et al.*, 2002; Vani & Varma, 1993; Antignus *et al.*, 1990; Inoue *et al.*, 1967). In Australia, CGMMV was first detected in the Northern Territory in 2014 (Tran-Nguyen *et al.*, 2015; Tesoriero *et al.*, 2016) and is now widespread there. Queensland (2015 and 2017) and Western Australia (2016) have also had outbreaks of CGMMV. CGMMV also detected in New South Wales in 2019 (https://www.dpi.nsw.gov.au).

CGMMV is an economically significant seed-transmitted virus, which causes yield losses of about 15% in cucurbitaceous crops (Antignus *et al.*, 2001; ISTA, 2010; Shang *et al.*, 2011). CGMMV can be transmitted through contaminated water and soil, and sap of the plants infected with the virus. Seed and soil transmissions are recognized as the primary sources for epidemic development of CGMMV disease (Tan *et al.*, 2000; Choi, 2001; Mandal *et al.*, 2008; Liu *et al.*, 2013; Nematollahi *et al.*, 2013). The early spread pattern in cucumber, which is different in the fruit-harvesting stage, shows that infected plants are scattered throughout the field (Choi, 2001; Mandal *et al.*, 2008).

Tomato brown rugose fruit virus (ToBRFV) is a novel tobamovirus, spreading rapidly since reported in Jordanian tomato greenhouses in 2015 (Salem *et al.*, 2016) and has posed an emerging threat to tomato and pepper production around the world in recent years (Chanda *et al.*, 2021). The wide distribution of ToBRFV has been attributed to the global movement of seed. The major resistance genes (*Tm-1, Tm-2, Tm-22*) in tomato that provide broad resistance to this group of viruses is not effective against ToBRFV (Luria *et al.*, 2017). Recent greenhouse experiments have shown ToBRFV is transmission in pollen carried by bumblebees (*Bombus terrestris*) (Levitzky *et al.*, 2019). Therefore, ToBRFV not only can be transmitted through contact, seeds, seedlings, grafts and cuttings, but also spreading by insect pollinators.

1.2.1.3. Yellow tailflower mild mottle virus

Yellow tailflower mild mottle virus (YTMMV) was first reported in 2014 from a wild plant of yellow tailflower (*Anthocercis littorea, Family Solanaceae*), a member of a genus endemic to Western Australia (WA) (Wylie *et al.*, 2014). Wild-infected plants may be difficult to identify from symptoms alone. It is the first member of the *Tobamovirus* genus to be identified only from the Australian flora. Its natural distribution amongst indigenous plants, and lack of reports of its occurrence elsewhere, suggest it is indigenous to Australia (Wylie *et al.*, 2015). The closest relatives of YTMMV are obuda pepper virus (OPV) and paprika mild mottle virus (PaMMV), both of which are transmitted naturally by contact in pepper (Hancinský *et al.*, 2020). The first detection of PaMMV was in *C. annuum* in the Nertherlands in 1977 and then in Bulgaria and Japan (Yordanova & Stoimenova, 2008; Hamada, *et al.*, 2003). This raises the question of the origin of YTMMV. Was it ‘marooned’ in Australia after the split up of the super-continent Gondwanaland, or did it arrive after the continent of Australia was formed?

Under experimental conditions the virus infected plants of *Solanum lycopersicum, S. betaceum, S. melongena, S. nigrum, C. annuum, Nicotiana* (19 species), and *Physalis* (3 species) (Li *et al.*, 2016; Wylie *et al.*, 2015). Many of the indigenous solanaceous species present in the WA are desert plants. Indigenous solanaceous plants include species of *Solanum, Nicotiana* (Fig 1.2) and *Anthocercis* (Frontispiece).

Figure 1.2 *Nicotiana benthamiana* plant (foreground) growing in Karijini National Park, Western Australia. Photo taken by Steve Wylie, July 2020

A survey of distribution and natural host range of YTMMV was carried out on 89 wild and cultivated plants from *Anthocercis littorea, Cuscuta epithymum, Datura inoxi, Hardenbergia comptoniana, Nicotiana rotundifolia, N. occidentalis subsp. obliqua, A. ilicifolia subsp. ilicifolia, A. gracilis, A. viscosa, N. occidentalis subsp. hesperis, Solanum lasiophyllum, S. oldfieldii*, an unidentified species of *Solanum*, and *C. annuum* in Western Australia, from Coral Bay in the north to Esperance in the south. The survey revealed nine solanaceous plant species that were naturally infected with YTMMV, including two *C. annuum* plants from a commercial planting located in Carnarvon, Western Australia (Wylie & Li, 2017).

The resilience of YTMMV virions was tested in dry leaf tissue over-time periods from one hour to one year under temperatures ranging from -80ºC to 160ºC (Koh *et al.*, 2018). Infectivity was maintained for at least a year when incubated at -80 or 22°C, or at fluctuating ambient temperatures of 0.8 to 44°C, but incubation under dry conditions at 160°C for >4 days eliminated infectivity.

1.2.2. Tobamovirus transmission between plants

Plant viruses spread naturally through four main transmission pathways: vectors, seed, pollen and contact (Hamelin *et al.*, 2016). Vectors provide the most important means of horizontal transmission from host to host for many virus species (Bragard, 2013). Vectors are taxonomically very diverse, with most being arthropods, but including some fungi and nematodes (Hull, 2014). The virus–vector interaction occurs at the protein level where a specific viral protein(s) interacts with a specific vector protein(s) (Froissart *et al.*, 2002).

The tobamoviruses are unusual among plant viruses in that vectors for them have not been described (Almeida *et al.*, 2018). Arthropods are known to transmit some tobamoviruses

mechanistically as virions on contaminated mouthparts (Hoggan, 1931; Walters, 1951; Newton, 1953; Chant, 1959; Rao & Varma, 1984), but this form of transmission is not vectored because no specialised protein-protein interaction occurs. Tobamoviruses utilise other means by which to transmit to new hosts. Many tobamoviruses are seed-borne, and so are able to transmit between generations within seed. As international trade in plant propagules has become a major industry, tobamoviruses have been transported around the world in contaminated seed (Revers & Garcia, 2015). Many are also pollen-borne, using bees or other pollinators to carry infected pollen to new hosts (Mink, 1993).

These modes of tobamovirus transmission are discussed below.

1.2.2.1. Contact: leaves, roots, humans

Tobamoviruses can be transmitted to new hosts by contact between plants. This mode of transmission makes human beings an agent of transmission of tobamoviruses in crops (Carlye & Scott, 2000). The virions are stable and survive in sap for many years and can remain infectious on contaminated surfaces such as farm equipment for long periods (Holdings *et al.*, 1975; Antignus, 2012; AUSVEG, 2017;). Virions persist on clothing and workers' hands. TMV is known to survive in cigarettes and cigars made from infected *N. tabacum* leaves and to be transmitted from the hands of a smoker to susceptible plants (Baker & Adkins, 2000). In natural settings, sublethal wounding of plant cells occurs during normal leaf abrasion in the wind and possibly root contact, as well as contact with animals (Franc & Bantarri, 2001; Sacristán, 2011).

Critical factors for successful transmission by contact include:

1. High concentrations of virus particles within the epidermal cells of infected plants.

2. The leaf is slightly damaged and enables virus particles to penetrate its cuticle and cells where the virus can replicate (Bawden, 1964; Matthews, 1981).

1.2.2.2. Seed transmission

Tobamoviruses such as TMV, ToMV and pepper mild mottle virus (PMMoV) can be transmitted on the surface of seeds harvested from infected plants. Many factors affect to seed transmission rates such as the host cultivar, the virus isolate, environmental conditions, the timing of infection, vector characteristics, and viral synergism which is happened by co-infection of two unrelated viruses, causing more severe symptoms or increased titres of one or both viruses (Simmons & Munkvold, 2014). Some tobamoviruses adhere to the outside surfaces of the seed and infect the emerging seedling. Tobamovirus infection of the internal seed layers, which rarely includes the embryo, may partially follow the direct invasion pathway of potyviruses such as pea seed-borne mosaic virus (PSbMV) to the pea embryo (Dombrovsky & Smith, 2017).

Incidences of seed transmission of specific viruses vary between host plant species and between cultivars. Infection of the vegetative tissues and the maternal testa occurred irrespective of the virus's capability to be transmitted via seeds. A high incidence of seed transmission occurred in direct relation to virus invasion of an immature embryo (Fabre *et al.*, 2014).

Even seed transmission at very low rates can have a significant effect on the epidemiology of tobamoviruses in crops. Virus in field plants can spread initially to neighbouring plants by contact and then by pollen at flowering time (Coutts *et al.*, 2009). Vertically-infected seedlings often do not exhibit symptoms of viral infection (Simmons & Munkvold, 2014).

1.2.2.3. Pollen transmission

Pollen and seed transmission are closely related (Mink, 1993; Hull, 2004). Pollen-transmitted viruses are generally also transmitted by seed, but not necessarily vice versa, e.g., broad bean stain virus is seed-transmitted but not pollen-transmitted (Brunt *et al.*, 1996). Considerably more research has focused on seed transmission because the seed is an important means of virus dissemination for several economically important vegetable and potential worldwide spread and fruit crops (Mink, 1993). Seed transmission occurs either through contamination of the seed surface or maternally derived seed parts, or, more commonly, through embryo infection. The embryo may become infected either directly during embryogenesis or indirectly via infection of the reproductive tissues (i.e., ovule, megaspore mother cell or pollen mother cell) before embryogenesis (Johansen *et al.*, 1994).

Pollen transmission of tobamoviruses is a more effective mechanism of long-distance transmission than contact because it does not require direct contact between closely-growing plants.

The tobamovirus CGMMV is transmitted by pollen under laboratory conditions (Liu *et al.*, 2014; AUSVEG, 2017). TMV is transmitted *via* pollen by bumblebees between tomato plants in greenhouses (Okada *et al.*, 2000). When a virus is transmitted by pollen, it may systemically-infect the plant through the fertilized flower (horizontal transmission), or more commonly, it may infect the ovule and thus the seedling that will grow from the seed that develops from the ovule (vertical transmission) (Hull, 2004).

1.3. Aims of this research

Currently we know YTMMV naturally infects indigenous host plants along a long coastal strip that extends 1600 km from Coral Bay in the north to Esperance in the south. How it achieved this wide distribution is unclear, but it was probably not by leaf contact alone. The main aim of this project is to study modes of transmission of yellow tailflower mild mottle virus, including contact by roots, mechanistically by insect feeding, and pollen- and seed-borne transmission. Initial work in our group showed there was uneven root transmission of the virus, but nothing is presently known about pollen and seed transmission for YTMMV. We will also undertake study to understand epidemiological aspects of a possible spillover event of YTMMV in a field trial and to study the influence of host plant species and age-at-infection on symptomology and potential crop losses. We are in the unusual position to study modes of transmission and to hypothesise about the risks of this wild-plant tobamovirus before it spills over to exotic solanaceous species in the region.

I. Modes of YTMMV transmission

- Investigate vertical transmission of YTMMV through seed (Chapter 2)
- Investigate transmission of YTMMV through pollen (Chapter 2)
- Investigate root contact and graft transmission of YTMMV (Chapter 3)
- Investigate mechanistic insect transmission of YTMMV (Chapter 4)

II. Influence of YTMMV infection on symptomology and yield of plants such as:

- *Solanum lycopersicum* 'Tigerella'
- *Capsicum annuum* 'Californian Wonder'

- *Capsicum annuum* ‘Jalapeno’? (Chapter 5)

Chapter 2: Seed and pollen transmission

2.1. Introduction

The term “seed transmission” refers to the passage of viruses through seeds to plants in the subsequent generation (Sastry, 2013). There are two distinct types of seed transmission of plant viruses: embryonic and non-embryonic. Viruses that undergo embryonic seed transmission are able to infect the embryo either directly from maternal tissue or indirectly from paternal pollen. There is usually a high rate of infection among new seedlings. Non-embryonic seed transmission occurs when viruses are carried on the surface of seeds, and not in the embryo.

Tobamoviruses are usually described as residing in the seed coat and the endosperm but not the embryo (Broadbent, 1965; Crowley, 1957; Demski, 1981; Nagai, 1974; Taylor *et al.*, 1961; Agarwal & Sinclair, 1996; Hull, 2002; Reingold *et al.*, 2015; Dombrovsky & Smith, 2017). This mechanism allows the seeds to remain infectious for a long period (Reingold *et al.*, 2015). When the seeds are contaminated, the virions found on the outer layers of the seed coat infect the cotyledons through wounds that form during germination, although this does not occur for all tobamovirus-contaminated seeds (Dombrovsky *et al.*, 2017). Ellis *et al*., (2020) detected TMV in embryos in tobacco seed, demonstrating that TMV undergoes embryonic seed transmission in tobacco. The information known about the location of tobamoviruses in infected seed is listed in Table 2.1.

Table 2.1 Tobamovirus virion location in the seed

Tobamovirus species	**Host**	**Location**	**References**
Tobacco mosaic virus	*Capsicum annuum*	Seed coat	Crowley, 1957; Sakamoto & Matsuo, 1972
Tobacco mosaic virus	*Solanum lycopersicum*	Seed coat, endosperm	Broadbent, 1965
Tomato mosaic virus	*S. lycopersicum*	Seed coat, endosperm	Broadbent, 1976
Tobacco mosaic virus	*Arabidopsis thaliana*	Seed coat	De Assis Filho & Sherwood, 2000
Cucumber green mottle mosaic virus	*Cucumis sativus*	Seed coat, endosperm	Shargil *et al.*, 2019
Tobacco mosaic virus	*Nicotiana tabacum*	Embryo	Ellis *et al.*, 2020
Tomato brown rugose fruit virus	*S. lycopersicum*	Seed coat, endosperm	Davino *et al.*, 2020
Pepper mild mottle virus	*C. annuum*	Seed coat	Genda *et al.*, 2005; Genda *et al.*, 2011

Other factors affecting rate of transmission are host cultivar, virus isolate, environment, and the lifecycle stage of the host when infection occurs (Sastry, 2013; Simmons & Munkvold, 2014). CGMMV, the most economically important of the five main cucurbit-infecting tobamoviruses (Gibbs *et al.*, 2015; Reingold *et al.*, 2016), can transmit by seed in eight different cucurbit crop species (Dombrovsky *et al.*, 2017). Seed transmission tends to reduce over time as seed is stored. For example, CGMMV seed transmission rate after one month in storage was 8%, reducing to 0.1% after five months (Demski, 1981; Nagai, 1981; Nishimura, 1962).

Chemical and physical measures can be used to control the seed transmission of tobamoviruses. However, chemical treatment alone is not sufficient to eradicate the virus from infected seeds, and physical treatment can have serious consequences on seed longevity depending upon the seed condition (Demski, 1981; Nagai, 1981; Rast & Stijger, 1987).

Pollen transmission is a means of long-distance tobamovirus transmission. Pollen transmission is divided into two possible modes of transmission: horizontal, where virions in the pollen infect somatic tissue and spread within the mother plant, and vertical, where the ovule or other parts of the seed are infected via the pollen (Mink, 1993; Card *et al.*, 2007; Isogai *et al.*, 2017). In vertical transmission, there are two possible routes the virus can take to infect the seed. The virus may enter the seed through the placenta from the mother plant's vascular system, or it may enter from the pollen tube from infected pollen. This virus-infected pollen may either originate from the mother plant (self-pollination) or from another plant (cross-pollination). It may even be possible for virus-infected pollen from another plant species to transmit a virus to the ovule. It should be noted that virus-infected pollen may also transmit viruses horizontally from an infected to an uninfected plant (Fig 2.1) (Matsushita *et al.*, 2018).

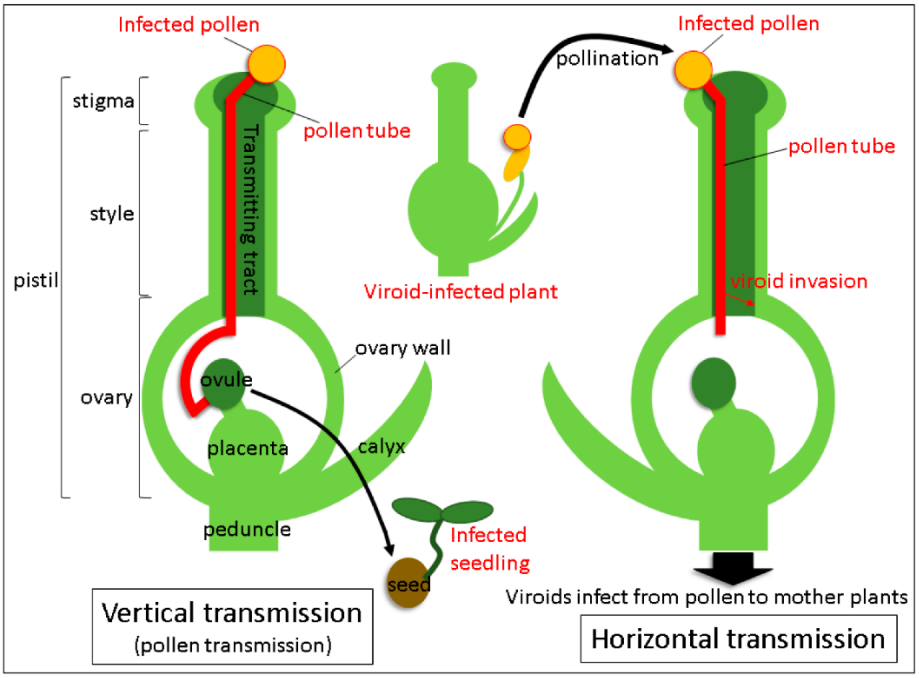


Figure 2.1 Two possible transmission routes of a viroid *via* pollen. Vertical transmission involves infection of seedlings in the subsequent generation via seed that has been infected by virus in pollen. Horizontal transmission occurs when virus-infected pollen transmits the virus to somatic cells of the recipient plant. This image describes the transmission of a viroid, but the same routes apply to tobamovirus-infected pollen (Matsushita *et al.*, 2018)

Pollen transmission occurs in viruses from several plant virus genera. Vertical transmission occurred more prequently than horizontal transmission. While more than 45 plants are vertically transmitted by pollen, 18 of these viruses are horizontally transmitted by pollen (Mink, 1993; Card *et al.*, 2007; Isogai *et al.*, 2020).

Some tobamovirus species were reported to be pollen transmitted. CGMMV was transmitted in pollen to cucumber with the rate from 17 to 51% of fruit compared with 33.3–100% for mechanically inoculated plants (Lui *et al.*, 2014). Under protected cropping conditions, CGMMV, TMV, and PePMV, were spread by pollinating bumble bees or honeybees (Darzi *et*

al., 2018; Lacasa *et al.* 2003; Okada *et al.*, 2000; Shipp *et al.*, 2008; De Assis Filho & Sherwood, 2000).

The experiments described in this chapter were designed to answer if YTMMV is transmitted through seed and pollen. We wished to determine if treatment of the external surface of the seed eliminated infection, and if horizontal and vertical transmission occurred at pollination.

Aims of the experiments:

1. Study YTMMV transmission by seed of solanaceae plants
2. Evaluate the effectiveness of commercial disinfectants against seed transmission of YTMMV
3. Determine whether YTMMV can be transmitted via pollen in *Nicotiana tabacum*

2.2. Materials and Methods

2.2.1. Virus

YTMMV inoculum used in all experiments of this project was collected originally from a mature yellow tailflower plant (*Anthocercis littorea* Labill.) growing close to the entrance to of Nambung National Park (GPS -30.60346, 115.15467), and located about 15 km to the south-east of the township of Cervantes in South-Western Australia (Wylie *et al.*, 2014). The isolate was named YTMMV-Cervantes and its genome sequence is retained in GenBank under accession KF495564. The isolate was maintained mechanically on *Nicotiana benthamiana* accession RA-4 and *Solanum betaceum* seedlings by manual inoculation after grinding infected leaves in 0.1 M sodium phosphate buffer (pH 7.0) and diatomaceous earth (Sigma). Plants were grown in a temperature-controlled and insect-proof greenhouse (22°C days, 17°C nights).

To avoid contamination of other plants with the virus, infected plants were maintained apart from uninfected plants, and handwashing before and after entering the facility was practiced.

2.2.2. RNA extraction, cDNA synthesis, and PCR amplification

500 µl Trizol Reagent (ThermoFisher) was used to extract RNA from 40 mg leaf samples, following the manufacturer's protocol, and then re-suspended in 30 µL of RNase-free water and stored at -20°C until reverse-transcription (RT) using random primers. Samples were prepared using GoScript reverse transcriptase (Promega) with a random primer (Table 2.1), that produces products of 574 nucleotide (nt) fragments of the coat protein (CP) gene was amplified using MP1R and CP1R with synthesize the coat protein region of 917 nt and 612 nt, respectively. PCR primers were MP1R and MP1F; CP1R and CP1F (Table 2.1) (Koh *et al.*, 2017).

PCR was conducted using GoTaq Green Mastermix (Promega) following the cycling steps of 95°C for 3 min; 25 cycles of 95°C for 30 s, 55°C for 45 s, 72°C for 90 s; and a final extension of 72°C for 10 min. The resulting amplification products were analyzed on a 1% TAE agarose gel.

Table 2.2 Primers used to detect YTMMV (Koh *et al.*, 2017)

Primer	Sequence (5' to 3')	Purpose/target	Expected size of amplicon (bp)
Random primer	CGTACAGTTAGCAGGCNNNNNNNN NNNN (Where N represents any nucleotide)	Synthesize cDNA	574
MP1F	ACGAGGCAATAGGGGAAGTT	Synthesize movement protein region	917
MP1R	GCAAACTGCTTAGGTGAAGTGA		
CP1F	CGCTTAAAGAGCGAATTGATG	Synthesize coat protein region	612
CP1R	CCAAACAGCCAAACCCTTC		

2.2.3. Seeds

Six solanaceous species/lines were used in this project, namely *N. benthamiana* accessions RA-4 and MtA-6 (Wylie *et al.*, 2015), *Solanum lycopersicum* 'Tigerella', *Capsicum annuum* 'Californian Wonder'; *Capsicum annuum* 'Jalapeno'; *Nicotiana tabacum* 'Wisconsin 38'. Plants were grown as below:

1. Soil: Potting mix (60:40 rotted bark: sand mix) to which 5 g each of lime and dolomite and 40 g each of slow-release NPK fertilisers Osmocote and Grower's Blue were added per 40 L.

2. Sowing: Seed of all of the above plants except those of *N. benthamiana* MtA-6 (which was treated with 10 ppm GA_3 overnight prior to sowing to break dormancy) was sown directly in pots filled with damp potting mix and then covered with a polythene bag to keep moisture in until germination, upon which time the polythene was removed.

3. Transplanting: Seedlings were transplanted at the 4-leaf stage.

4. Producing seeds for transmission experiments: To produce virus-infected seeds of *N. benthamiana* accessions RA-4 and MtA-6, *S. lycopersicum* 'Tigerella', *C. annuum* 'Californian Wonder'; *C. annuum* 'Jalapeno'; *N. tabacum* 'Wisconsin 38' we inoculated seedlings (usually at the 6-8 leaf stage) but later and for *N. benthamiana* accessions RA-4 and *C. annuum* 'Jalapeno' (before flowering) in order to keep infected plant still alive and produce seeds, confirmed presence of YTMMV in plants by symptoms and RT - PCR test, and collected seeds from fruit and dried in room temperature.

2.2.4. Seed transmission experiment

2.2.4.1. Is YTMMV transmitted by seed?

One thousand seeds of uninoculated and inoculated plants of *N. benthamiana* accessions RA-4 and MtA-6, *S. lycopersicum* 'Tigerella', *C. annuum* 'Californian Wonder', *C. annuum* 'Jalapeno', and *N. tabacum* 'Wisconsin 38' were collected. Five hundred seeds were harvested from YTMMV-infected plants and 500 seeds harvested from uninfected plants of each species. Seeds were divided into 20 groups of 50 seeds per group and sown separately. All seeds were sown directly (without any treatment) except seeds of *N. benthamiana* MtA-6, which were treated with gibberellic acid (as above). Seedlings numbers were recorded 7 days post-

germination (dpg). Fifty seedlings per species were selected randomly and then tested for the presence of YTMMV by RT- PCR (Table 2.1).

2.2.4.2. Do commercial disinfectants protect against seed transmission of YTMMV?

1250 seeds per species of uninoculated and inoculated plants of *N. benthamiana* accessions RA-4 and MtA-6, *S. lycopersicum* 'Tigerella', *C. annuum* 'Californian Wonder', *C. annuum* 'Jalapeno', and *N. tabacum* 'Wisconsin 38' were used.

Disinfection treatments were:

1) Water: The seeds were washed three times in sterile distilled water before sowing.

2) Bleach and ethanol (EtOH): Seeds were soaked in bleach (1% sodium hypochlorite) for 2 min, then the bleach was replaced with a solution of 75% EtOH. After 1 min, the ethanol was poured off, and the seeds were washed five times in sterile distilled water.

Five hundred seeds were from uninfected plants, and these were divided into two treatments: 250 seeds were used for controls (treatment 1) and 250 seeds were treated by bleach + EtOH (treatment 2). Of the remaining 750 seeds from infected plants, 250 seeds were used for controls (treatment 3), 250 seeds were treated with water (treatment 4), and 250 were treated by bleach + EtOH (treatment 5). The 1250 seeds were divided into 25 groups of 50 seeds per group. Seedlings were counted at 7 dpg and 50 seedlings per species were collected randomly and tested for the presence of YTMMV by RT-PCR (Table 2.1).

2.2.5. Pollen transmission

Twenty *N. tabacum* ‘Wisconsin 38’ plants (sixteen plants for cross pollination and four plants for control – self-pollinated) were grown in 5 L pots in laboratory conditions free of natural pollinators. And ten YTMMV-infected *N. tabacum* ‘Wisconsin 38’ plants were produced by manual inoculation and maintained in the separated greenhouse. To eliminate self-pollination, flowers were emasculated before anthers dehisced. Pollination was done by collecting pollen from dehisced anthers to a petri dish with a cotton bud. Pollination of emasculated flowers was done by transferring pollen on a new cotton bud. Each flower was pollinated once only and then covered by a new paper bag until fruits were mature.

Figure 2.2 Flowers of *Nicotiana tabacum* ‘Wisconsin 38’ plants were isolated by paper bags after pollination

Three weeks after pollination, plants were tested for the presence of YTMMV. There were two possible outcomes: 1) plants remained virus-free virus yet transmitted the virus in seed (vertical transmission), and 2) plants became systemically-infected with YTMMV (horizontal transmission). Seedlings from seeds of fruits in both situations were checked for YTMMV. The number of seeds from each fruit was counted. Seeds from each fruit were sown separately and then seedlings were collected in groups of 100 seedlings per group. RT–PCR assays were used to evaluate the virus vertical and horizontal transmission rate. The data for control was analysed from 10 fruits of four uninfected YTMMV plants including the criteria such as number of seeds per fruit, seed germination rate and seedlings transmission rate.

2.3. Results

2.3.1. Seed germination

YTMMV infection did not significantly affect seed germination rates (Fig 2.3). Germination rates varied between species, the highest rate of 94.4%, was seen in *S. lycopersicum* 'Tigerella' and *N. tabacum* 'Wisconsin 38', while *N. benthamiana* MtA-6 showed the lowest rate of 10%. The surface treatments of water and bleach plus ethanol did not significantly affect seed germination (Figs 2.3-2.9).

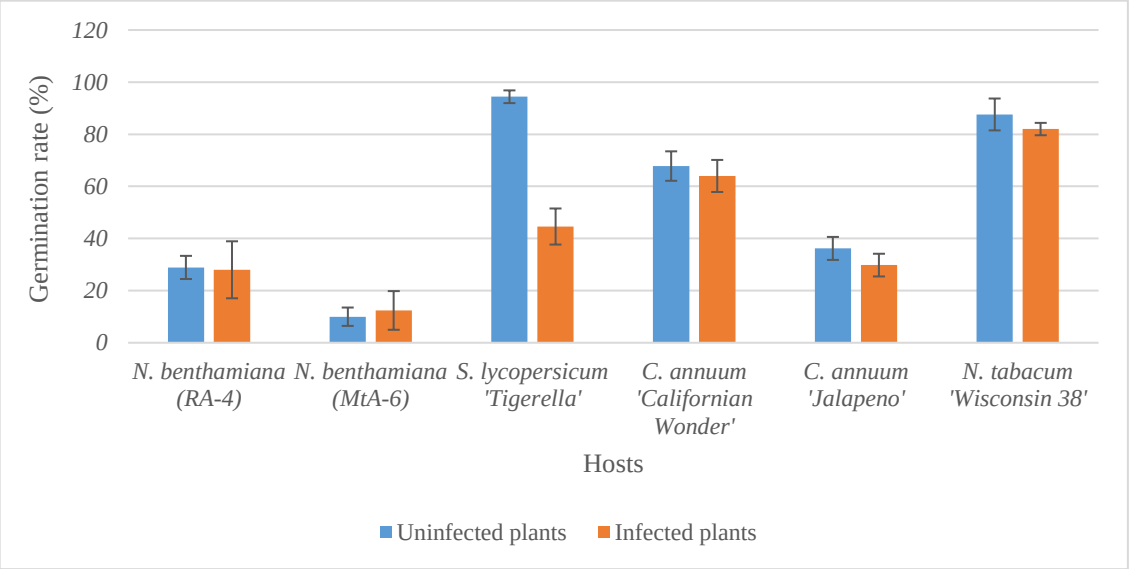


Figure 2.3 Comparison of germination rates of seed harvested from YTMMV-free (uninfected) plants (in blue) and YTMMV-infected plants (in orange) of six species/accessions

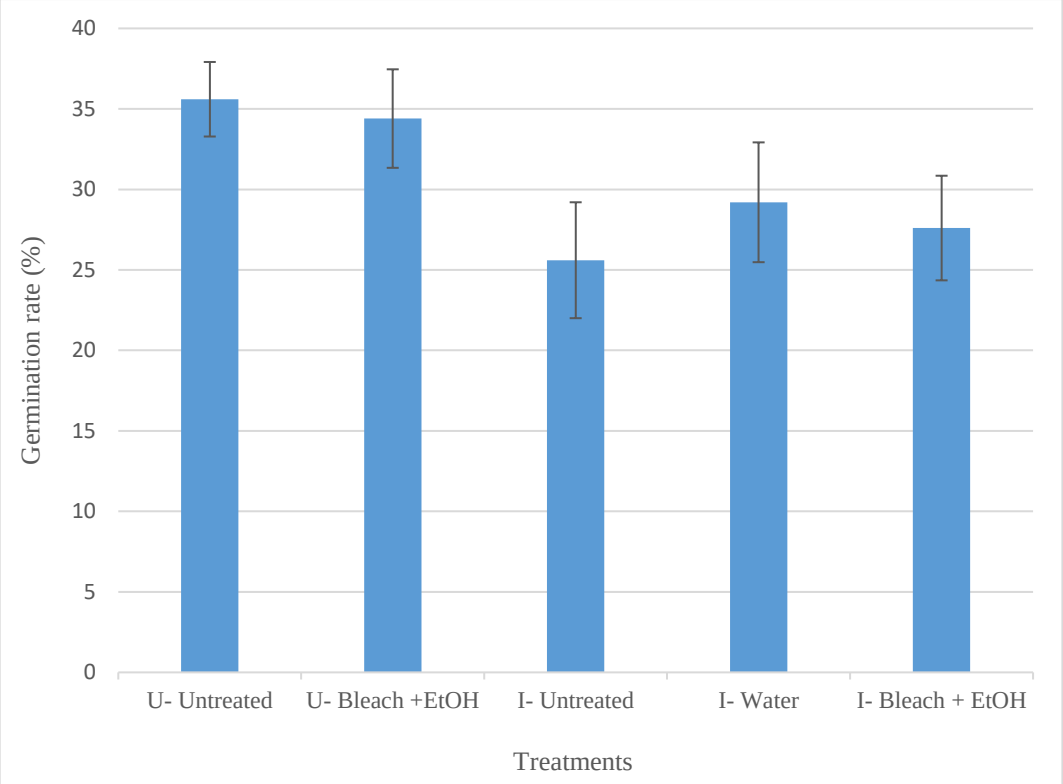


Figure 2.4 Comparison of the germination rate of *Nicotiana benthamiana* RA-4 seeds harvested from YTMMV-free plants and YTMMV-infected plants under five different treatments

Treatment 1 (U-Untreated): Seeds from uninfected plants, no treatment (control – uninfected seed – untreated chemical)

Treatment 2 (U-Bleach+EtOH): Seeds from uninfected plants treated by bleach and ethanol

Treatment 3 (I-Untreated): Seeds from infected plants germinated naturally (control – infected seed – untreated chemical)

Treatment 4 (I-Water): Seeds from infected plants and cleaned by water before sowing

Treatment 5 (I-Bleach+EtOH): Seeds from infected plants treated by bleach and ethanol

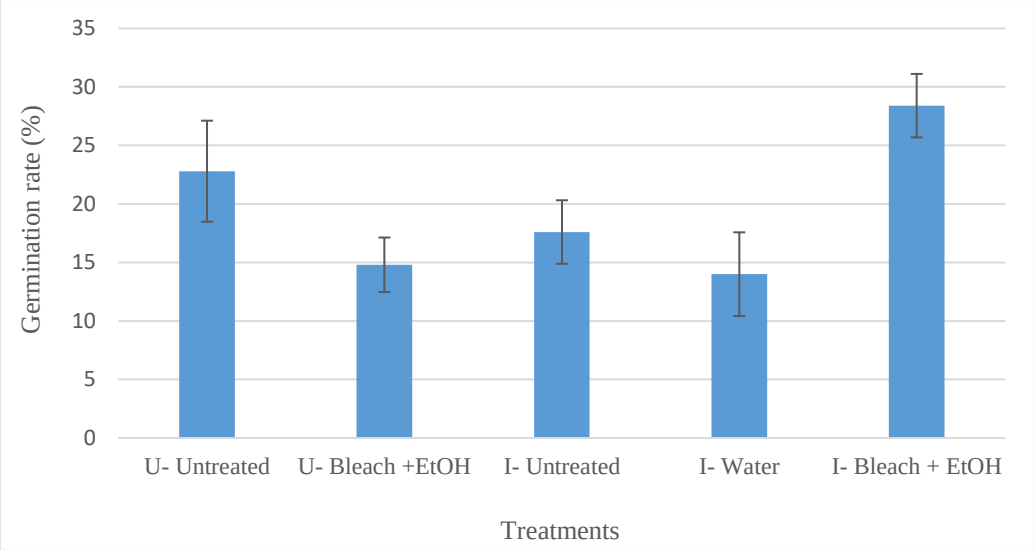


Figure 2.5 Comparison of the germination rate of *Nicotiana benthamiana* MtA-*6* seeds harvested from YTMMV free plants and YTMMV infected plants under five different treatments

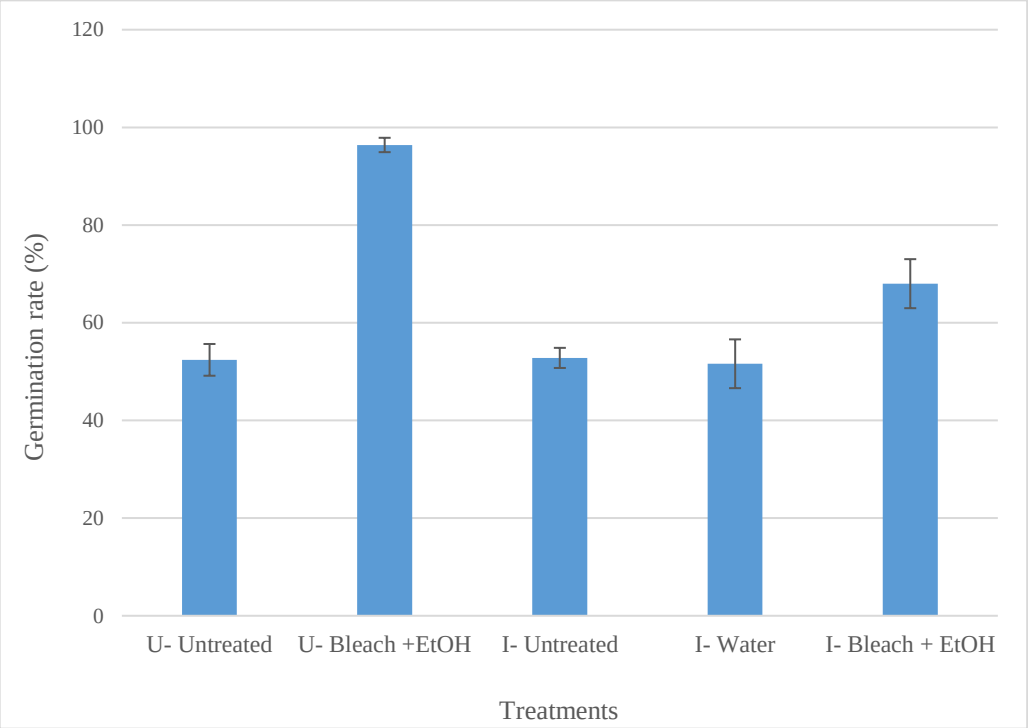


Figure 2.6 Comparison of the germination rate of *Sonalum lycopersicum* 'Tigerella' seeds harvested from YTMMV free plants and YTMMV infected plants under five different treatments

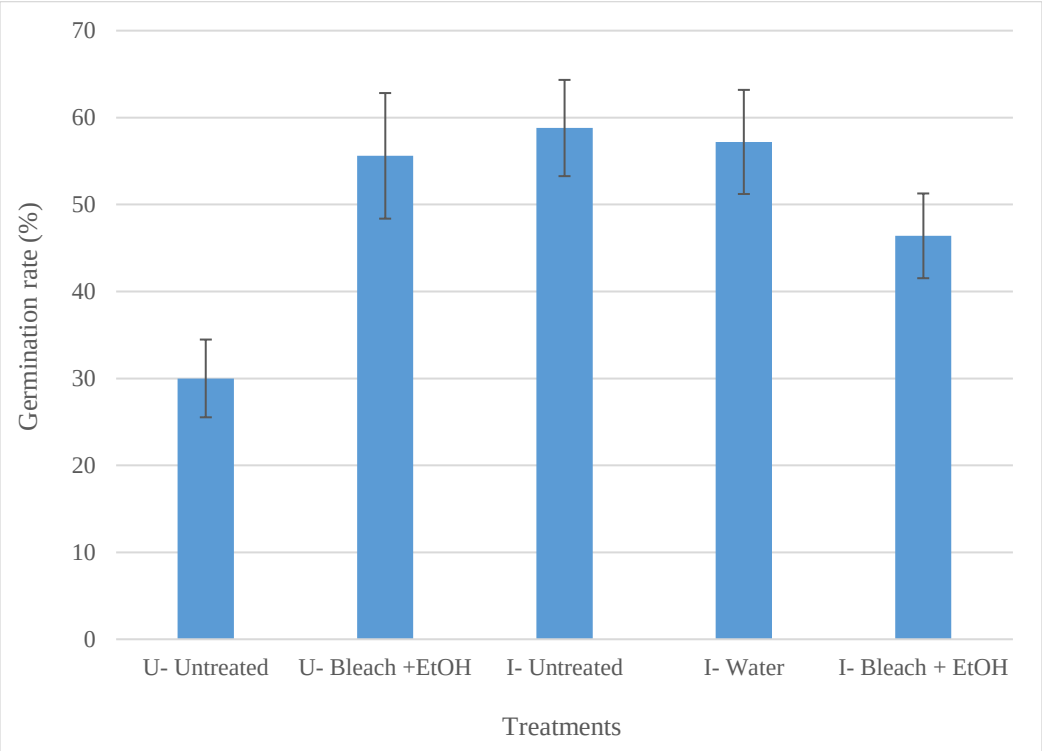


Figure 2.7 Comparison of the germination rate of *Capsicum annuum* 'Jalapeno' seeds harvested from YTMMV free plants and YTMMV infected plants under five different treatments

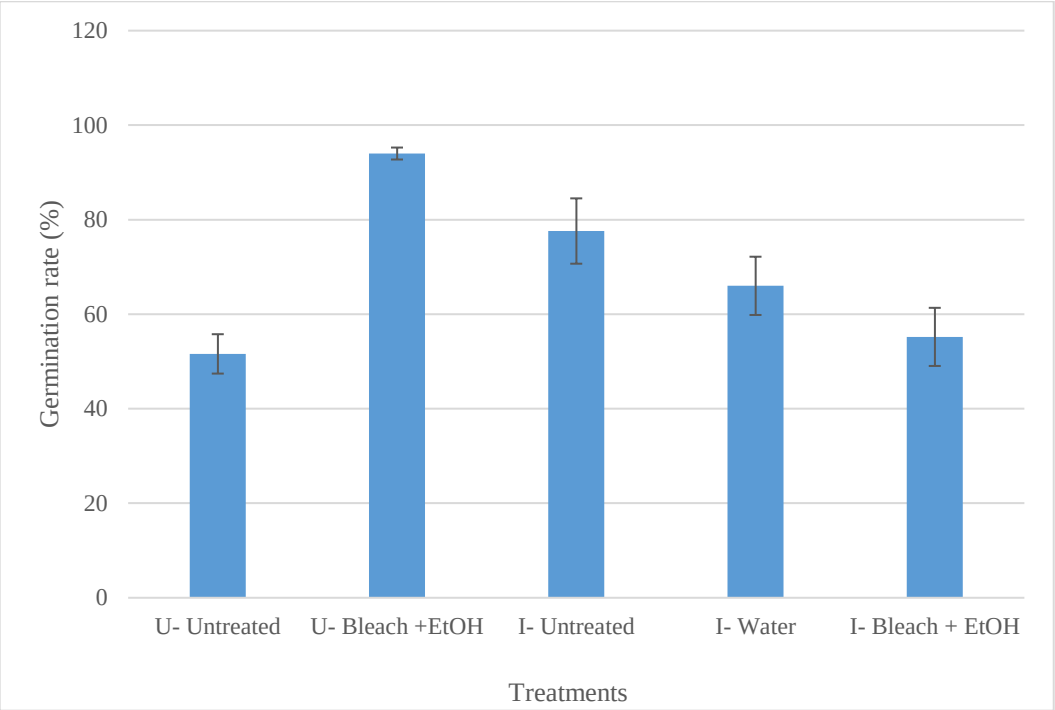


Figure 2.8 Comparison of the germination rate of *Capsicum annuum* 'Californian Wonder' seeds harvested from YTMMV free plants and YTMMV infected plants under five different treatments

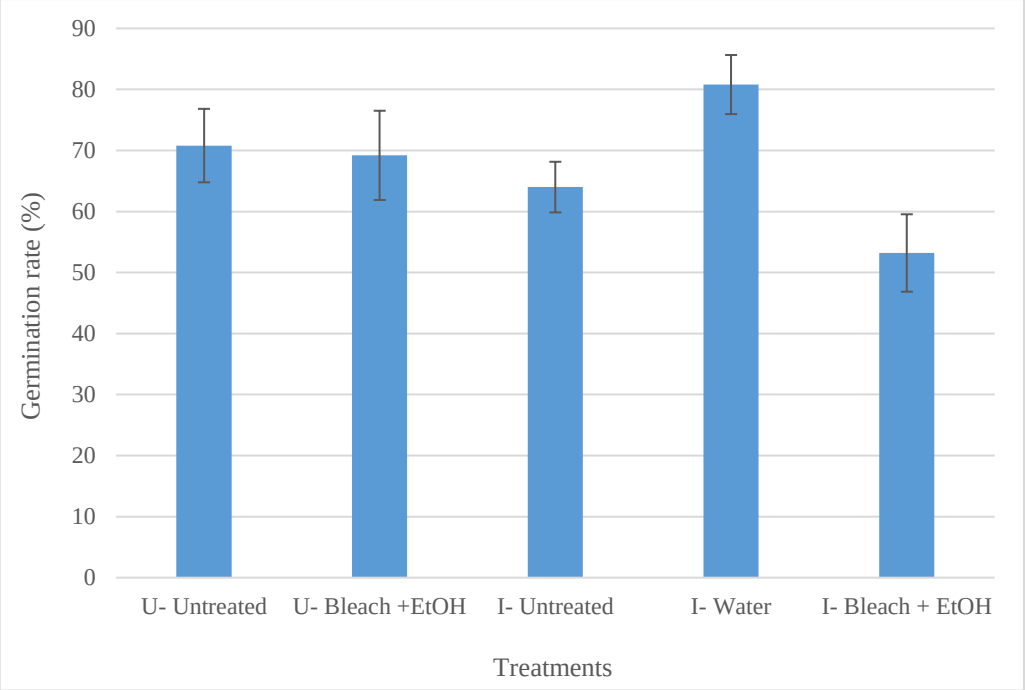


Figure 2.9 Comparison of the germination rate of *Nicotiana tabacum* 'Wisconsin 38' seeds harvested from YTMMV free plants and YTMMV infected plants under five different treatments

2.3.2. Seed transmission

YTMMV transmission rates through seed were highest in *N. benthamiana* RA-4 and *S. lycopersicum* 'Tigerella' plants at 40% and 36%, respectively, and lowest in *N. tabacum* 'Wisconsin 38' at 2.5% (Fig 2.10). Surface treatment of seeds with bleach and ethanol did not significantly change the seed transmission (Fig 2.11). Seed transmission of YTMMV occurred in all five species tested. Bleach and ethanol did not eradicate YTMMV from infected seeds.

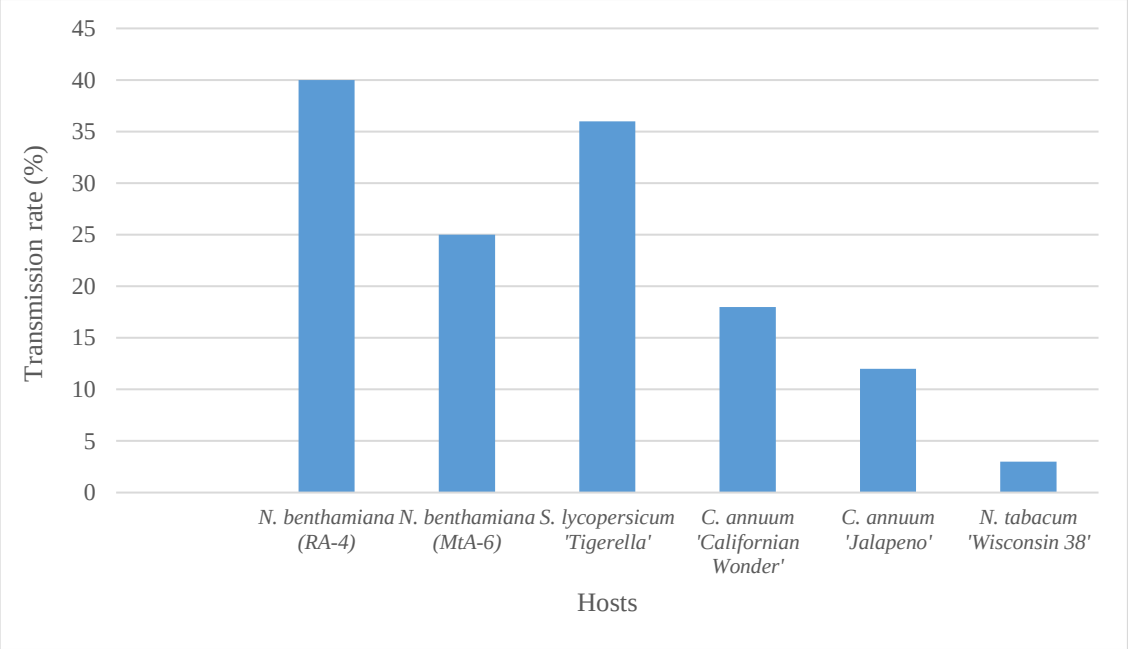


Figure 2.10 Comparison of seed transmission rates of YTMMV to six varietes/accessions species

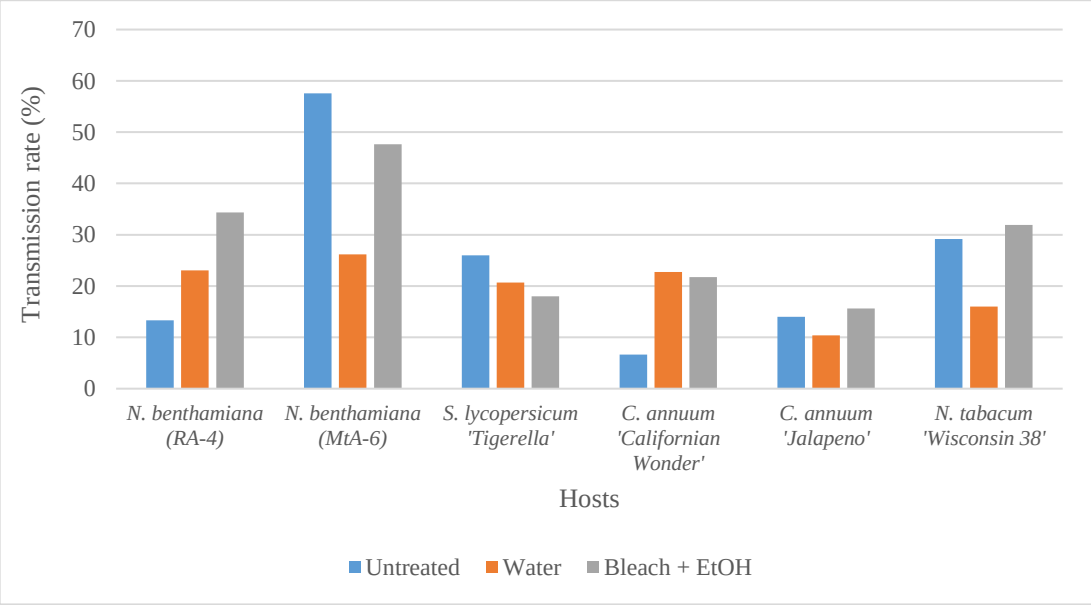


Figure 2.11 Comparison of effects of treatments on seed transmission rates of YTMMV to six species/accessions

2.3.3. Pollen transmission

After 16 virus-free *N. tabacum* 'Wisconsin 38' plants were pollinated with pollen from virus-infected plants, 26 fruits were produced in which YTMMV was present in the seedlings grown from the seeds. Of these 26 fruits, 20 were the result of vertical transmission because the mother plants remained uninfected, while six fruits (from two plants) were the result of horizontal and vertical transmission, where the mother plant was systemically-infected by pollination.

2.3.3.1. Vertical transmission

Twenty fruits from virus-pollinated plants that were not systemically-infected were collected. The data including number of seeds per fruit, germination rate and transmission rate were shown in Table 2.3 and compared to the control in the Table 2.4 Virus transmission occurred in less than 1% (0% of 9 fruits; 0.2% of 6 fruits; 0.4% of 2 fruits; 0.6% of 1 fruit; 0.8% of 1 fruit; 1% of 1 fruit) of the seed in the fruits in which vertical transmission occurred. Transmission did not occur in all fruits pollinated with virus-infected pollen.

Table 2.3 Pollen transmission of YTMMV to seed of twenty fruits derived from YTMMV-uninfected *Nicotiana tabacum* 'Wisconsin 38' flowers that were fertilised with pollen sourced from YTMMV-infected parent plants. Pollinated plants were not systemically infected with YTMMV, confirming vertical transmission

Fruit number	1	2	3	4	5	6	7	8	9	10
Number of seeds per fruit	3000	2889	1333	556	978	2897	2444	2333	3111	2556
Number of seedlings that germinated	2952	2224	1097	470	940	2750	2300	2300	2500	2230
Rate of germination (%)	98.4	76.9	82.2	84.5	96.1	94.9	94.1	98.5	80.3	87.2
Number of seedlings tested by RT- PCR	500	500	500	470	500	500	500	500	500	500
Number of seedlings positive for YTMMV	1	3	0	2	0	0	0	1	0	0
Rate of vertical virus transmission (%)	0.2	0.6	0	0.4	0	0	0	0.2	0	0

Table 2.3 Continued

Fruit number	11	12	13	14	15	16	17	18	19	20
Number of seeds per fruit	3100	1625	2211	2989	1878	3089	1211	3433	3522	878
Number of seedlings that germinated	2523	1449	1847	2667	1625	2973	1099	3196	3271	716
Rate of germination (%)	81.3	89.1	83.5	89.2	86.5	96.2	90.7	93.0	92.8	81.5

Number of seedlings tested by RT- PCR	500	500	500	500	500	500	500	500	500	500
Number of seedlings positive for YTMMV	0	1	5	4	1	1	0	2	1	0
Rate of vertical virus transmission (%)	0	0.2	1	0.8	0.2	0.2	0	0.4	0.2	0

Table 2.4 Comparison of the data of twenty *Nicotiana tabacum* 'Wisconsin 38' fruits from Table 2.3 to the control

	Fruit number (range from 1-20)	Control (range from 1-10)
Number of seeds per fruit	556 - 3522	1263 - 3175
Number of seedlings that germinated	470 - 3271	1051 - 2978
Rate of germination (%)	76.9 - 98.5	81.9 – 96.7
Number of seedlings tested by RT- PCR	470 - 500	500
Number of seedlings positive for YTMMV	0 - 5	0
Rate of vertical virus transmission (%)	0 - 1	0

2.3.3.2. Horizontal transmission

Flowers of virus-free mother plants were fertilised by pollen sourced from YTMMV-infected father plants. Both horizontal and vertical transmission of virus can occur after pollination. There were six fruits produced from two these infected plants (Tables 2.5, 2.6).

Table 2.5 Pollen transmission of YTMMV to seed of six fruits derived from YTMMV-uninfected *Nicotiana tabacum* 'Wisconsin 38' flowers that were fertilised with pollen sourced from YTMMV-infected parent plants. The result after cross pollination is the pollinated plants become infected with YTMMV, horizontal transmission occurs

Fruit number	1	2	3	4	5	6
Number of seeds per fruit	3189	1889	2922	1867	2214	3546
Number of seedlings	2846	1441	2575	1572	1993	3214
Rate of germination (%)	89.2	76.2	88.1	84.1	90.0	90.6
Number of seedlings tested RT- PCR	500	500	500	500	500	500
Number of seedlings positive for YTMMV	2	2	0	0	2	1
Rate of transmission (%)	0.4	0.4	0	0	0.4	0.2

Table 2.6 Comparison of the data of six fruits of *Nicotiana tabacum* 'Wisconsin 38' from Table 2.5 to the control

	Fruit number (range from 1- 6)	Control (range from 1-10)
Number of seeds per fruit	1867 - 3546	1263 - 3175
Number of seedlings that germinated	1441 - 3214	1051 - 2978
Rate of germination (%)	76.2 – 90.6	81.9 – 96.7
Number of seedlings tested by RT- PCR	500	500
Number of seedlings positive for YTMMV	0 - 2	0
Rate of vertical virus transmission (%)	0 – 0.4	0

2.4. Discussion

Tobamoviruses are considered a major threat to a range of economically-important plant species and their cultivars, especially those in the families Cucurbitaceae and Solanaceae. Cucurbits are threatened internationally by CGMMV, and in localities by KGMMV, ZGMMV and CFMMV. The most important tobamoviruses in the solaneceous crops are TMV, TMGMV, ToMV, PMMoV and tomato brown rugose fruit virus (ToBRFV). Currently, YTMMV is not a tobamovirus of concern in solanaceous crops and amenity plants, and the hope is it remains this way. However, it is important that we understand aspects of its biology, transmission being a critical factor in its potential for spillover. Most, if not all, tobamoviruses are seed-borne, and even at very low rates this is of concern to growers because contact between plants can transmit the virus quickly throughout a closely-planted crop from a few foci of infection. In this study we confirmed that YTMMV resembles other tobamoviruses in that it too is seed-borne. Pollen transmission also appears to be a trait shared by most, if not all, tobamoviruses. Here we also confirmed YTMMV is a pollen-transmissible virus.

These experiments confirmed that YTMMV is transmitted in the seed of all the host species tested: *N. benthamiana* accessions RA-4 and MtA-6, *S. lycopersicum* 'Tigerella', *C. annuum* 'Californian Wonder', *C. annuum* 'Jalapeno', and *N. tabacum* 'Wisconsin 38'. Our confirmation of YTMMV as seed-borne in at least some of its hosts reveals that it is potentially capable of being spread by the international trade in vegetable seed should it emerge in plants being grown for seed.

The lowest YTMMV seed transmission rate was 2.5% in *N. tabacum* 'Wisconsin 38' and the highest rate was 57.5 % of *N. benthamiana* (MtA-6). This is quite similar to other tobamovirus species and expresses the role of interactions with the host in rates of

seed transmission. For example, seed transmission rates of PMMoV in two *C. annuum* cultivars and one *C. frutescens* genotype were 30.4%, 100%, 18.6%, respectively (Jarret *et al*., 2008), strongly suggesting a host factor regulating transmission by seed.

A ToMV seed transmission rate of 16% of tropical soda apple mosaic virus (TSAMV) in *Solanum viarum* (Adkins, 2007), or the low seed transmission rate which recorded in ToBRFV of 2.8% in cotyledons and 1.8% in the third true leaf of *S. lycopersicum* (Davino *et al.*, 2020).

Treatment by bleach and ethanol was not effective at eliminating seed transmission of YTMMV, showing that the virus was protected from these treatments, likely because virions were located within the seed coat. No significant difference was observed in germination between YTMMV-infected seeds and non-infected seed, with the expection of *S. lycopersicum* 'Tigerella'. This is in contrast to some other research. For instance, 4.2% sodium hypochlorite was reported as one of solutions in reduction but not elimination of PMMoV in contaminated pepper seed (Crowley, 1957; Lamb *et al.*, 2001; Stommel *et al.*, 2021). A slight reduction was recorded when applying sodium hypochlorite 2% treatment for 20 min and 4% in 30 min to TMV-infected tomato seed (Milinko, 1956; Broadbent, 1965). Sodium hypochlorite concentrations of 5.25-6% were suggested by Bratsch (2018) to treat ToMMV-infected seed.

Chemical treatment of the external seed surfaces was ineffective at reducing or eliminating YTMMV in the host species tested. Heat treatments have been utilized to reduce the incidence of PMMoV and other tobamoviruses in contaminated pepper seed and ToBRFV in tomato (Stommel *et al.*, 2021; Salem *et al.*, 2021). Ozone treatment was used to against viral pathogens on various substrates. Stommel *et al.*, (2020) applied ozone treatment PMMoV-infected *C. annuum* seed to eliminate reduce

infection rate of seedlings. Heat and ozone treatments are possible methods of reducing YTMMV transmission in seeds.

The effects of YTMMV infection on potato, *S. tuberosum*, are unknown. Potato production contributes $619 million or 15% of the total value of vegetables in Australia, and followed by tomatoes ($547 million) (https://www.agriculture.gov.au/abares/research-topics/surveys/vegetables. While Australian tomato production is only approximately 30 percent as large as potato production by volume, it is nearly 90 percent as significant when measured by value (https://ausveg.com.au/resources/economics-statistics/australian-vegetable-production-statistics/). Control of viruses is centered on the use of virus-free seeds.

Seed transmission in annual plants is a means by which the virus can survive when the host plant dies, leaving only the seed as propagules to begin the next generation. In addition, seed transmission, in conjunction with secondary spread by insect vectors, can result in the introduction of viruses into new areas and can produce viral disease epidemics (Dinant & Lot, 1992). Furthermore, seeds cannot be treated easily and major management practices are not known and applied efficiently by farmers. These are the reasons seed-borne viruses cause huge loses in crop production and quality (Paylan, 2011).

In order to clarify the mechanism of YTMMV transmission and spread of seedborne virus disease, the location of this virus inside the seed should be researched. Fluorescence is applied widely to tobamovirus research. For example, by using fluorescence microscopy, Genda *et al.*, 2005 observed two things about PMMoV in *C. annuum*: 1) PMMoV was present in the epidermis and parenchyma but not in the endosperm or embryo; 2) The virus was restricted to the surface of the epidermis and

parenchyma. Other application is implemented by Salem *et al.* (2021) to determine the localization of ToBRFV within tomato seeds.

Modes of transmission of YTMMV via pollen were studied. Pollen collected from systemically-infected pollen donor plants was infected with YTMMV, although we did not test the rate of infection of pollen grains. Virus-infected pollen was capable of infecting both the whole recipient plant through horizontal transmission and the recipient ovule through vertical transmission.

There were three different leaf infection outcomes recorded:

1) The leaves were infected with YTMMV because the whole plant had become infected *via* YTMMV-infected pollen.

2) The leaves were uninfected with YTMMV because no part of the plant had become infected via YTMMV-infected pollen.

3) The leaves were uninfected with YTMMV but the ovule had become infected, thereby transmitting the virus to the seed from a plant free of systemic virus infection.

To distinguish between outcomes 2 and 3, seedlings germinating from the seed of the recipient plant were tested for YTMMV. Where no seedlings were infected, it was assumed outcome 2 had occurred, and pollen free of virus had fertilized the ovule. Where some seedlings were infected, it was assumed outcome 3 had occurred, and virus carried in the pollen tube had infected the ovule.

Transmission of YTMMV by pollen-borne virus was confirmed in *N. tabacum* 'Wisconsin 38'under laboratory conditions. This result enables us to consider the

hypothesis that YTMMV is spread over long distances from infected plants to uninfected plants through pollination. Pollen grains are probably carried by arthropod pollinators, but birds and other creatures are also potential pollinators (Ortega-Olivencia *et al.*, 20025; Castellanos *et al.*, 2006). Further research is required to investigate whether pollen transmission of YTMMV occurs naturally.

Pollen transmission may occur between species. For instance, raspberry bushy dwarf virus (RBDV) can be horizontally transmitted to *Torenia fournieri* plants by penetration of germinating pollen tubes originating from RBDV-infected *Rubus idaeus* (Isogai *et al.*, 2014). Bees may visit flowers of several plant species, thereby transmitting viruses to vulnerable species other than the original host species.

Transmission did not occur in all fruits pollinated with virus-infected pollen. In *N. tobacum* plants pollinated with virus-infected pollen, there was no virus detection in 11 fruits, equal to 42.3% of escapes. The reason may be because an insufficient number of virions reached the ovary or the virus does not always survive the pollination process (Liu *et al.*, 2014; Isogai *et al.*, 2017). This result confirms those of Mink (1993), who found that vertical transmission was less common than vertical transmission through ovules. Future research should use quantitative real-time RT-PCR to determine the rate of pollen infection by YTMMV.

Although the rate of YTMMV seed infection of *N. tabacum* 'Wisconsin 38' via pollen occurred at a low rate (1%) comparing the high rate of the same species from seed transmission (from 3 % to 29%). Zucchini yellow mosaic virus (ZYMV) (genus Potyvirus) seed infection via pollen occurred at a rate of 0.13 % (Harth *et al.*, 2017). Within potyviruses the evidence suggests that vertical transmission *via* the maternal

parent through the ovule or embryonic tissue is less common (Simmons & Munkvold, 2014).

The transmission of YTMMV from leaf to flower, pollen to seed, and seed to seedlings was demonstrated in this study. YTMMV is transmitted *via* pollen and is seed-borne in *N. tabacum* and is seed-borne in *N. benthamiana* accessions RA-4 and MtA-6, *S. lycopersicum* 'Tigerella', *C. annuum* 'Californian Wonder'; *C. annuum* 'Jalapeno'; *N. tabacum* 'Wisconsin 38'.

YTMMV transmission *via* seed and pollen is confirmed by RT-PCR tests of seedlings. Another test that could be done is to visualise YTMMV particles in the cells of seed or pollen using Transmission Electron Microscopy (TEM). TEM study would enable localisation of virus particles in the organ of interest and might help to explain why plants differ in transmission rates.

Chapter 3: Yellow tailflower mild mottle virus transmission by root and graft contact

3.1. Introduction

Tobamovirus species are reportedly transmitted by foliage contact, seed and soil contamination. Transmission of tobamoviruses occurs by root contact and root-grafting (Schwarz *et al.*, 2010). Tobamoviruses such as CGMMV, ToMV and TMV are infectious in water and are able to transmit between plants through root contact (Beijerinck, 1898; Paludan, 1985; Jacobi & Castello, 1991; Vani & Varma, 1993; Pares *et al.*, 1992; Pares *et al.*, 1996). Antignus *et al.* (2005) studied cucumber fruit mottle mosaic virus (CFMMV) on cucumber plants and concluded that virus invasion occurred in the root cells is *via* wounds inflicted through the disturbance of the root system during transplanting.

Koh *et al.* (2018) showed infrequent YTMMV transmission by root contact between plants growing in a hydroponics system. These authors found that root transmission of YTMMV between infected and uninfected plants growing in the same pot, where the leaves were not in contact, was uncommon and inconsistent. When transmission did occur, the virus could be detected in the leaves of plants, but the usual symptoms of leaf infection (stunting, curling, mosaic, mottle) were absent for the 45 days in which plants were observed. Roots from uninoculated plants that were in contact with inoculated plants tested positive for YTMMV, but the shoots of the same plants did not.

Grafting is a standard practice for many perennial fruit trees and vines, but also for some annuals, especially tomatoes and some cucurbits. Grafting technology has evolved into a unique component in the production of several solanaceous and cucurbitaceous vegetables for disease management and for improvement of crop productivity (Guan *et al.*, 2012). By choosing resistant rootstock, some of the well-known examples include controlling tristeza on citrus, fire blight and collar rot on

apples (Mudge *et al.*, 2009). Where the scion or rootstock is virus-infected, the virus may cross the graft union to the uninfected tissue.

Cucurbits are commonly grafted either on intraspecific rootstocks (Cohen *et al.*, 2007) or interspecific rootstocks (Lee & Oda, 2010). In Japan and Korea, grafted seedlings were used approximately 92% and 95% of the area cultivated with watermelon, respectively (Lee *et al.*, 2010). Importantly, cucurbit grafting could reduce viral infection from contaminated soil (Cohen *et al.*, 2007; Cohen *et al.*, 2017; Edelstein *et al.*, 2017; Smith *et al.*, 2018). For example, it is estimated that grafted tomatoes account for 20% to 40% of worldwide tomato production, and grafting is increasing to control soil-borne pathogens and to mitigate abiotic stresses (Lee *et al.*, 2010).

Among vegetable crops, tomato, eggplant, sweet pepper, watermelon, melon, and cucumber are commonly and economically grafted in Asia, Europe, and North America (Gaion *et al.*, 2018). Naturally-occurring grafts are uncommon and mainly found in roots (Dijkstra & de Jager, 1998).

Koh *et al.* (2018) showed the potential of YTMMV transmission *via* root in hydroponics system in an inconsistent manner. Because transmission through roots is less studied than for above-ground organs, we will further evaluate YTMMV distribution in the infected plant and in grafted plants.

Aims of the research described in this chapter were to study:

1. YTMMV distribution in the infected plant.
2. Movement of virus from shoots to roots and vice versa in grafting experiments.
3. Further test the role of root contact in YTMMV transmission.

3.2. Materials and Methods

N. benthamiana accessions (RA-4 and MtA-6) plants infected by YTMMV isolate Cervantes were used as the source of virus. RNA extraction, cDNA synthesis, PCR amplification and primers used to detect YTMMV by RT-PCR were as described in Chapter 2. The methods of planting and inoculating *N. benthamiana* accessions RA-4 and MtA-6, *N. tabacum* 'Wisconsin 38', and *S. lycopersicum* 'Tigerella' are described in Chapter 2.

3.2.1. Virus distribution

N. benthamiana accessions RA-4 and MtA-6 and *N. tabacum* 'Wisconsin 38' plants were used as the indicators to investigate virus distribution in infected plants. While *N. tabacum* 'Wisconsin 38' exhibited mild responses to YTMMV infection, young seedlings of *N. benthamiana* RA-4 plants deteriorated very quickly. Therefore, inoculation of *N. benthamiana* RA-4 plants was conducted on older seedlings at the 5-7 leaf stage.

At 60 days post-inoculation (dpi), tissue samples were tested by RT-PCR for the presence of YTMMV. One leaf sample and two root samples for each plant were tested from each of twelve *N. benthamiana* and nine *N. tabacum* 'Wisconsin 38' plants using virus-specific primers as described previously (Table 2.1, Chapter 2). Two root samples were collected from opposite sides of the root ball (if present), or if roots were stunted, from upper and lower parts of the root (Figure 3.1).

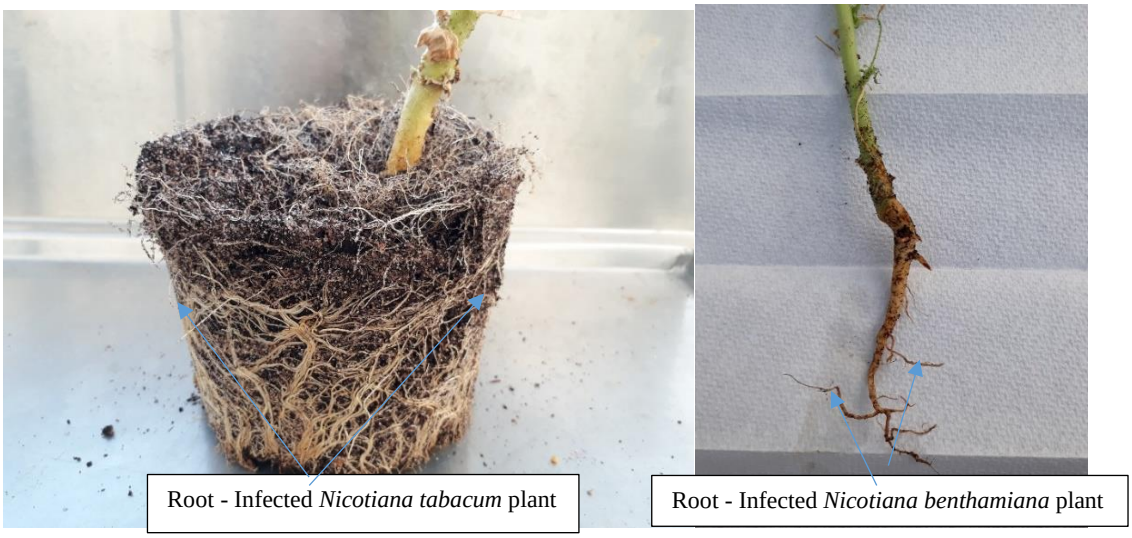


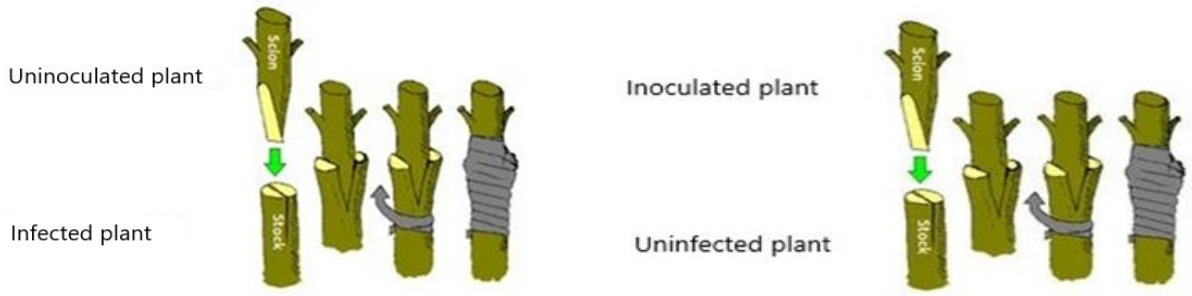
Figure 3.1 Development of root system in YTMMV-infected plants of *Nicotiana tabacum* 'Wisconsin 38' (left) and *Nicotiana benthamiana* RA-4 (right) and the positions where root samples were collected from the roots

3.2.2. Grafting

S. lycopersicum cv Tigerella plants were used as scions and rootstocks for the grafting experiment. Virus-free seed was sown in potting mix and transplanted into pots at the third true leaf stage. Some plants were mechanically inoculated with sap from YTMMV-infected *N. benthamiana* RA-4 plants. Infected plants and uninfected plants were grown in different glasshouse compartments to avoid inadvertent cross-infection. RT-PCR (as above) was used to test leaves and roots samples of inoculated plants.

Tomato plants were 6-weeks old when prepared for grafting. Rootstocks were prepared by removing the shoots above the two basal leaves and then creating a vertical cut of 1.5–2 cm at the center of the stem. Scions (5-7 cm in length) were prepared by removing leaves and trimming the base of the scion to a wedge. The scion/rootstock junction was wrapped with Parafilm® (American National Can.). Eighteen uninfected scions were grafted to eighteen YTMMV-infected rootstocks (treatment 1), and

eighteen YTMMV-infected scions were grafted to eighteen uninfected rootstocks (treatment 2) (Fig 3.2). The treatments were repeated twice (repeats 1 and 2).



Treatment 1 Treatment 2

Figure 3.2 Both YTMMV-infected and uninfected rootstocks and scions of *Solanum lycopersicum* 'Tigerella' plants were grafted to test transmission to and from roots

Grafted plants were grown for four weeks (Fig 3.3). After four weeks, leaf, stem and root samples were collected and tested for YTMMV by RT-PCR.

Figure 3.3 One day after grafting (left) and 15 days after grafting (right) *Solanum lycopersicum* 'Tigerella' plants where axillary buds have germinated

3.2.3. Root contact

Three experiments were done:

1) In experiment 1, decaying roots of an infected plant cut at ground level 7 days previously were present in the soil when *N. benthamiana* RA-4 seedlings were

transplanted into the soil. Seedlings were tested for YTMMV transmitted to the live roots from the dead roots.

2) In experiment 2

Treatment 1: two *N. benthamiana* RA-4 seedlings were grown in the same pot. As they matured, one of the two plants was inoculated with YTMMV. The uninfected plant was covered with a polythene bag to prevent leaves touching.

Treatment 2: resembled treatment 1, but there were two uninfected seedlings and one inoculated seedling covered with a polythene bag to prevent leaves touching in each pot. *N. benthamiana* RA-4 and *C. annuum* 'Jalapeno' were used.

3.2.3.1. Experiment 1: infection from decaying roots

After YTMMV infection was confirmed in seedling *N. benthamiana* RA-4 plants, the above-ground parts of the plant were removed, and virus-free seedlings were planted in the same soil. Symptoms were observation and leaf samples were collected after 35 days. Plants were tested for the presence of YTMMV by RT-PCR.

3.2.3.2. Experiment 2: infection from live roots

3.2.3.2.1. Treatment 1 (one infected plant and one uninfected plant grown on the same pot)

N. benthamiana RA-4 seedlings were grown with two plants per pot. One plant was mechanically inoculated with YTMMV. The uninoculated plant was covered by a polythene bag. Symptoms were observed and leaf and root samples collected at 35 dpi. Roots of uninoculated plants were tested for YTMMV.

Figure 3.4 Root transmission experiment. One *Nicotiana benthamiana* RA-4 plant was inoculated with YTMMV and the other one was uninoculated. The polythese bag prevented shoot/leaf contact

3.2.3.2.2. Treatment 2 (one infected plant and two uninfected plants grown on the same pot)

N. benthamiana RA-4 and *C. annuum* 'Jalapeno' were used as indicator plants in experiment 3. Three seedlings were grown in one pot. At the vegetative stage, one plant was inoculated manually with YTMMV and then covered by a polythene bag. Two weeks after inoculation the leave of infected plants were tested by RT-PCR to confirm the infected source plants. Root samples of both infected and uninfected (tested) plants were collected at 35 dpi and tested for the presence of YTMMV by RT-PCR test. Length of roots at 35 dpi of both infected and tested plants was measured.

3.3. Results

3.3.1. Distribution of YTMMV within the leaves and roots

YTMMV was always detected in the leaf of *N. tabacum* 'Wisconsin 38' (Table 3.1) and *N. benthamiana* (Table 3.2) plants tested, and in the two root samples of *N. tabacum* 'Wisconsin 38' plants, but were not uniformly-distributed in roots of *N. benthamiana* plants.

Table 3.1 Results of RT-PCR tests of leaves and roots of nine inoculated *Nicotiana tabacum* 'Wisconsin 38' plants at 60 dpi. There were three samples per plant including one young leaf sample and two root samples collected in two different positions on the pot

Plant number	1	2	3	4	5	6	7	8	9
Leaf	+	+	+	+	+	+	+	+	+
Root – position 1	+	+	+	+	+	+	+	+	+
Root – position 2	+	+	+	+	+	+	+	+	+

Presence (+) of virus in the leaves and roots of plants were recorded

Table 3.2 Results of RT-PCR tests of leaves and roots of twelve infected *Nicotiana benthamiana* plants including ten *N. benthamiana* RA-4 plants (pots 1 - 10) and two *N. benthamiana* MtA-6 plants (pots 11 and 12). One leaf and two root samples per plant

Plant number	1	2	3	4	5	6	7	8	9	10	11	12
Leaf	+	+	+	+	+	+	+	+	+	+	+	+
Root – position 1	+	+	+	-	-	+	+	-	+	+	+	-
Root – position 2	-	NT	+	-	-	+	+	-	+	+	-	-

Presence (+) or absence (−) of virus in the leaves and roots of plants were recorded;
NT: not tested

3.3.2. Grafting

S. lycopersicum 'Tigerella' is a suitable rootstock in grafting. However, of these eighteen plants for each experiment, only five to six scions of survived and grew in the first treatment compared to seventeen in the repeat treatment. *S. lycopersicum* 'Tigerella' is mild response to the YTMMV. There was no symptom by observation. The results for two repeats of the first and second grafting treatments are shown in the Tables 3.3 and 3.4, respectively. Our data (Tables 3.3 and 3.4) show that YTMMV was transmitted *via* grafting in *S. lycopersicum* 'Tigerella' in both directions, from leaf to root and from root to leaf. Also, from these data (Table 3.3 and 3.4) expressed the same result about virus distribution in *S. lycopersicum* 'Tigerella'to the *N. benthamiana* and *N. tobacum* that when plants become infected with YTMMV it always appeared on the leaf, but was inconsistently present in the root, except to *N. tabacum* 'Wisconsin 38'.

3.3.2.1. Treatment 1

When infected rootstocks were grafted to uninfected scions, YTMMV was transferred from root to stem, leaf. Although the virus was not detected in the roots in pots 7, 8, 9, 14, 16, 17, 19, 20, and 23. These results were similar to the results of the virus distribution experiment. The virus was present in stems and leaves. YTMMV was transmitted from roots to scion (Table 3.3).

Table 3.3 YTMMV transmission through graft union in *Solanum lycopersicum* from root to scion: the uninfected scions were grafted to infected rootstocks. Six plants (number 1 – 6) and seventeen plants (number 7 - 23) were grafted successfully in repeats 1 and 2

Plant	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23
Leaf of tested plant	+	+	+	+	+	+	-	+	+	+	NT	+	-	+	+	-	+	+	NT	NT	+	+	NT
Stem of tested plant	NT	NT	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Root of infected plant	+	+	+	+	+	+	-	-	-	+	+	+	+	-	+	-	-	+	-	-	+	+	-

Presence (+) or absence (−) of virus in the leaves and roots of plants were recorded; NT: not tested

3.3.2.2. Treatment 2

When the scion of infected plants was grafted to uninfected rootstocks, YTMMV transferred from the stem to the root. The virus was not always detected in roots (Table 3.4).

Table 3.4 YTMMV transmission through graft union in *Solanum lycopersicum* from scion to root: The infected scion was grafted to rootstock of uninfected rootstock. Five plants (number 1 – 5) and seventeen plants (number 6 - 22) were grafted successfully in repeats 1 and 2

Plant	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
Leaf of tested plant	NT	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Stem of tested plant	NT	+	+	+	+	+	NT	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Root of tested plant	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	+	+

Presence (+) or absence (-) of virus in the leaves, stems and roots of plants were recorded; NT: not tested

3.3.3. Root contact

3.3.3.1. Experiment 1

RT-PCR was applied to test YTMMV from the leaf samples of tested plants. No YTMMV was detected from leaf samples on 19 *Nicotiana benthamiana* RA-4 plants grown in soil containing dead roots of infected plants.

3.3.3.2.Experiment 2

3.3.3.2.1. Treatment 1

There was no detection of YTMMV on 19 root samples from 19 *N. benthamiana* RA-4 plants which were grown in the same pot with YTMMV-infected plants where no leaf contact between plants was permitted.

3.3.3.2.2. Treatment 2

At 35 dpi, *C. annuum* ‘Jalapeno’ YTMMV-infected roots were still growing well but *N. benthamiana* infected roots were small and weak. The size of the root system of infected plants was very small and short compared to uninfected plants (Fig. 3.5; 3.6).

Roots of infected *N. benthamiana* RA-4 and *C. annuum* ‘Jalapeno’ plants all survived and grew (Fig 3.6, 3.7; 3.8) although infection resulted in smaller root systems. The highest values of length root of infected *N. benthamiana* and *C. annuum* were 6 cm and 15 cm, respectively compared to the uninfected plants of these two species of 48 cm and 52 cm, respectively.

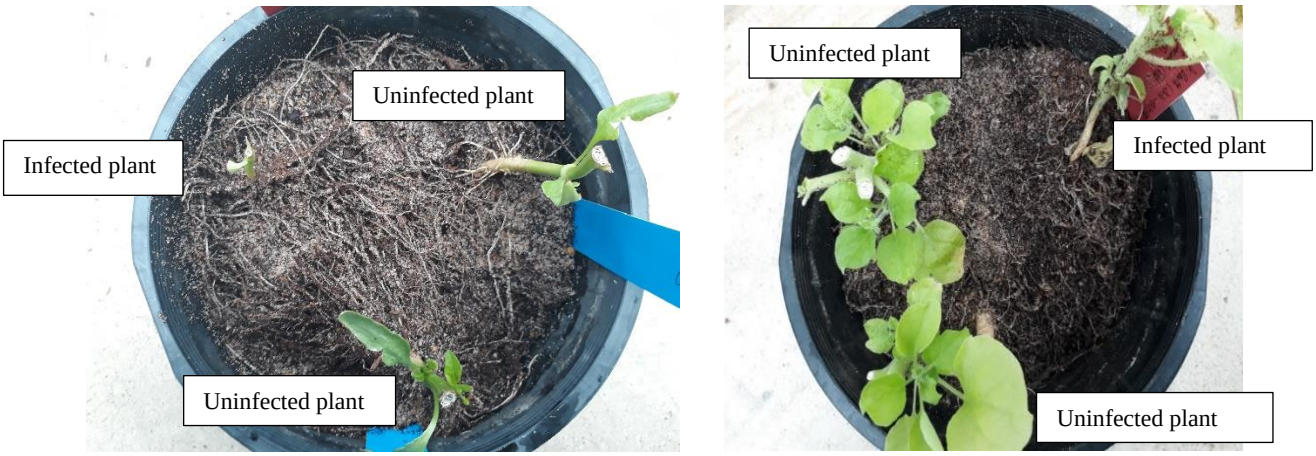


Figure 3.5 Roots status and root contact images of infected and uninfected *Nicotiana benthamiana* RA-4 plants (right) and *Capsicum annuum* 'Jalapeno' plants (left) at 35 dpi

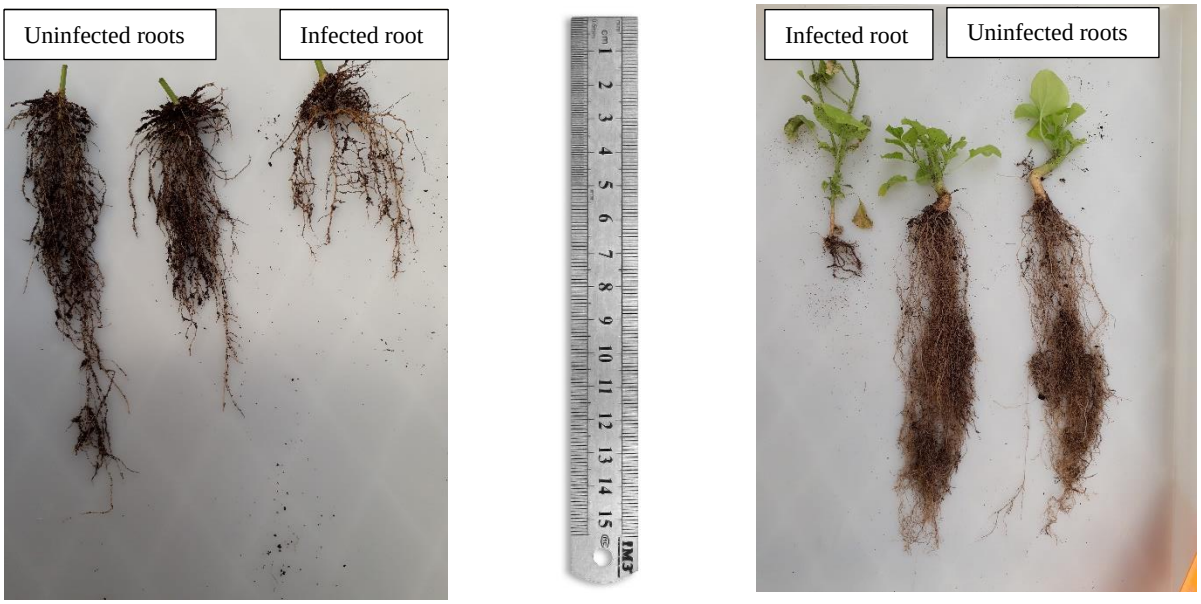


Figure 3.6 Roots of two uninfected and one infected plant of *Capsicum annuum* 'Jalapeno' growing in the same pot (left) and *Nicotiana benthamiana* RA-4 (right). Length of roots of infected plant always shorter than that of uninfected plants

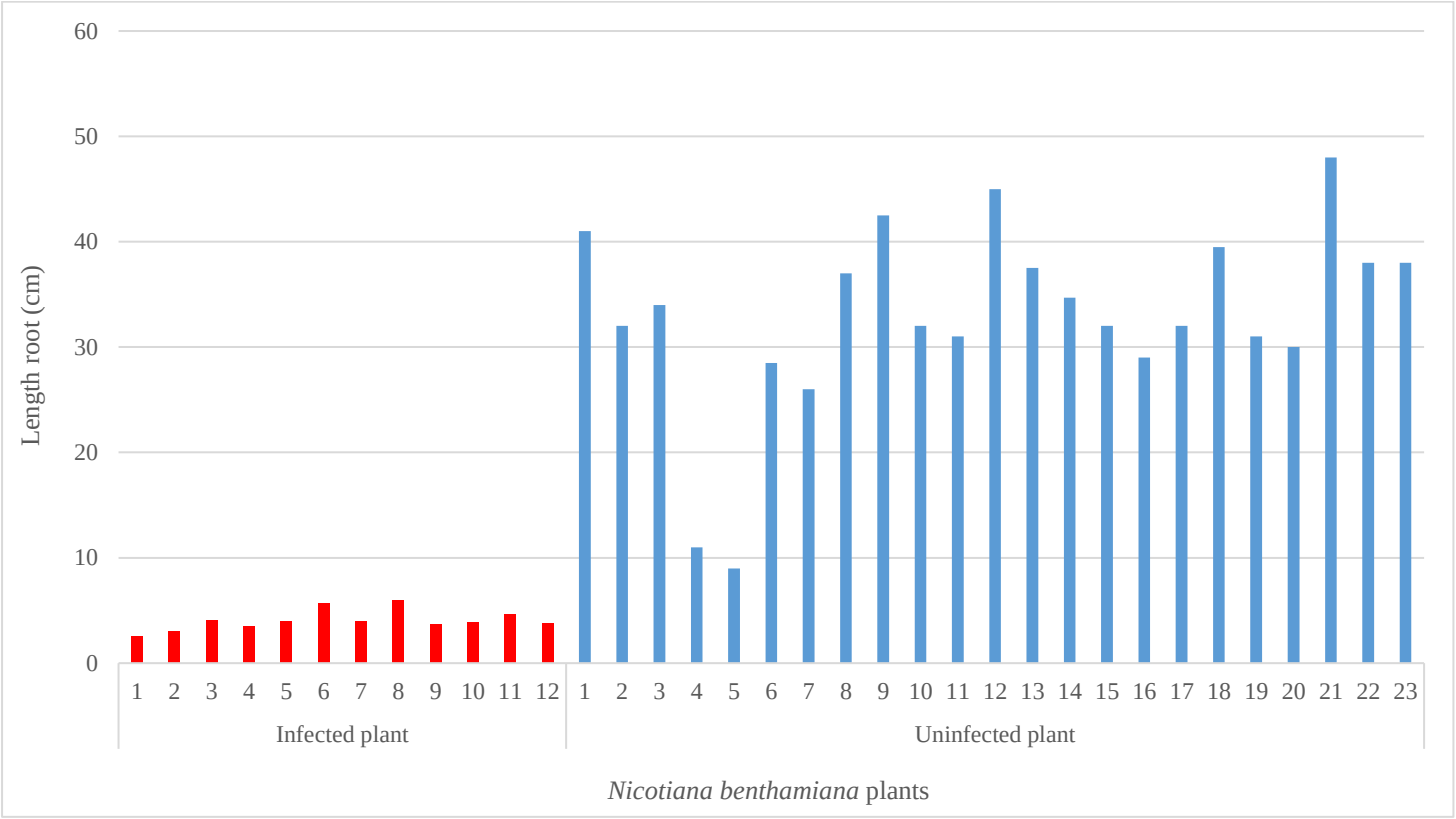


Figure 3.7 Comparison the length roots (cm) of *Nicotiana benthamiana* infected plants and uninfected plants when one infected plant grown the same pot with two uninfected plants. There were 12 infected plants (equal 12 pots) and 23 uninfected plants (there was only one uninfected plant, number 21 belong to pot 11 recorded

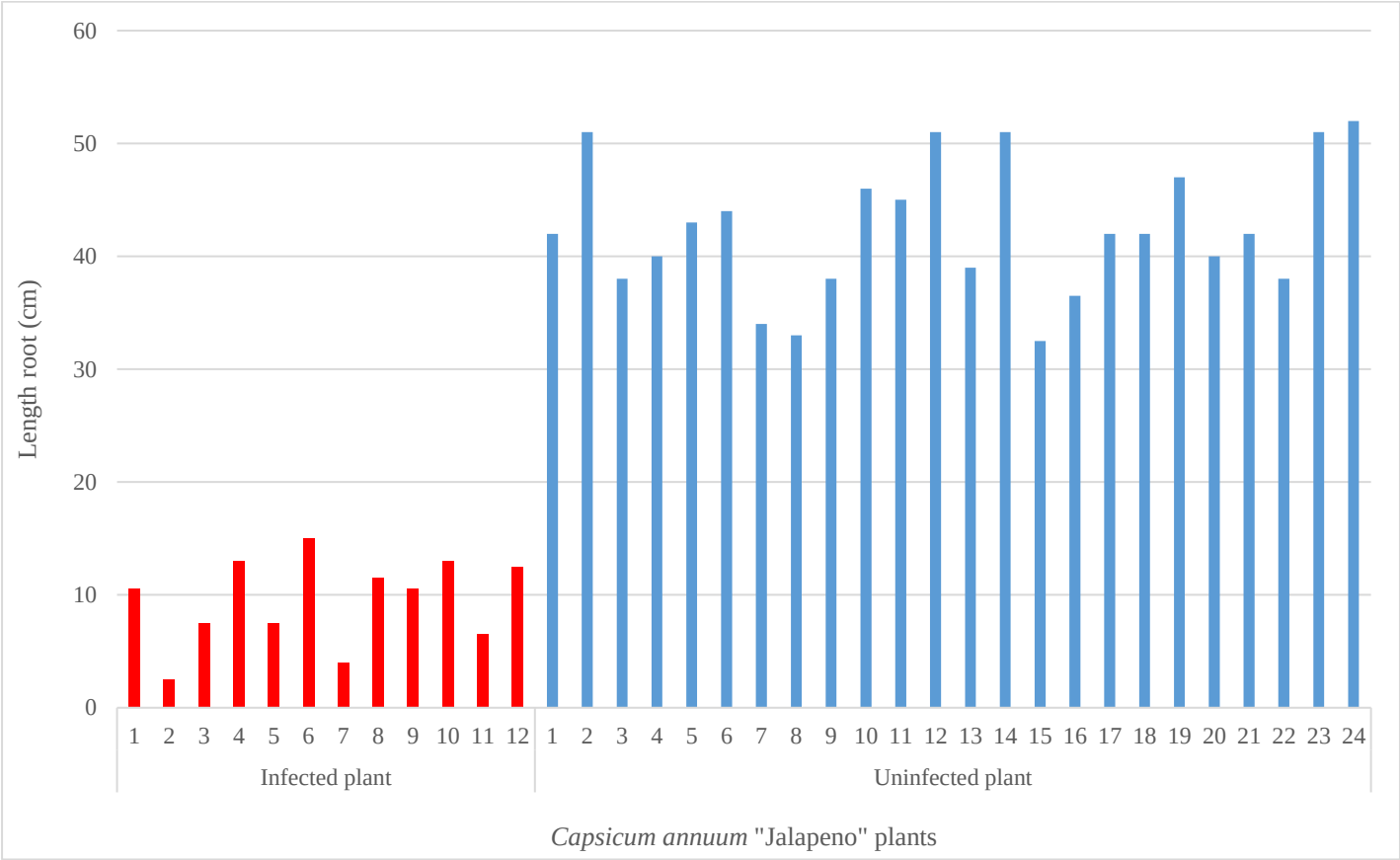


Figure 3.8 Comparison the length roots (cm) of *Capsicum annuum* 'Jalapeno' infected plants and uninfected plants when one infected plant grown the same pot with two uninfected plants. There were 12 pots including 12 infected plants and 24 uninfected plants

Presence of YTMMV in the roots of infected plants of *N. benthamiana* RA-4 and *C. annuum* ‘Jalapeno’ are shown in Table 3.7. The virus was not always detected in roots of YTMMV-infected plants.

One pot had two uninfected plants and one infected plant in it. We considered that root transmission had occurred if the virus was detected in least one root of an uninfected plant. Tables 3.8 and 3.9 showed contact transmission *via* roots of *N. benthamiana* RA-4 and *C. annuum* ‘Jalapeno’ plants. Root transmission in both species was 83% (10 per 12 pots) and 50% (6 per 12 pots) for *N. benthamiana* and *C. annuum* ‘Jalapeno’, respectively.

Table 3.5 Virus detection on the roots of infected *Nicotiana benthamiana* RA-4 and *Capsicum annuum* 'Jalapeno' plants. YTMMV was not uniformly distributed in roots of infected plants. Plant infection was confirmed by a leaf RT-PCR test for each plant

Pot	1	2	3	4	5	6	7	8	9	10	11	12
Infected *Nicotiana benthamiana* plants	+	+	NS	NS	+	NS	+	+	NS	NS	NS	-
Infected *Capsicum annuum* 'Jalapeno' plants	+	+	-	-	-	-	+	-	+	-	+	-

Presence (+) or absence (-) of virus in the roots were recorded. NS: not sampled because plant was dead

Table 3.6 Virus transmission to roots of uninoculated *Nicotiana benthamiana* plants. In one pot were two tested plants and one infected plant. If the virus was detected in at least one uninoculated plant, we considered root transmission occurred

Pot	1		2		3		4		5		6		7		8		9		10		11		12	
Tested plants	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
	-	+	+	+	+	+	+	+	-	-	-	+	+	-	NS	+	+	+	+	+	-	-	+	+

Presence (+) or absence (-) of virus in the roots were recorded; NS: not sampled

Table 3.7 Virus transmission to roots of tested *Capsicum annuum* 'Jalapeno' plants. Each pot had two uninfected plants and one infected plant. If the virus was detected in at least one uninoculated plant, we consider that root transmission occurred

Pot	1		2		3		4		5		6		7		8		9		10		11		12	
Tested plants	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
	+	-	NS	+	-	-	-	-	+	-	-	-	-	-	-	+	-	-	+	-	-	+	-	-

Presence (+) or absence (-) of virus in the roots were recorded. NS: not sampled

3.4. Discussion

We tested whether YTMMV was transmitted through roots in soil. YTMMV was transmitted at 50% and 83% for *C. annuum* 'Jalapeno' and *N. benthamiana*, respectively. Both roots and shoots of infected plants were tested by RT-PCR and we found that YTMMV was unevenly distributed within the roots of plants of *N. benthamiana*, *C. annuum* and *S. lycopersicum*. Surprisingly, shoots of plants infected by root contact did not exhibit symptoms typical of YTMMV infection, even though virus was present in the shoots. This phenomenon happened in *N. tobaccum* and *S. lycopersicum* plants that usually show mild symptoms of YTMMV infection, and in plants of *N. benthamiana* and *C. annuum* that exhibit severe symptoms.

The results of this study suggest that YTMMV is transmitted through roots although the rate in which this occurs may vary with host species. In a hydroponics system, Park *et al.* (1999) indicated that TMV could be transmitted from tobacco, tomato and hot pepper plants to healthy plants mainly *via* natural root-tip grafting.

The results of this study showed that RT-PCR assays of the leaves for YTMMV were more reliable than the roots, because distribution was less even in roots for unknown reasons. Similar results were found with CGMMV in grafted watermelon (Chen *et al.*, 2009; Yoon *et al.*, 2008; Komuro *et al.*, 1971). More extensive study is required to determine possible mechanisms.

Analysis of virus distribution of other tobamovirus species in infected plants revealed that while hibiscus latent fort pierce virus moved from the place of inoculation to the roots first and then toward the bottom (oldest) leaves of the plants (Kamenova & Adkins, 2004), TMV also moved from the place of inoculation to the roots first but then

toward to the young, apical leaves before infecting the middle-aged and older leaves (Hull, 2002). Although it is often assumed that viruses infected systemically and detected in all leaves, the distribution of virus in infected plants has not been widely studied.

The distribution of a virus in infected plants depends on several factors such as host genes, viral genes, the host defense system, and environmental factors (Hull, 2002). In this experiment YTMMV was detected in all leaf samples of infected *N. tobacum, N. benthamiana, C. annuum, S. lycopersicum* plants, but only in all root samples of *N. tobacum* plants. Host-encoded factors may be at play in root distribution of YTMMV.

We did not detect transmission to seedlings planted in soil containing the roots of infected plants. Other tobamoviruses are reported to be stable under some circumstances in soil and crop residues in the soil, and to be transmissible (Gülser *et al.*, 2008; Candemir *et al.*, 2012). Similarly, YTMMV virions are highly resilient (Koh *et al.*, 2018). CGMMV had a survival of 50% in soil without host plants for 17 months and had high infectivity in the debris of infected plants over one year (Park *et al.*, 2010). Li *et al.* (2016) suggested that contaminated soil, pruning and irrigation could transmit CGMMV at different efficiencies. Broadbent (1965) confirmed that tomato plants can acquire TMV through their roots from debris in the soil. Thus, they are capable of infecting newly established plants through roots injuries. It seems likely that YTMMV could be transmitted in the soil to injured roots, but this may occur uncommonly. These experiments should be done by also planting seed in YTMMV-contaminated soil to test if transmission occurs at germination.

The effects of virus-infection on root systems are not well-studied. Here, the effects on roots were mirrored by effects on shoots. For instance, *N. benthamiana* RA-4 shoots are severely affected by YTMMV infection, as are the roots.

Chapter 4: Mechanistic transmission of YTMMV during insect feeding

4.1. Introduction

The interactions between vector, virus and plant have important epidemiological implications. Understanding this three-way relationship is integral to understanding epidemiology and for developing control strategies (Krenz *et al.*, 2015).

Many plant viruses are transmitted by arthropods, with aphids in the subfamily Aphidinae (Order: Homoptera) being the most important, transmitting nearly 30% of all plant virus species described to date (Brault *et al.*, 2010), including some large virus groups such as potyviruses (Ng & Perry, 2004; Herrbach *et al.*, 2016). Other large groups such as begomoviruses are transmitted by whiteflies (Zerbini *et al.*, 2017), and thrips are vectors of orthotospoviruses and ilarviruses (King *et al.*, 2012; Bujarski *et al.*, 2019).

Specific interactions between virus and vector factors occur regardless of the type of virus/vector association. The different modes of viral transmission by vectors include non-persistent, semi-persistent and persistent, whereby the transmission window to disseminate the virus to a new host plant after feeding on an infected plant by the vector lasts from seconds to the lifetime of the vector, depending on the transmission mode.

Non-persistent, non-circulative viruses bind to the mouthpart/stylet of the vector through protein-protein interactions, including the viral capsid or a helper-component or a combination of both (Ng & Falk, 2006). They are not transmissible from the gut. They enter the plant when saliva enters the cells/phloem.

Semi-persistent, non-circulative viruses are retained within the insect foregut. Semi-persistent viruses are internalised in the insect by binding to the chitin lining the gut,

but do not move into other tissues (Blanc *et al.*, 2011). They are regurgitated into the plant during feeding.

Persistent viruses are taken up and retained in insect tissues and are characterized by invading the salivary glands (Hogenhout *et al.*, 2008). Persistent viruses can be further being divided into circulative non-propagative, and circulative propagative (Bragard *et al.*, 2013).

When viruses are transmitted mechanistically by the contaminated mouthparts of herbivores, including arthropods, this form of virus transmission is not considered to be vectored. With chewing insects, such non-specific transmission mostly occurred when insect mouthparts were contaminated with infective sap and ceased as soon as this sap was cleaned away during feeding (Hoggan, 1934; Orlob, 1963; Smith, 1965; Walters *et al.*,1951). Tobamoviruses are not vectored by insects *per se* (Fulton *et al.*, 1987), but there are reports of mechanistic transmission by insects in the older literature. For example, aphids (Hoggan, 193I), grasshoppers (Walters, 195I) and mealy-bugs (Newton, 1953) have all been reported to transmit TMV. Chant (1959) showed that Galerucid beetles (*Ootheca mutabilis*) transmitted TMV from Bengal bean (*Mucuna aterrima*) to Bengal bean and cowpea (*Vigna unguiculata*), and from cowpea to Bengal bean. Similarly, cucumber leaf beetles (*Raphidopalpa fevicollis*) transmited CGMMV in bottle gourd (*Lagenaria siceraria*) (Rao & Varma, 1984).

The aim of the current experiment was to examine vector transmission of YTMMV through feeding by the sucking insect green peach aphid (GPA) (*Myzus persicae*) and the chewing insect cotton bollworm (*Helicoverpa armigera*) under laboratory conditions. We also tested transmission under field conditions by any mechanistic

means by interplanting infected and uninfected plants in plots and allowing wild insects, molluscs, birds etcetera to interact with the plants over time.

4.2. Materials and Methods

4.2.1. Laboratory experiment: Feeding by green peach aphids and cotton bollworms

4.2.1.1.Plants

N. benthamiana (RA-4), *C. annuum* 'Jalapeno', and *Solanum betaceum* (tamarillo) plants were selected as hosts because green peach aphids and cotton bollworms feed on leaves of these species, and symptoms of YTMMV infection are clearly visible.

When plants were four weeks old they were manually inoculated with YTMMV as described in Chapter 2. When symptoms became apparent, they were moved to a growth chamber for experiments with insects.

4.2.1.2. Insects

Cultures of GPAs and cotton bollworms were obtained from existing cultures maintained at Murdoch University.

4.2.1.2.1. Green peach aphid culture

Green peach aphids were initially maintained on *N. tabacum* 'Wisconsin 38' plants in a growth chamber with a 16/8h (light/dark) cycle. Aphids were brushed onto a petri dish to be transferred to virus-virusuninfected plants of *N. benthamiana* RA-4, *S. betaceum* and *C. annuum* 'Jalapeno' plants using a small paintbrush. All pots were placed on the trays and placed inside an insect-proof cage.

4.2.1.2.2. Cotton bollworm culture

Foods for larvae and adults were prepared according to the formulae below (Table 4.1, 4.2). We put 15–20 cocoons per cage. Once adult moths appeared, equal numbers of

male and female were kept in a cage. Larvae were kept in individual cages with artificial diet cubes.

Table 4.1 Larval diet

Fraction	Components	Amount
A	Chickpea flour	150 g
	Ascorbic acid	2.3 g
	Sorbic acid	0.75 g
	Yeast extract	24 g
	Vitamin mixture	5 mL
	Antifungal solution	1.3 mL
	Streptomycin (1 g/ml stock)	200 µL
	Sterile distilled water	200 mL
B	Agar (powder)	8.625 g
	Sterile distilled water	350 mL

Method:

1. Prepare fraction A separately in 1000 mL beaker.
2. Prepare fraction B in separate flask/beaker and boil it in microwave till the Agar powder is completely digested in water. Make sure that agar doesn't boil out from the container.
3. Pour the fraction B in fraction A and blend immediately using hand blender or a spatula.
4. Once both fractions are completely mixed and smooth slurry is formed, pour the mixture into individual Petri dishes and keep it open for solidification.

Table 4.2 Adult diet

Components	Amount
Sucrose (10%)	50 g
Vitamin Mixture	20 mL
Antifungal Solution	2 mL
Sterile distilled water	Make up to 500 mL

4.2.1.3. Insect transmission in laboratory conditions

4.2.1.3.1. Transmission by green peach aphid

Step 1: Sixty aphids per each host plant species (*N. benthamiana* (RA-4), *C. annuum* 'Jalapeno', *S. betaceum*) were collected from uninfected plants of *N. benthamiana* (RA-4), *C. annuum* 'Jalapeno' and *S. betaceum* and placed in petri dishes, which were sealed by plastic wrap. Aphids were fasted for 1 h.

Step 2: After fasting aphids were transferred to the infected plants of the same species for 3 h (treatment 1), 5 h (treatment 2), 7 h (treatment 3) or 24 h (treatment 4) (the same species).

Step 3: After feeding from the infected plants, 10 aphids were transferred to an uninfected (tested) plant of the same species. And then, after 2 days the aphids were removed from tested plants.

Six replicate plants were used for each species.

Plants were maintained in aphid-proof cages. Control of this experiment was carried out on two plants per each species. Twenty aphids which collected from uninfected *N. benthamiana* (RA-4) plants and placed in a petri dish, sealed by plastic wrap and fasted for 1 h and then transferring ten aphids per one *N. benthamiana* (RA-4) plant. The similar steps were applied to *C. annuum* 'Jalapeno', *S. betaceum.*

The note in this experiment is transferring aphids in the same species from maintaining culture to feeding in the infected plant and then to tested plant to ensure that this aphid can eat and growth and maintain in these plant species (already adapted). The aim of this experiment was to ensure aphids were adapted to the test plant species.

The experiment was repeated twice. After 5 d since removing the aphids from tested plants, symptoms were observed daily in three weeks (to the date leaf samples collected). Leaf samples were collected after 21 d and RT-PCR assays with YTMMV-specific primers (Chapter 2) were used to detect the presence of YTMMV.

Table 4.3 YTMMV transmission experiments testing green peach aphids as the agent of transmission

Treatment	Species	Starvation time (h)	Feeding time on infected plants (h)
1	*Nicotiana benthamiana* (RA-4) *Capsicun annuum* 'Jalapeno' *Solanum betaceum*	1	3
2	*N. benthamiana* (RA-4) *C. annuum* 'Jalapeno' *S. betaceum*	1	5
3	*N. benthamiana* (RA-4) *C. annuum* 'Jalapeno' *S. betaceum*	1	7
4	*N. benthamiana* (RA-4) *C. annuum* 'Jalapeno' *S. betaceum*	1	24

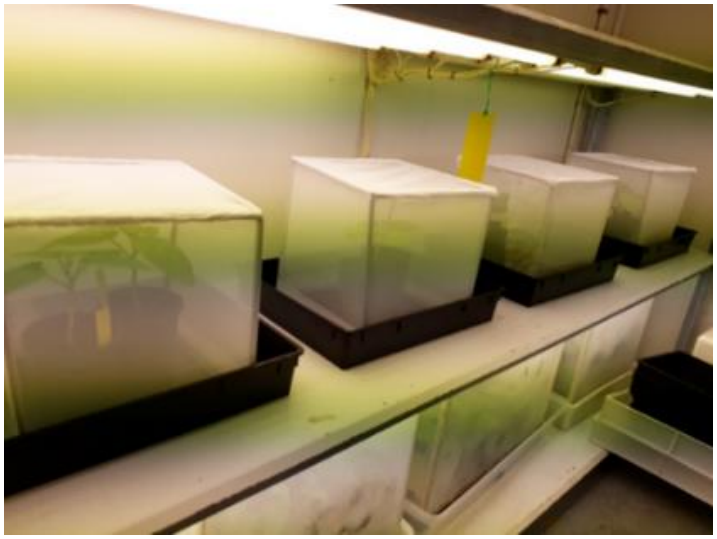


Figure 4.1 *Solanum betaceum* plants in insect cages in controlled-temperature room

4.2.1.3.2. Transmission by Cotton bollworm

Step 1: Larva from 4th and 5th instar (the highest feeding stage with very high feeding rate) were selected and fasted for 12 h (this time is enough to increase their larvas feeding and eats anything after starvation).

Step 2: After fasting, larvae were transferred to YTMMV-infected plants of *N. benthamiana* (RA-4), *C. annuum* 'Jalapeno' and *S. betaceum* for 12 h (treatment 1); 24 h (treatment 2); 48 h (treatment 3).

Step 3: After feeding on the infected plants, larvae were collected separately by species and transferred to tested plants of the same species to ensure these plant species were adapted food to this cotton bollworm (three larvae per plant). After two days feeding on the tested plants, larvas were removed.

Control of this experiment was carried out on two plants per each species and only included two steps (larvas were fasted and then transferred to uninfected (control) plants and then removed after two days feeding). There were six replicate plants per species. Plants were maintained in cages.

The experiment was repeated twice. Tested plants were observed daily for symptoms in three weeks (to the date samples collected) since 5d removing the larvas. Leaf samples were collected after 21 d and RT-PCR assays with YTMMV-specific primers were used to detect the presence of YTMMV.

Table 4.4 YTMMV transmission experiment using larvae of cotton bollworm

Treatments	Species	Starvation time (h)	Feeding time on infected plants (h)	Feeding time on tested plants (h)
1	*Nicotiana benthamiana* (RA-4)	12	12	24
	Capsicum annuum 'Jalapeno'			36
	Solanum betaceum			72
2	*N. benthamiana* (RA-4)	12	24	24
	C. annuum 'Jalapeno'			36
	S. betaceum			72
3	*N. benthamiana* (RA-4)	12	48	24
	C. annuum 'Jalapeno'			36
	S. betaceum			72

4.2.2. Testing transmission of YTMMV in field plots under natural conditions

4.2.2.1. Plant sources

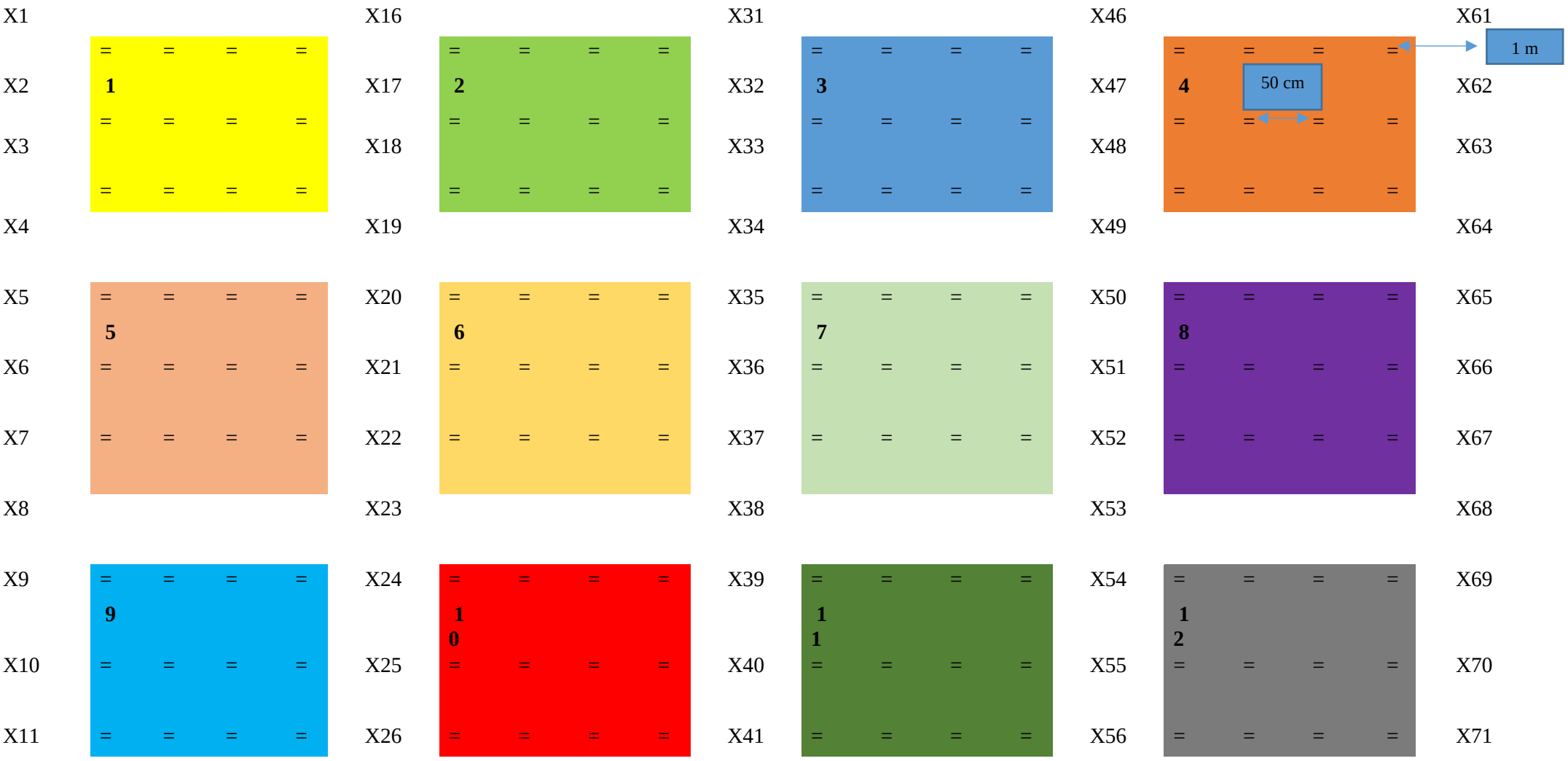
Seven species were used to test transmission of YTMMV in the field: *N. benthamiana* RA-4, *S. lycopersicum* 'Tigerella', *C. annuum* 'Californian Wonder', *C. annuum* 'Jalapeno', *N. tabacum* 'Wisconsin 38', *S. betaceum*, *S. melongena* 'Long Purple'. All infected and tested plants were prepared and transferred in 2 L pots in a glasshouse before moving them to the field. Infected plants of all seven species were produced by manual inoculation with YTMMV-Cervantes isolate when they were from five to six weeks of age. Leaf samples were tested by RT-PCR (Chapter 2) to confirm the presence of YTMMV after two weeks since inoculated. The numbers of infected and tested plants are listed in Table 4.5.

Table 4.5 Plant species used in the field to test for natural transmission of YTMMV

Plant species	Numbers of infected plants	Numbers of uninfected plants	Total plants
Capsicum annuum 'Jalapeno'	13	35	48
Nicotiana benthamiana (RA-4)	24	62	86
Solanum betaceum	11	30	41
C. annuum 'Californian Wonder'	5	16	21
S. melongena 'Long Purple'	5	14	19
N. tabacum 'Wiscosin 38'	3	10	13
S. lycopersicum 'Tigerella'	14	25	39
Total plants	75	192	267

4.2.2.2. Field experimental design

The 192 uninfected (tested) plants (pots) were placed into 16 plots (Fig. 4.2). Each plot was organized by 12 tested plants which selected randomly from the source, the detail of species and location are listed in the Table 4.6. Infected plants were arranged between the plots (Table 4.7). The distance between pots (tested plants) was 50 cm, and between tested plant (pot) and infected plant (pot) was 1 m (Fig 4.2).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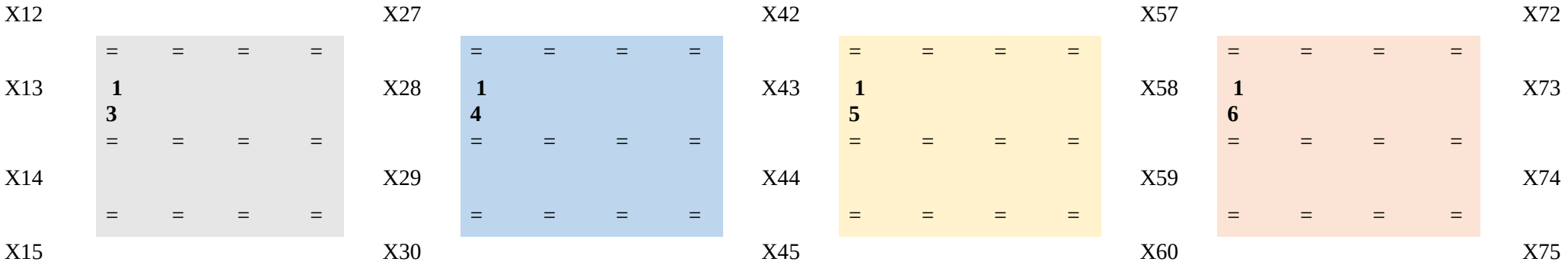

Figure 4.2 Field plot design to test for transmission of YTMMV under field conditions. Infected source plants (75 plants) are shown as X. Uninfected plants are shown as a dash. There are 16 plots equal to 16 different colours, one plot has 12 uninfected tested plants

Table 4.6 Uninfected plant species and their position in all 16 plots (equal 16 diferrent colours) to test for transmission of YTMMV under field conditions

1)

Capsicum annuum 'Jalapeno'	*Solanum melongena* 'Long Purple'	*S. lycopersicum* 'Tigerella'	*C. annuum* 'Jalapeno'
C. annuum 'Californian Wonder'	*Nicotiana benthamiana* (RA-4)	*S. lycopersicum* 'Tigerella'	*S. lycopersicum* 'Tigerella'
Nicotiana benthamiana (RA-4)	*S. betaceum*	*C. annuum* 'Jalapeno'	*N. benthamiana* (RA-4)

2)

N.benthamiana (RA-4)	*S. melongena* 'Long Purple'	*S. lycopersicum* 'Tigerella'	*C. annuum* 'Jalapeno'
C. annuum 'Jalapeno'	*N. benthamiana* (RA-4)	*S. lycopersicum* 'Tigerella'	*S. betaceum*
C. annuum 'Californian Wonder'	*S. lycopersicum* 'Tigerella'	*S. lycopersicum* 'Tigerella'	*N. benthamiana* (RA-4)

3)

N. benthamiana (RA-4)	*S. betaceum*	*N. benthamiana* (RA-4)	*C. annuum* 'Californian Wonder'
S. melongena 'Long Purple'	*S. lycopersicum* 'Tigerella'	*S. lycopersicum* 'Tigerella'	*S. betaceum*
C. annuum 'Jalapeno'	*N. benthamiana* (RA-4)	*C. annuum* 'Jalapeno'	*N. benthamiana* (RA-4)

4)

N. benthamiana (RA-4)	*S. melongena* 'Long Purple'	*S. lycopersicum* 'Tigerella'	*C. annuum* 'Jalapeno'
C. annuum 'Jalapeno'	*N. benthamiana* (RA-4)	*N. benthamiana* (RA-4)	*S. betaceum*
S. betaceum	*N. tabacum* 'Wiscosin 38'	*C. annuum* 'Californian Wonder'	*N. benthamiana* (RA-4)

5)

C. annuum 'Jalapeno'	*S. melongena* 'Long Purple'	*C. annuum* 'Jalapeno'	*N. benthamiana* (RA-4)
C. annuum 'Californian Wonder'	*N. benthamiana* (RA-4)	*S. betaceum*	*S. lycopersicum* 'Tigerella'
N. benthamiana (RA-4)	*N. tabacum* 'Wiscosin 38'	*N. benthamiana* (RA-4)	*S. betaceum*

6)

S. betaceum	*S. lycopersicum* 'Tigerella'	*S. lycopersicum* 'Tigerella'	*S. betaceum*
C. annuum 'Jalapeno'	*N. benthamiana* (RA-4)	*N. benthamiana* (RA-4)	*C. annuum* 'Jalapeno'
N. benthamiana (RA-4)	*C. annuum* 'Californian Wonder'	*S. melongena* 'Long Purple'	*N. benthamiana* (RA-4)

7)

S. betaceum	*N. benthamiana* (RA-4)	*S. melongena* 'Long Purple'	*N. benthamiana* (RA-4)
N. benthamiana (RA-4)	*N. tabacum* 'Wiscosin 38'	*S. betaceum*	*C. annuum* 'Jalapeno'
C. annuum 'Jalapeno'	*C. annuum* 'Jalapeno'	*C. annuum* 'Californian Wonder'	*N. benthamiana* (RA-4)

8)

C. annuum 'Jalapeno'	*S. melongena* 'Long Purple'	*S. betaceum*	*N. benthamiana* (RA-4)
N. tabacum 'Wiscosin 38'	*S. betaceum*	*C. annuum* 'Jalapeno'	*S. lycopersicum* 'Tigerella'
N. benthamiana (RA-4)	*C. annuum* 'Californian Wonder'	*N. benthamiana* (RA-4)	*N. benthamiana* (RA-4)

9)

N. benthamiana (RA-4)	*N. benthamiana* (RA-4)	*N. tabacum* 'Wiscosin 38'	*C. annuum* 'Californian Wonder'
S. lycopersicum 'Tigerella'	*S. betaceum*	*C. annuum* 'Jalapeno'	*N. benthamiana* (RA-4)
C. annuum 'Jalapeno'	*S. melongena* 'Long Purple'	*N. benthamiana* (RA-4)	*S. betaceum*

10)

C. annuum 'Jalapeno'	*N. benthamiana* (RA-4)	*N. benthamiana* (RA-4)	*C. annuum* 'Californian Wonder'
S. melongena 'Long Purple'	*S. betaceum*	*S. lycopersicum* 'Tigerella'	*N. benthamiana* (RA-4)
N. benthamiana (RA-4)	*N. tabacum* 'Wiscosin 38'	*C. annuum* 'Jalapeno'	*S. betaceum*

11)

N. benthamiana (RA-4)	*S. betaceum*	*N. benthamiana* (RA-4)	*C. annuum* 'Californian Wonder'
S. lycopersicum 'Tigerella'	*C. annuum* 'Jalapeno'	*N. tabacum* 'Wiscosin 38'	*C. annuum* 'Jalapeno'
S. betaceum	*S. melongena* 'Long Purple'	*N. benthamiana* (RA-4)	*N. benthamiana* (RA-4)

12)

N. benthamiana (RA-4)	*C. annuum* 'Jalapeno'	*N. benthamiana* (RA-4)	*S. betaceum*
N. tabacum 'Wiscosin 38'	*S. melongena* 'Long Purple'	*S. lycopersicum* 'Tigerella'	*C. annuum* 'Jalapeno'
N. benthamiana (RA-4)	*S. betaceum*	*C. annuum* 'Californian Wonder'	*N. benthamiana* (RA-4)

13)

N. benthamiana (RA-4)	*N. benthamiana* (RA-4)	*S. lycopersicum* 'Tigerella'	*N. tabacum* 'Wiscosin 38'
S. betaceum	*S. lycopersicum* 'Tigerella'	*C. annuum* 'Jalapeno'	*N. benthamiana* (RA-4)
C. annuum 'Jalapeno'	*S. melongena* 'Long Purple'	*N. benthamiana* (RA-4)	*C. annuum* 'Californian Wonder'

14)

N. benthamiana (RA-4)	*S. lycopersicum* 'Tigerella'	*C. annuum* 'Jalapeno'	*N. benthamiana* (RA-4)
S. lycopersicum 'Tigerella'	*S. lycopersicum* 'Tigerella'	*S. melongena* 'Long Purple'	*S. betaceum*
N. benthamiana (RA-4)	*C. annuum* 'Jalapeno'	*C. annuum* 'Californian Wonder'	*N. benthamiana* (RA-4)

15)

N. benthamiana (RA-4)	*C. annuum* 'Jalapeno'	*N. benthamiana* (RA-4)	*N. benthamiana* (RA-4)
S. lycopersicum 'Tigerella'	*S. betaceum*	*S. lycopersicum* 'Tigerella'	*S. betaceum*
C. annuum 'Jalapeno'	*C. annuum* 'Californian Wonder'	*N. benthamiana* (RA-4)	*C. annuum* 'Jalapeno'

16)

N. benthamiana (RA-4)	*S. lycopersicum* 'Tigerella'	*C. annuum* 'Jalapeno'	*N. benthamiana* (RA-4)
S. betaceum	*S. lycopersicum* 'Tigerella'	*N. benthamiana* (RA-4)	*S. betaceum*
C. annuum 'Jalapeno'	*S. lycopersicum* 'Tigerella'	*S. lycopersicum* 'Tigerella'	*N. benthamiana* (RA-4)

Table 4.7 Lists and position of 75 infected plants (X1-X75) of *Nicotiana tabacum* 'Wiscosin 38'; *Capsicum annuum* 'Californian Wonder'; *C. annuum* 'Jalapeno'; *Solanum melongena* 'Long Purple'; *N. benthamiana* RA-4; *S. lycopersicum* 'Tigerella', *S. betaceum* to test for transmission of YTMMV under field conditions

X1	*C. annuum* 'Jalapeno'	X16	*N. benthamiana* (RA-4)	X31	*S. betaceum*	X46	*N. benthamiana* (RA-4)	X61	*C. annuum* 'Jalapeno'
X2	*N. benthamiana* (RA-4)	X17	*S. lycopersicum* 'Tigerella'	X32	*C. annuum* 'Jalapeno'	X47	*N. benthamiana* (RA-4)	X62	*S. melongena* 'Long Purple'
X3	*S. betaceum*	X18	*N. benthamiana* (RA-4)	X33	*C. annuum* 'Californian Wonder'	X48	*S. lycopersicum* 'Tigerella'	X63	*N. benthamiana* (RA-4)
X4	*N. benthamiana* (RA-4)	X19	*C. annuum* 'Jalapeno'	X34	*N. benthamiana* (RA-4)	X49	*S. lycopersicum* 'Tigerella'	X64	*C. annuum* 'Jalapeno'
X5	*C. annuum* 'Jalapeno'	X20	*S. melongena* 'Long Purple'	X35	*S. lycopersicum* 'Tigerella'	X50	*S. betaceum*	X65	*N. benthamiana* (RA-4)
X6	*C. annuum* 'Californian Wonder'	X21	*S. betaceum*	X36	*C. annuum* 'Jalapeno'	X51	*N. benthamiana* (RA-4)	X66	*S. lycopersicum* 'Tigerella'
X7	*N. benthamiana* (RA-4)	X22	*C. annuum* 'Jalapeno'	X37	*N. benthamiana* (RA-4)	X52	*C. annuum* 'Californian Wonder'	X67	*S. lycopersicum* 'Tigerella'
X8	*S. betaceum*	X23	*N. benthamiana* (RA-4)	X38	*S. lycopersicum* 'Tigerella'	X53	*S. betaceum*	X68	*N. benthamiana* (RA-4)
X9	*C. annuum* 'Jalapeno'	X34	*S. lycopersicum* 'Tigerella'	X39	*N. benthamiana* (RA-4)	X54	*N. benthamiana* (RA-4)	X69	*S. betaceum*
X10	*N. tabacum* 'Wiscosin 38'	X25	*S. lycopersicum* 'Tigerella'	X40	*S. betaceum*	X55	*S. melongena* 'Long Purple'	X70	*S. lycopersicum* 'Tigerella'
X11	*N. benthamiana* (RA-4)	X26	*N. benthamiana* (RA-4)	X41	*N. tabacum* 'Wiscosin 38'	X56	*S. lycopersicum* 'Tigerella'	X71	*N. benthamiana* (RA-4)
X12	*S. melongena* 'Long Purple'	X27	*C. annuum* 'Californian Wonder'	X42	*N. benthamiana* (RA-4)	X57	*S. betaceum*	X72	*C. annuum* 'Californian Wonder'

X13	*S. lycopersicum* 'Tigerella'	X28	*C. annuum* 'Jalapeno'	X43	*S. lycopersicum* 'Tigerella'	X58	*N. benthamiana* (RA-4)	X73	*S. lycopersicum* 'Tigerella'
X14	*N. benthamiana* (RA-4)	X29	*S. betaceum*	X44	*N. benthamiana* (RA-4)	X59	*N. tabacum* 'Wiscosin 38'	X74	*S. betaceum*
X15	*C. annuum* 'Jalapeno'	X30	*N. benthamiana* (RA-4)	X45	*S. melongena* 'Long Purple'	X60	*C. annuum* 'Jalapeno'	X75	*C. annuum* 'Jalapeno'

4.2.2.3. Collecting samples

The leaves of tested plants were collected after 8 weeks since the field experiment exposed. However, many plants did not grow well under the natural condition. Some of them died before collecting samples. This experiment finished when collecting the fruits of tested plants such as *N. tabacum* fruits. The experiment was repeated twice.

4.3. Results

No symptoms were recorded and no any RT-PCR test detection to all leaf samples which collected from both transmission experiments of GPAs and cotton bollworm. No virus transmission occurred that was caused by GPAs or cotton bollworm larvae under any of the experimental conditions tested.

Figure 4.3 *Solanum betaceum* plants after feeding by cotton bollworm for 72 h

In the field experiment, a number of insects and snails were recorded on the plants (Fig 4.4). The numbers of uninfected plants were chosen to test the presence of YTMMV by RT-PCR tests as described as the Table 4.8. Total samples per each repeat was 80 and selected randomly in the total 192 tested plants. It is difficult to realize the symptom because of weather effected. No any transmission was confirmed.

Table 4.8 Numbers of RT-PCR tests by species in the field for presence of YTMMV after 8 weeks of exposure to source plants

Species	*Capsicum annuum* 'Jalapeno'	*Nicotiana benthamiana* (RA-4)	*Solanum betaceum*	*C. annuum* 'Californian Wonder'	*S. melongena* 'Long Purple'	*N. tabacum* 'Wiscosin 38'	*S. lycopersicum* 'Tigerella'
Total samples (80)	22	6	8	12	6	10	16


A
B


C
D

Figure 4.4 Insects and molluscs colonised both virus source plants and uninfected plants in the field. Snails were in *Capsicum annuum* 'Jalapeno' (Picture A), *Nicotiana tabacum* 'Wisconsin 38' (Picture B & D), *Solanum melongena* 'Long Purple' (Picture H); Ladybug in *N. tabacum* 'Wisconsin 38' (Picture E); in *S. lycopersicum* 'Tigerella' (Picture C), *N. tabacum* 'Wisconsin 38' (Picture D, F); *N. benthamiana* RA-4 (Picture G)

4.4. Discussion

Previous studies showed transmission of tobamoviruses occurred by insects, probably through mechanistic transmission of virions still adhering to mouthparts or other parts of the arthropod after feeding on infected plants (Hoggan, 1931; Walters, 1951; Newton, 1953; Chant, 1959; Rao & Varma, 1984). We tested two insect species, one sucking (aphid) and one chewing (moth), to test if there was mechanistic or vectored transmission of YTMMV under laboratory conditions. Although green peach aphid (order Homoptera) is present in the wild in Western Australia where this research was done (https://grdc.com.au), cotton bollworm (order Lepidoptera) is not. No transmission was observed for either species, which doesn't preclude mechanistic YTMMV transmission happening if greater numbers of replicates were tested.

Mechanistic horizontal transmission of YTMMV by other animals in the environment, including but not limited to arthropods, molluscs, nematodes, mammals and birds is also possible. We endeavoured to test for horizontal transmission by wild agents in the field by providing ample sources of infection in the form of infected source plants spaced throughout the fields of uninfected plants. We observed snails, aphids, ladybugs on both the infected and uninfected plants in the field. Although rabbits lived around our field plot, they were excluded to prevent the test plants being eaten. Horizontal transmission of YTMMV was not detected in any uninfected bait plant. A limitation of this study was that vertical transmission *via* pollen, perhaps transferred between plants by honey bees or native bees, was not tested. Thus, if pollinators transmitted infected pollen to the uninfected plants, the seed could be infected but not the mother plant (Darzi *et al*., 2017). In this case, YTMMV would not have been detected because we were focused on horizontal transmission of virus in the leaves. Further experimentation

into vertical transmission between plants *via* pollen, whether wind-borne or pollinator-borne, in the field is a potential mode of transmission of this virus, and so should be tested. Very little is known about tobamovirus transmission in wild systems (Jones, 2018).

In addition to early reports (above) of apparently mechanistic tobamovirus infections caused by invertebrates, Castello *et al.* (1995) discovered infectious virions of ToMV in fog and clouds. They proposed a new mechanistic route of transmission by ToMV, that of wet aerosols as the source of infections of red spruce (*Picea rubens*) in the USA. Although they failed to identify site(s) of infection, they proposed it could enter through pores in needles or roots. Although it is possible that aerosol-borne transmission of YTMMV particles from infected coastal host plants such as *Anthocercis* species occurs, we hypothesise that this would most likely be a rare transmission route because, unlike red spruce plants that have huge surface areas and grow together in large numbers in coastal forests, the hosts of YTMMV are relatively small plants that are naturally usually widely spaced. Another neglected route of possible transmission of tobamoviruses in wild environments is parasitic plants that establish vascular bridges between plants, such as *Cuscuta* species, which occur widely in the study area. There are some ~4500 species of parasitic plants (Gogoi *et al*., 2021) described internationally and very little is known about the viromes of most of them

Although we cannot rule out mechanistic transmission of YTMMV by different creatures and parasitic plants, and possibly by air-borne aerosols, we consider these routes to be unlikely, or at least minor routes of transmission of this virus between plants. Testing these routes in the field is challenging, especially if the agents of

transmission are unknown, but an absorbing area of study to understand the relative importances of different routes of transmission used by the tobamoviruses.

Insects were not tested for freedom of virus by RT-PCR before applying them in the transmission experiments. They were maintained on virus-free plants in insect-proof cages in a separate building from the virus-infected to infected plants. We consider these precautions sufficient to prevent inadvertent contamination. Similarly, the symptoms of the infected plants of *N. benthamiana* (RA-4), *C. annuum* 'Jalapeno', *S. betaceum* were clear. The uninfected control plants were maintained in the separate room.

Chapter 5: Potential implications of YTMMV spillover to horticultural production

5.1. Intoduction

Tomato (*Solanum lycopericum*) evolved in western parts of South America and Central America and was introduced to Europe by the Spanish in the sixteenth century following their colonization of Mexico (Hanssen & Lapidot, 2012; Knapp & Peralta, 2016). Tomato has a wide climatic tolerance and is now grown in both tropical and temperate regions around the world (Hanssen & Lapidot, 2012). According to the FAOSTAT, in 2020, tomato production of Australia was 297,474 tons harvested from the area of 3917 ha, with an average yield of 75.9 tons/ha. Tomato presents a limited caloric supply but is a source of carotenoids, dietary fiber, minerals, vitamins (mainly vitamin A), and flavonoids (Nagai *et al.*, 2019).

Pepper and chilli (*Capsicum* species) are also important crop plants in family Solanaceae that originated in South and Central America. The genus *Capsicum* comprises about 40 species, five of which are cultivated (Eshbaugh, 2012). China and Turkey are the world's largest producers of *Capsicum* species (Yaldiz *et al.*, 2010). The yield of chili and pepper in Australia has increased in recent years, 21.9; 23.6 and 25.7 tons/ha in 2017, 2018, 2019, respectively, but decreased to 19.7 ton/ha in 2020 (FAOSTAT). Production of chillies and pepper in Australia was 40,774 tons harvested from the area of 2058 ha (FAOSTAT).

Damaging tobamoviruses identified from tomato, capsicum and chili include TMV, ToMMV, ToMV, tobacco mild green mosaic virus (TMGMV), PMMoV, and ToBRFV (Dombrovsky & Smith, 2017; Wetter, 1984). These viruses can cause severe economic losses in both field and greenhouse-grown crops. For instance, yields of capsicum decreased by 35-55% in the USA (Villalon, 1981) and by 40% (late infection) to 80% (early infection) in Argentina (Feldman *et al.*,1969) because of tobamovirus infections.

ToMV and ToMMV are both considered major viral threats to tomato (Nagai *et al.*, 2019). To respond to the risk of ToMMV (Li *et al.*, 2017), Australia has implemented emergency measures to prohibit infected tomato and capsicum seed entering the country.

The aim of this chapter is to investigate the influence of YTMMV infection on yield in tomato and capsicum cultivated varieties under experimental conditions.

5.2. Materials and Methods

5.2.1. Plants

S. lycopersicum ‘Tigerella’, *C. annuum* ‘Californian Wonder’ and *C. annuum* ‘Jalapeno’ cultivars were selected to evaluate the potential implications of YTMMV infection on plant growth and fruit yield of these crop varieties.

5.2.2. Experimental design

Experiments were done under laboratory conditions. Plants were grown in temperature-controlled and insect-proof greenhouses (22°C day and 17°C night). Uninfected plants and infected plants of the same varieties were grown under the same environmental conditions.

YTMMV isolate Cervantes was used to challenge plants. Inoculation was done at three developmental stages: Stage 1. four-leaf stage (seedling); Stage 2. 8-10 leaf stage (vegetative); Stage 3. pre-flower stage. Each experiment was done twice.

Before each stage of inoculation, every plant was tested by RT-PCR using YTMMV-random and specific primers (see Table 2.1, Chapter 2) to confirm it was free of virus. The method described previously (Chapter 2) was used to confirm the presence of YTMMV in the infected plants after two weeks of inoculation and also to mock-inoculated control plants.

5.2.2.1. Solanum lycopersicum 'Tigerella'

Table 5.1 Treatments of *Solanum lycopersicum* 'Tigerella' plants. The experiment was replicated twice. The first experiment included 40 infected plants and 40 uninfected plants compared to 36 infected plants and 36 uninfected plants in the repeat experiment. Tomato fruits were harvested when the skin changed to red colour in the period of 44 to 48 days

Inoculated stage	Replicate	Inoculation date (days post sowing – dps)	Infected plants	Uninfected plants (control)	Harvesting time
1. Seedling	1	30 days	13	13	44 days
	2	28 days	12	12	48 days
2. Vegetative	1	37 days	13	13	44 days
	2	35 days	12	12	48 days
3. Pre-flowering	1	43 days	14	14	44 days
	2	42 days	12	12	48 days

5.2.2.2. Capsicum annuum 'Californian Wonder'

Table 5.2 Treatments of *Capsicum annuum* 'Californian Wonder' plants. The experiment was replicated twice. The first experiment included 30 infected plants and 30 uninfected plants compared to 60 infected plants and 60 uninfected plants in the repeat experiment. Capsicum fruits were harvested in one time including both red and green colour

Inoculated stage	Replicate	Inoculation date (days post sowing – dps)	Infected plants	Uninfected plants (control)	Harvesting time
1. Seedling	1	31 days	10	10	One time
	2	33 days	20	20	One time
2. Vegetative	1	45 days	10	10	One time
	2	46 days	20	20	One time
3. Pre-flowering	1	56 days	10	10	One time
	2	57 days	20	20	One time

5.2.2.3. Capsicum annuum 'Jalapeno'

Table 5.3 Treatments of *Capsicum annuum* 'Jalapeno' plants. The experiment was replicated twice. The first experiment included 30 infected plants and 30 uninfected plants compared to 48 infected plants and 48 uninfected plants in the repeat experiment. Chili fruits were harvested in two months when the skin changed to red colour

Inoculated stage	Replicate	Inoculation date (days post sowing – dps)	Infected plants	Uninfected plants (control)	Harvesting time
1. Seedling	1	31 days	10	10	64 days
	2	36 days	16	16	65 days
2. Vegetative	1	68 days	10	10	64 days
	2	68 days	16	16	65 days
3. Pre-flowering	1	93 days	10	10	64 days
	2	98 days	16	16	65days

5.2.3. Infection damage

There were five measurements to evaluate damage on infected plants: height (cm), number of branches, number of fruits, weight fruit (gram), and length fruit (cm) (except to fruits of *S. lycopersicum* 'Tigerella' because of small size). Data of height, number of branches were recorded at the harvesting stage. The periods when fruit data was collected were 44-48 days for *S. lycopersicum* 'Tigerella', one time for *C. annuum* 'Californian Wonder' and 64-65 days for *C. annuum* 'Jalapeno'. Fruits of *S. lycopersicum* 'Tigerella' and *C. annuum* 'Jalapeno' were harvested and recorded when their colour was red. All fruits, red and green, of *C. annuum* 'Californian Wonder' were havvested at one time and data recorded.

5.2.4. Data analysis

5.2.4.1. Software

The statistical software package SPSS 22.0 (IBM, Armonk, NY, USA) was used to analyse the data.

5.2.4.2. Group analysis

Data was on height (cm), number of branches, number of fruits, weight fruit (gram), and length fruit (cm) between infected plants of three stages of inoculation and uninfected plants. Tomato fruit of this species is small so length of fruit was not recorded. The data were analysed as six groups (Appendix 1). The aim was to compare the difference between control (uninfected plant) and plants infected at three growth stages.

1) Group 1: Comparison of average height (cm), number of branches, number of fruits, weight of fruit (g), and length fruit (cm) of uninfected plants (control) between the first and the second replicate in stage 1/ stage 2/ stage 3 of inoculation.
2) Group 2: Comparison of average height (cm), number of branches, number of fruits, weight fruit (g), and length fruit (cm) of infected plants between the first and the second replicate in stage 1/ stage 2 / stage 3 of inoculation – three tables per species of *S. lycopersicum* 'Tigerella' and *C. annuum* 'Californian Wonder'.
3) Group 3: Comparison of average height (cm), number of branches, number of fruits, weight fruit (g), and length fruit (cm) of between uninfected plants (control) and infected plants in stage 1 / stage 2 / stage 3 of inoculation - three tables per species of *S. lycopersicum* 'Tigerella', *C. annuum* 'Californian Wonder' and one table for *C. annuum* 'Jalapeno' in stage 3 of inoculation.
4) Group 4: Comparison of average height (cm), number of branches, number of fruits, weight fruit (g), and length fruit (cm) of between all uninfected plants (control) and infected plants in stage 1 / stage 2 / stage 3 of inoculation – three tables per each species of *S. lycopersicum* 'Tigerella', *C. annuum* 'Californian Wonder' and one table for *C. annuum* 'Jalapeno' in stage 3 of inoculation.
5) Group 5: Comparison of average height (cm), number of branches, number of fruits, weight fruit (g), and length fruit (cm) of between all uninfected plants (control) and infected plants of stage 1 / stage 2 / stage 3 of inoculation in first replicate – three tables per each species of *S. lycopersicum* 'Tigerella', *C. annuum* 'Californian Wonder'.
6) Group 6: Comparison of average height (cm), number of branches, number of fruits, weight fruit (g) between all uninfected plants (control) and infected plants

of stage 1 / stage 2 / stage 3 of inoculation in the second replicate – three tables of each species of *S. lycopersicum* ‘Tigerella’, *C. annuum* ‘Californian Wonder’.

5.3. Results

5.3.1. *Solanum lycopersicum* 'Tigerella'

Four measurements were: average height (Fig 5.1), number of branches (Fig 5.2), number of fruits (Fig 5.3), weight fruit (Fig 5.4). The symptoms of YTMMV infection on *S. lycopersicum* 'Tigerella' was mild (Fig 5.5); all the measures resembled those of virus-free plants, and no clear symptoms of infection were visible.

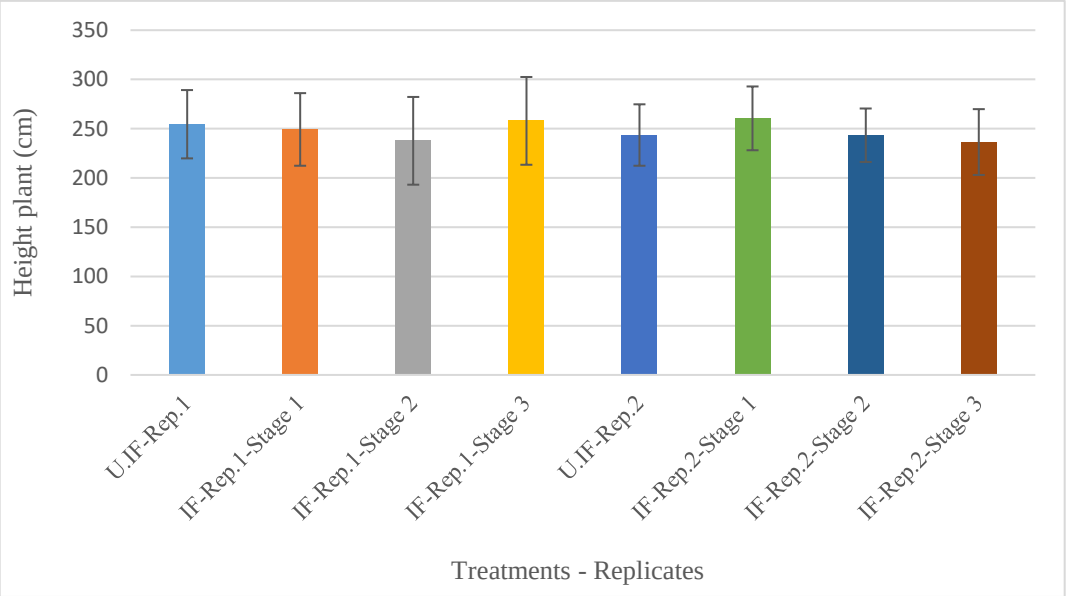

Figure 5.1 Comparison of the average height of *Solanum lycopersicum* 'Tigerella' plants between uninfected plants and infected plants in three stages of inoculation between two replicates

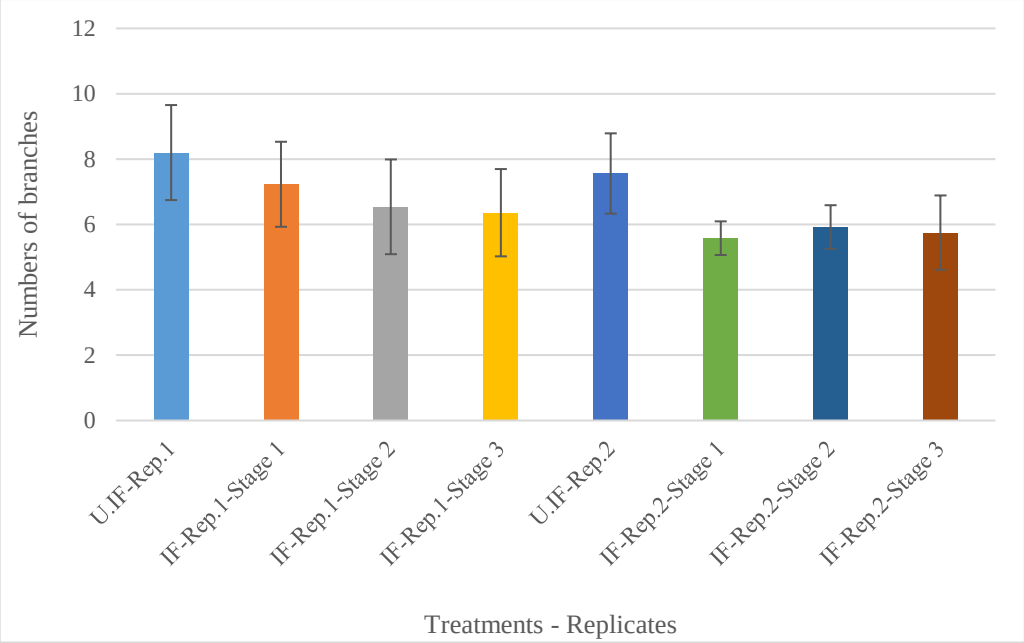

Figure 5.2 Comparison of the average numbers of branches per *Solanum lycopersicum 'Tigerella'* plant between uninfected plants and infected plants in three stages of inoculation between two replicates

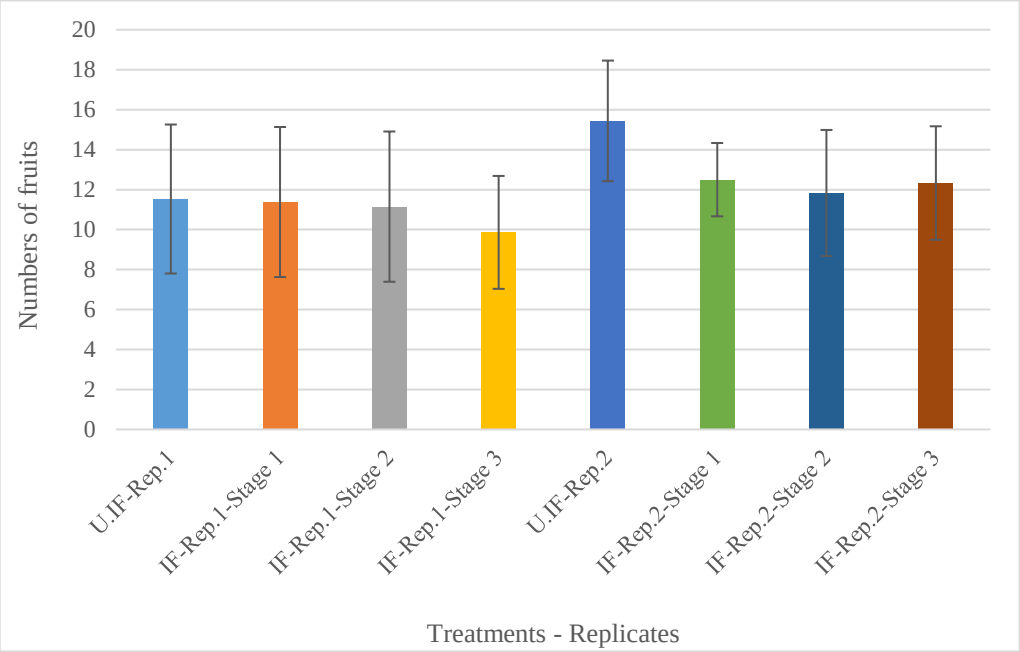

Figure 5.3 Comparison of the average numbers of fruits per of *Solanum lycopersicum* 'Tigerella' plants between uninfected plants and infected plants in three stages of inoculation between two replicates

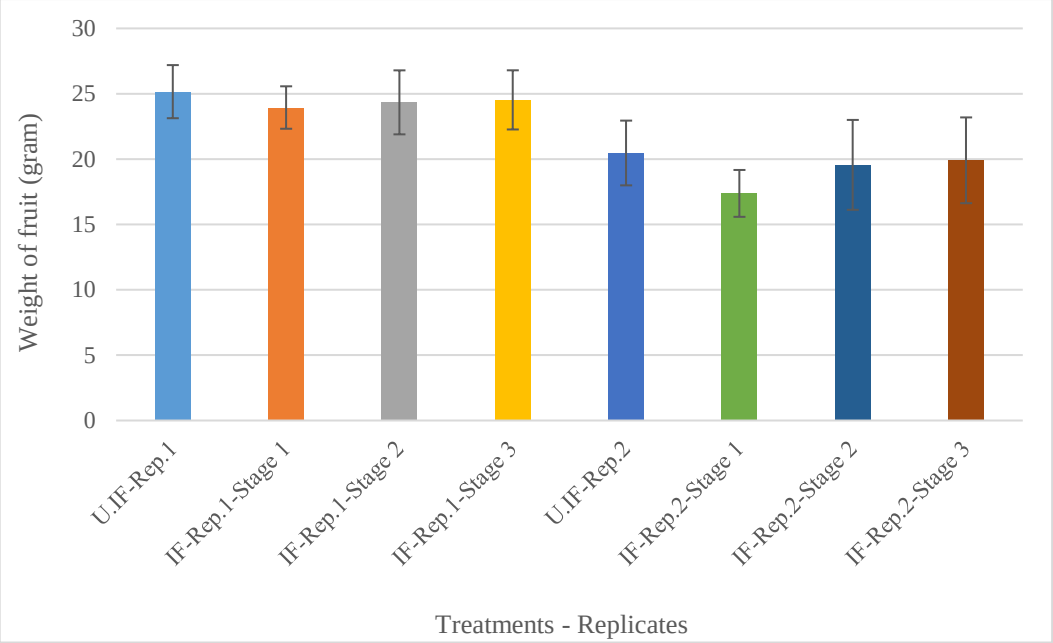

Figure 5. 4 Comparison of the average weight fruit of *Solanum lycopersicum* 'Tigerella' plants between uninfected plants and infected plants in three stages of inoculation between two replicates

Figure 5.5 There is no disease symptom observed from the *Solanum lycopersicum* 'Tigerella' infected plants (right) and the same growth when compared to uninfected plants (left) (The infected plants were inoculated by YTMMV at the seedling stage)

5.3.2. Capsicum annuum ‘Californian Wonder’

Five criteria were used to evaluate the effect of YTMMV to *C. annuum* ‘Californian Wonder’, namely, height (Fig. 5.6), number of branches (Fig. 5.7), number of fruits (Fig. 5.8), weight of fruit (Fig. 5.9), length of fruit (Fig. 5.10). The average number of fruits of *C. annuum* ‘Californian Wonder’ infected plants was the same between three inoculation stages, but lower than average number of fruits of uninfected plants.

Infection at the seedling stage of growth affected subsequent development of the plant, including fruit numbers and quality (Fig 5.8; 5.9; 5.10; 5.12). Infection at the two later stages of vegetative and pre-flowering also affected fruit quality, but to a lesser extent (Fig 5.9; 5.10).

This did not happen to all criteria, however, the data of stage 1 inoculation of height, number of fruits, weight of fruit, length of fruit were lower than that of the other stages of inoculation as well as to the control. Although the symptoms are very clear in the stage one inoculation (Fig. 5.11), the infected *C. annuum* ‘Californian Wonder’ plants still grew and produced fruits. However, the disease expressed very strongly in some fruits Fig. 5.12.

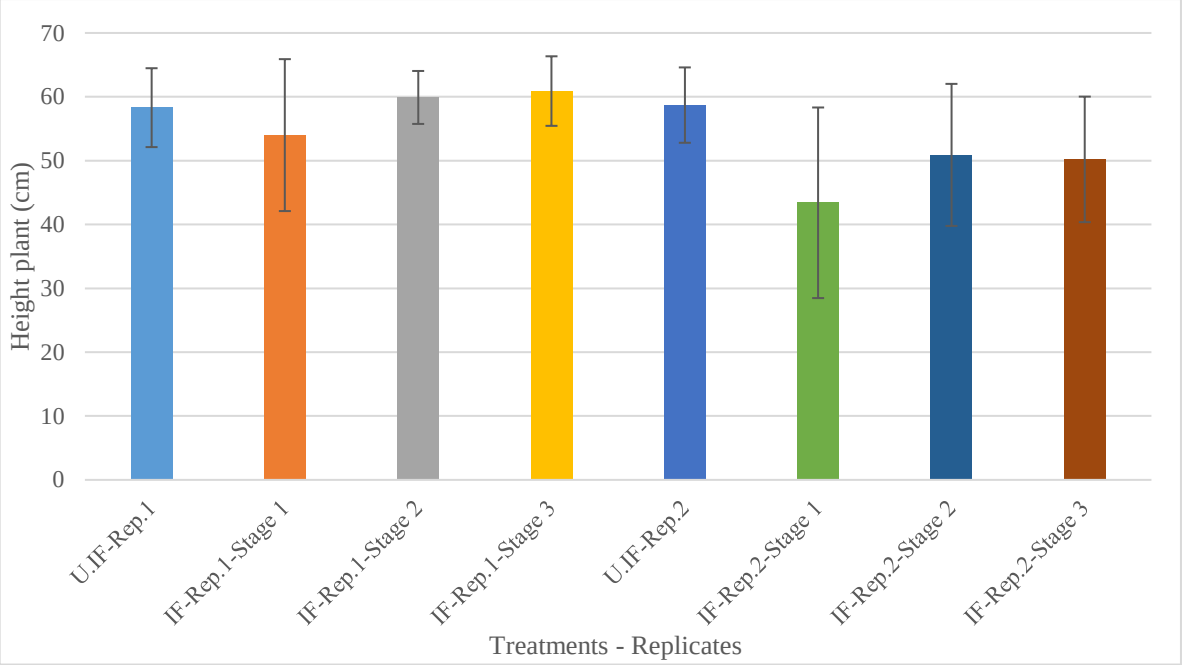

Figure 5. 6 Comparison of the average height of *Capsicum annuum* 'Californian Wonder' plants between uninfected plants and infected plants in three stages of inoculation between two replicates

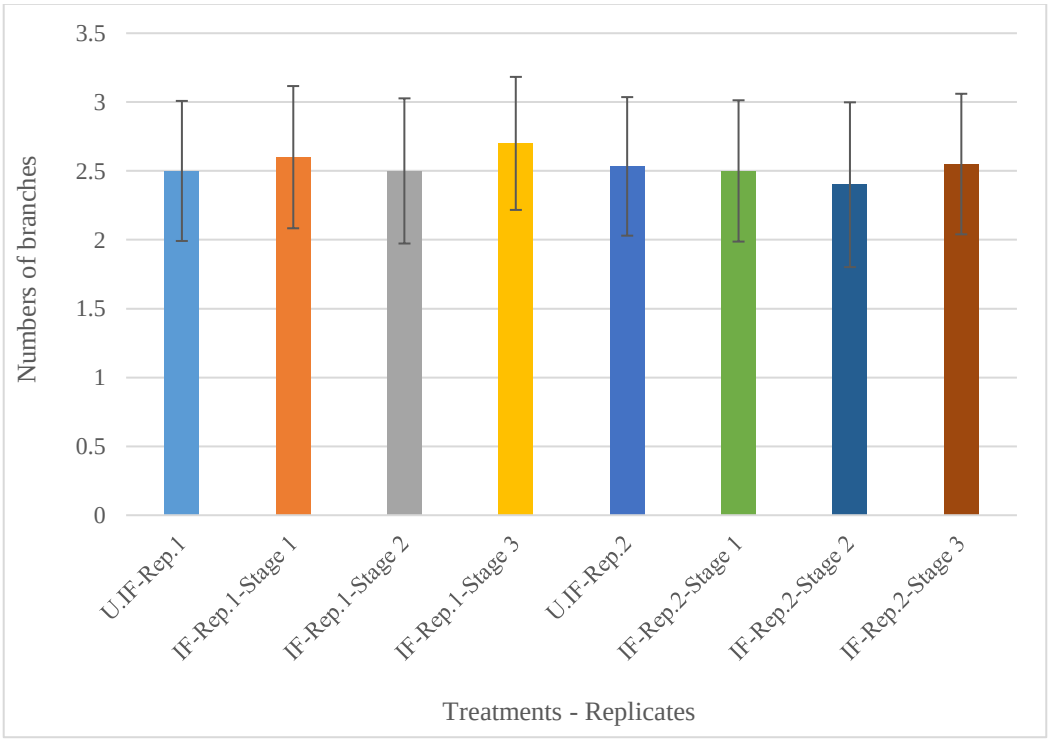

Figure 5.7 Comparison of the average numbers of branches per *Capsicum annuum* 'Californian Wonder' plants between uninfected plants and infected plants in three stages of inoculation between two replicates

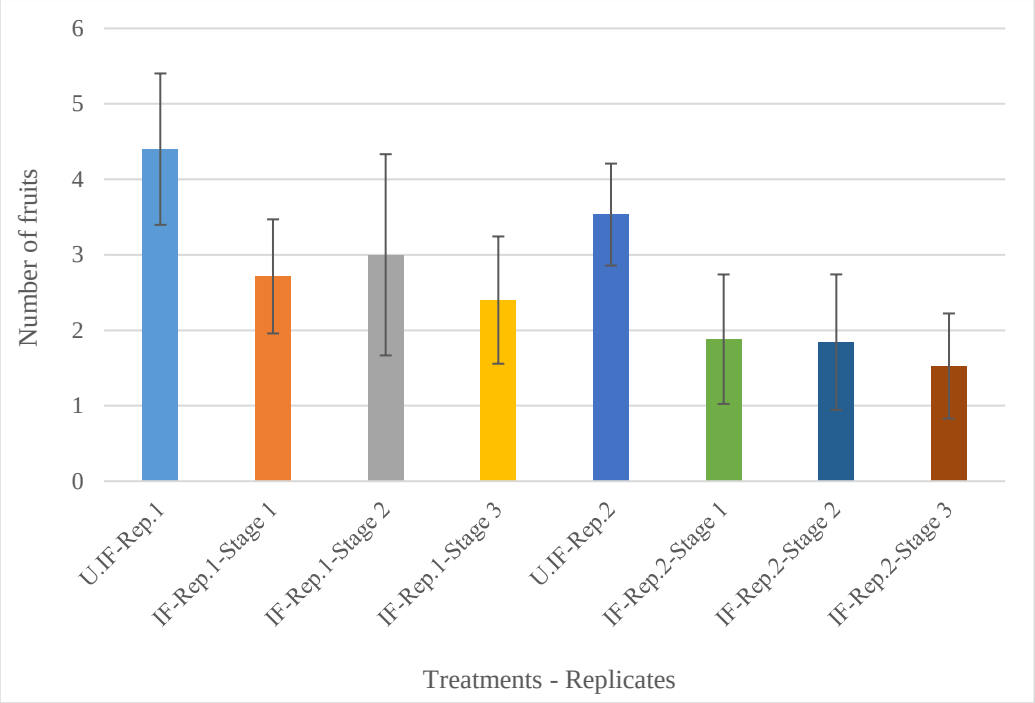

Figure 5.8 Comparison of the average number of fruits per *Capsicum annuum* 'Californian Wonder' plants between uninfected plants and infected plants in three stages of inoculation between two replicates

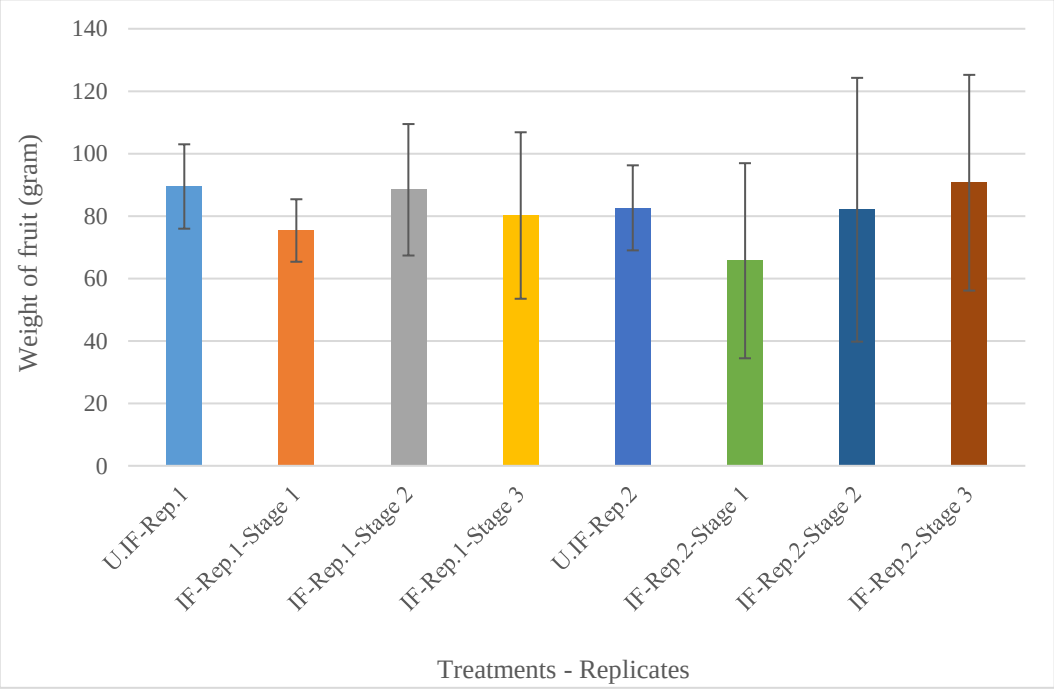

Figure 5. 9 Comparison of the average weight of fruit of *Capsicum annuum* 'Californian Wonder' plants between uninfected plants and infected plants in three stages of inoculation between two replicates

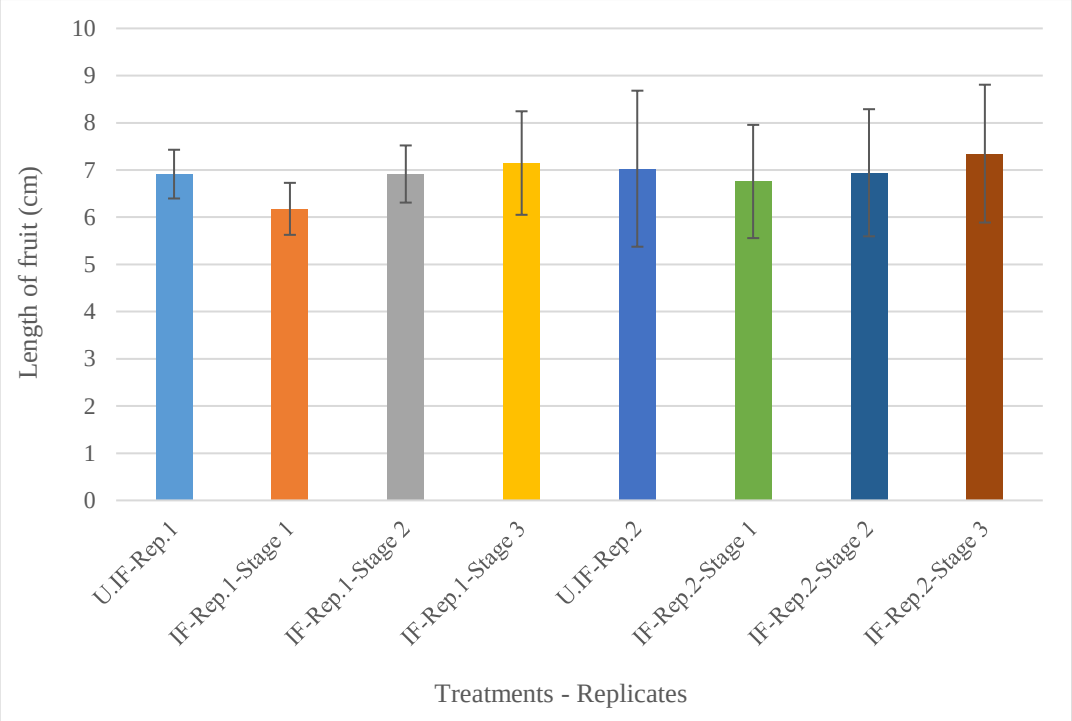

Figure 5.10 Comparison of the average length of fruit of *Capsicum annuum* 'Californian Wonder' plants between uninfected plants and infected plants in three stages of inoculation between two replicates

Figure 5.11 *Capsicum annuum* 'Californian Wonder' uninfected plants (left) and plants 6 days post inoculation (right) showing necotic patches and leaf distortion

Figure 5.12 *Capsicum annuum* 'Californian Wonder' fruits in the harvesting stage: fruits in uninfected plants (left) compared to the infection symptoms of deformation, necrotic spots on fruits of infected plants (right)

5.3.3. *Capsicum annuum* 'Jalapeno'

The response of *C. annuum* 'Jalapeno' to YTMMV depended on the time of inoculation. Symptoms were severe on young *C. annuum* 'Jalapeno' plants. The average height, numbers of branches, numbers of fruits, average weight of fruit, average length of fruit are shown in Figures 5.13; 5.14; 5.15; 5.16; 5.17, respectively. Although two infected plants from stage 1 inoculation remained alive, no fruit was produced and all inoculated plants in the second experiment (repeat) died after three weeks post-inoculation. All infected plants of stage 2 inoculation died. In the stage 3 of inoculation, not every plant died, (Fig. 5.18).

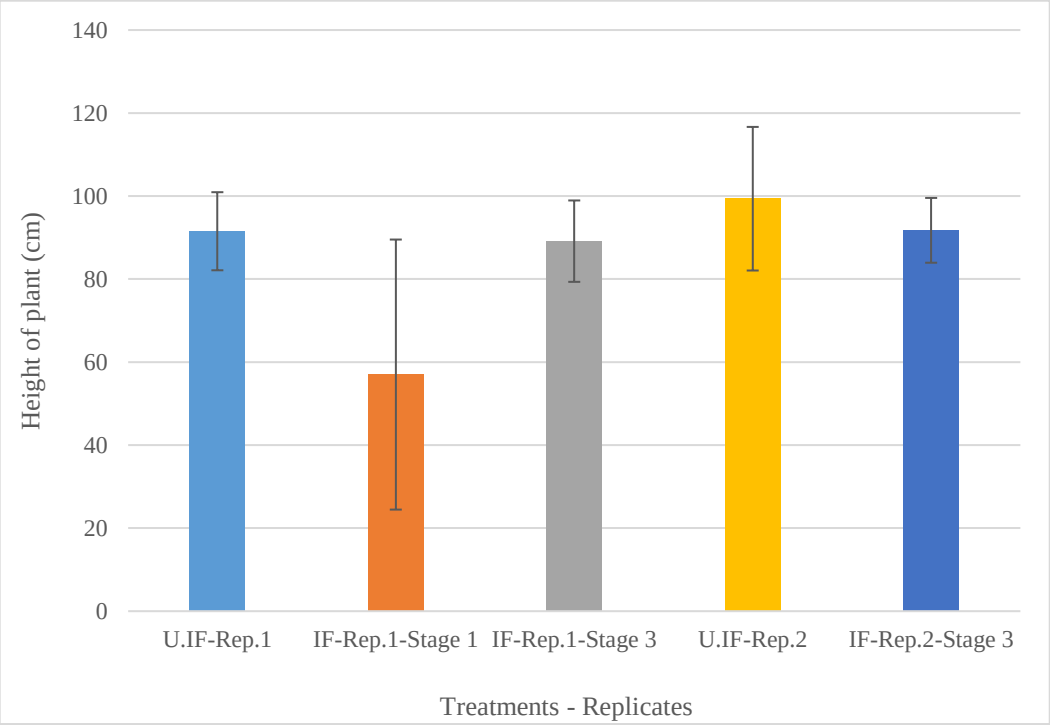

Figure 5.13 Comparison of the average height of *Capsicum annuum 'Jalapeno'* plants between uninfected plants and infected plants in three stages of inoculation between two replicates

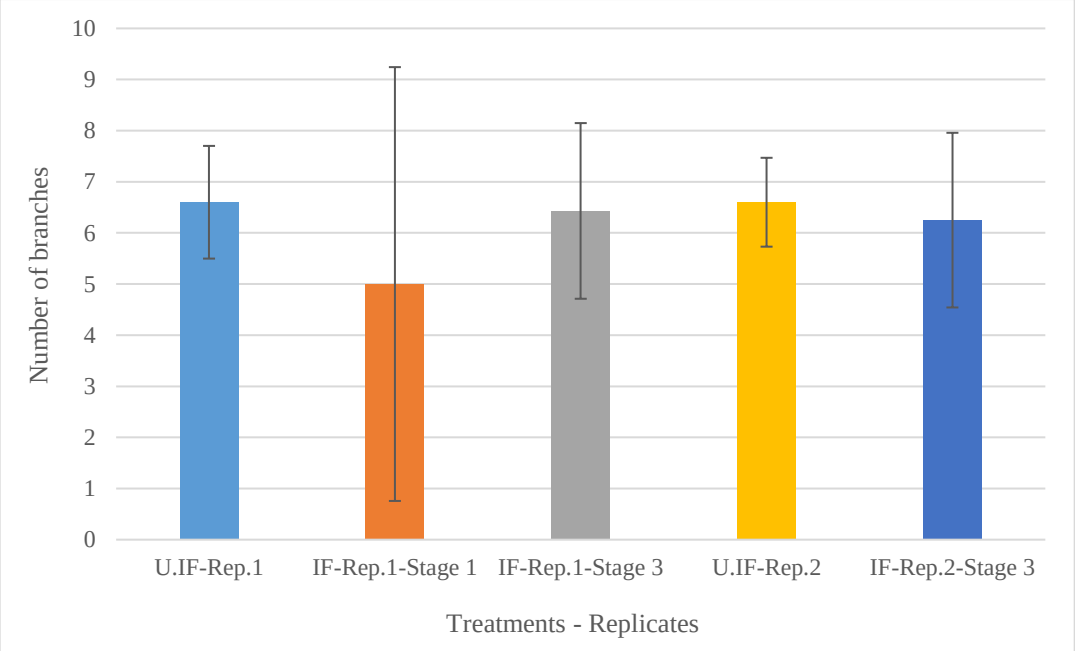

Figure 5.14 Comparison of the average number of branches per *Capsicum annuum 'Jalapeno'* plant between uninfected plants and infected plants in three stages of inoculation between two replicates

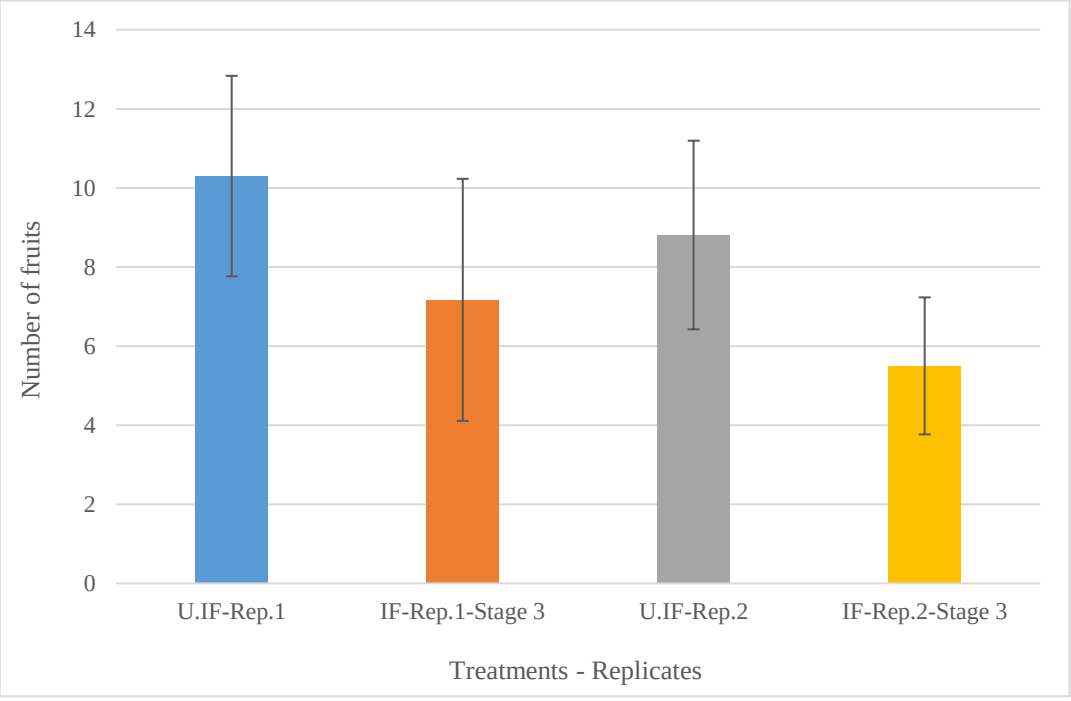

Figure 5.15 Comparison of the average number of fruits per *Capsicum annuum 'Jalapeno'* plant between uninfected plants and infected plants in three stages of inoculation between two replicates

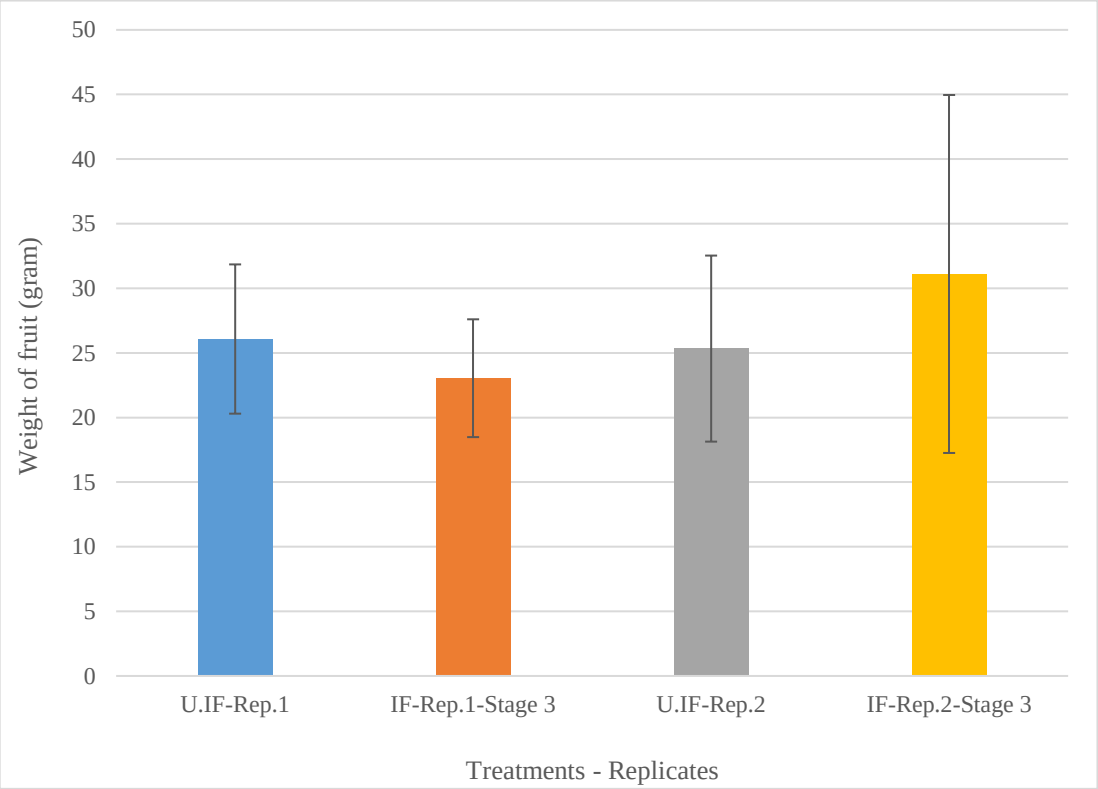

Figure 5.16 Comparison of the average weight fruit of *Capsicum annuum 'Jalapeno'* plants between uninfected plants and infected plants in three stages of inoculation between two replicates

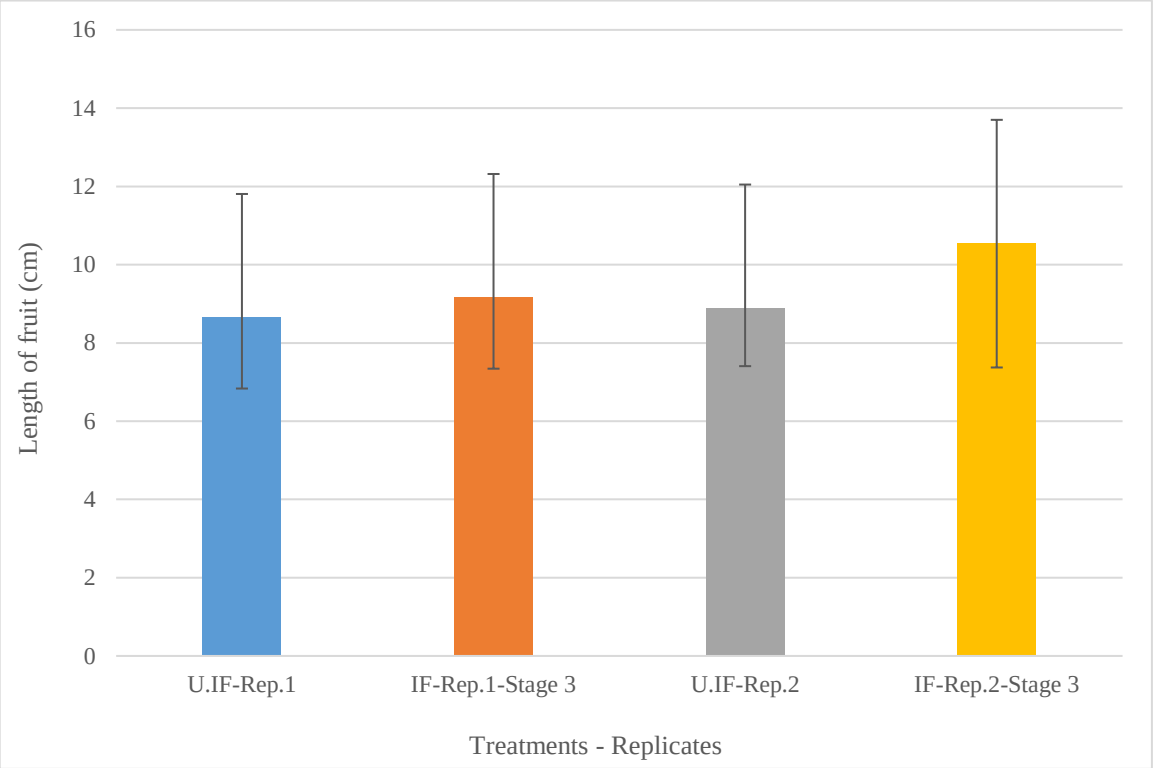

Figure 5.17 Comparison of the average length of fruits of *Capsicum annuum 'Jalapeno'* plants between uninfected plants and infected plants in three stages of inoculation between two replicates

Figure 5.18 The pictures of the uninfected plants (control) in the left (A, C, E) compared to the infected plants (B, D, F) of *Capsicum annuum* 'Jalapeno' after 10 days post inoculation of each stage: seedling, vegetative, pre –flowering, respectively. The symptoms of fruits of infected plants compared to the virus free (G)

5.4. Discussion

Responses by the plants tested of genus *Solanum* were generally milder than those of genus *Capsicum* when infected with YTMMV.

The plant height, number of branches, number of fruits, weight of fruit, length of fruit from YTMMV-infected *S. lycopersicum* plants were similar to those of uninfected plants at all inoculation stages. The Tigerella cultivar of tomato we tested exhibited mild symptoms that may not be noticed by growers. If this species is used as an asymptomatic vehicle by which the virus is spread, then the risk is that it could emerge from tomato and cause significant damage to other solanaceous crops, or infect other solanaceous plants such as *Solanum nigrum*, which also exhibit relatively mild symptoms and could act as a reservoir of YTMMV (Koh *et al.*, 2017). There are over 83,000 tomato accessions stored in germplasm collections (Figas *et al.*, 2015) and the effects of YTMMV infection on all but one of them is unknown. No doubt some will respond with more severe symptoms than cv Tigerella.

Of interest is that *C. annuum* appears to be severely infected, with large black lesions appearing on fruits, when fruits are produced. Such obvious symptoms alert growers to the presence of the virus, and plants will be removed from fields. As with tomatoes, more varieties should be tested.

Although of the same species, the two cultivars of *C. annuum* we tested responded similarly to YTMMV infection only when at the pre-flowering stage. While almost all infected *C. annuum* 'Jalapeno' plants died when plants were inoculated at the young and vegetative stages, *C. annuum* 'Californian Wonder' plants developed and produced fruits of reduced number, size and weight. The reasons for the differential responses of

varieties of the two genera are unclear but probably related to tobamovirus tolerance genes introgressed during breeding.

The effect of plant age at infection by pathogenic viruses is well known. For instance, Matthews (1970) and Chant (1960) showed that the earlier a virus infects a plant, the more severe the symptoms and lower the commercial yeild. Gilmer *et al.*, 1974 showed that inoculation of cowpea cultivars with the comovirus cowpea yellow mosaic virus (CpMV) 7-days after emergence reduced yield by 40% - 60% compared to the 10% - 15% loss in yield when plants were inoculated at the flowering stage. The tobamovirus CGMMV induced yield losses of up to 15% in cucumber crops when infected at the seedling stage. However, plants six weeks old when infected exhibited less yield loss (Fletcher, 1969).

We did not test response to infection under different air temperatureas and soil moisture regimes, and this should be done. Clearly, there is potential for yield loss under commercial conditions, but if transmission occurs by pollen, the plants will be mature and damage will be less than if infected as young seedlings.

Furthermore, only one virus isolate, YTMMV isolate Cervantes, was used to challenge plants here. Although other YTMMV isolate, YTMMV isolate Kalbarri (GenBank Accession KJ683937) is close genetically to YTMMV isolate Cervantes and the symptom revealed small differences in virulence on solanaceous hosts (Li *et al.*, 2016).

Resistance genes are powerful tools to control plant viral diseases. However, viruses can sometime gain the ability to overcome those resistance gene(s) through changes in their genomes. For example, ToBRFV was able to break *Tm-2*-mediated resistance in tomato that had lasted 55 years (Maayan *et al.*, 2018). A resistance-breaking strain of

PMMoV was capable of infecting previously-resistant varieties (Hamada *et al.* 2007; Tsuda *et al.* 1998). On the other hand, the TMV-resistance gene *N* has proved highly durable. *N* was introgressed into *N. tabacum* from *N. glutinosa* and continues to provide resistance to TMV in that species (Lewis *et al.*, 2005). The effect of tobamovirus resistance genes against YTMMV are unknown and should be tested.

Chapter 6: General Discussion

The wild-plant tobamovirus yellow tailflower mild mottle virus (YTMMV) was studied to determine the modes of its transmission between plants and the potential risk it poses to solanaceous plants of horticultural value. YTMMV is unusual among tobamoviruses in that it was first identified in a wild plant, not a crop, and that it was described in Australia, not a continent previously known to host indigenous tobamoviruses. In the wild, YTMMV was detected in widely-spaced plants of several solanaceous species along a 1600 km coastal strip. It is assumed that it occupies a larger area and may infect many of the species of indigenous solanaceous plants found throughout Australia, although, to our knowledge, no one has looked for it. The study of viruses of wild plants almost everywhere in the world is a neglected area, virtually unfunded by governments. This is understandable because research dollars are limited and viruses of cultivated crops can threaten international food security. Most viruses of wild plants will never be identified and named because they will forever remain in those wild plants and not become bothersome or threatening to the incomes of commercial producers.

However, a small proportion, probably a fraction of a percent of the millions of (mostly undescribed) plant viruses that undoubtedly exist in the planet's wild flora (Roossinck *et al.*, 2015), will spillover into crops, some to remain latent and unsuspected, others to cause minor losses in production. An even smaller proportion of these will cause substantial or devastating losses. Similar scenarios exist in fauna. The spillover of SARS-CoV-2 from an animal to a human initiated the Covid pandemic that began while I was undertaking my own experiments to try to understand how YTMMV might spillover to crops. This terrible event reminded me of the importance of my work, but

also of the futility in attempting to predict which of the potentially millions of wild viruses might one day cause terrible losses in crops or farm animals or humans. To predict which viruses will spillover and cause substantial disease and death is an impossible and futile exercise. Potentially we could identify and study members of the genera or families of viruses that have previously spilled-over, such as tobamoviruses as I have done, but there is a risk that to concentrate only on groups from which known pathogens have arisen may blind us to other groups with as yet unrealized spillover and destructive potential. High-throughput sequencing and bioinformatics have enabled us to identify large numbers of viruses in organisms that show no apparent symptoms of infection in their hosts, revealing that viruses may be part of the beneficial microbial flora of many species (Jo *et al*., 2017). As our ability to identify viruses has increased greatly, are understanding of how these viruses interact with their hosts has not kept pace with their discoveries. In this study we have attempted to study some aspects of the biology of one wild-plant virus, YTMMV. The time and effort involved in such detailed study of one virus is prodigious, and cannot possibly be achieved for every virus discovered by high-throughput approaches.

It is probable that many undescribed viruses exist in the indigenous flora and fauna of Australia. Some may indeed emerge and infect crops, and resources will be expended to understand how to control them, but the remainder will probably never be identified nor studied. It is uncertain if my study of YTMMV will ever be of commercial value. As far as we know, ZGMMV has not emerged into commercial crops at a level where it has come to the attention of growers and biosecurity authorities. Currently, the value of this study has been as a training tool for this young virologist. If YTMMV does emerge, I hope that my research will be of value in controlling it. My most important findings were:

1) YTMMV is transmitted in seed produced from an infected parent and the rate is species/variety-dependent. Host genetics effects seed transmission rate of YTMMV to these six solanaceae species. The other factors controlling rate of transmission are currently unknown.

   At least some of the virions including TMV, ToMMV, CGMMV are located within the seed coat; surface-sterilisation of whole seeds did not prevent infection of seedlings that emerged from the seed. Thus, surface treatment of the seed of *Solanum, Capsicum, Nicotiana* will probably not be an effective means of control, but other species should be considered individually. The national and international trades in seed is a possible route of dissemination of YTMMV.

2) YTMMV is transmitted both vertically and horizontally *via* pollen. Thus, bees and other pollinators may spread this virus from plant to plant as they do with other tobamoviruses such as ToBRFV, CGMMV (Darzi *et al.*, 2018; Levitzky *et al.*, 2019; Chanda *et al.* 2021). Transmission of YTMMV occurred after pollination

3) Transmission of YTMMV through root contact between plants growing close together occurs, and may be a factor in commercial production, but is probably not a major transmission route in widely-spaced wild plants.

4) YTMMV infection caused minimal symptoms and yield loss in the tomato and tobacco varieties tested, but more severe symptoms or death in two *N. benthamiana* accessions and two *C. annuum* varieties. The factors controlling these very different responses in the plants tested are likely to be genetic ones. For example, the *N* gene of tobacco confers resistance to TMV in transgenic tomato (Whitham *et al.*, 1996). And the R genes *Tm-2* and *Tm-2*^{2} (*Tm-2*^{a}) introduced by introgression resulted in resistance to TMV and ToMV in tomato,

respectively (Pelham, 1966; Hall, 1980; Meshi *et al.*, 1989; Lanfermeijer *et al.*, 2005; de Ronde *et al.*, 2014). However, tomato cultivars certified to harbor the *Tm-2*^{2} resistance gene are susceptible to ToMMV (Luria *et al.*, 2017).

We studied YTMMV in the controlled environments of the laboratory and one field trial, not in the wild *per se*, so our findings do not necessarily apply to transmission of the virus in its natural environment—the vast solanaceous flora of Australia. It is hypothesized by some, e.g., Roossinck and Garcia-Arenal (2015), that indigenous viruses naturally infecting their wild hosts induce few, if any, symptoms. Co-existence over evolutionary time may limit or eliminate severe pathogenesis, and in some cases, a mutualistic relationship evolves (Fraile & Garcia-Arenal, 2016). Indeed, there was no obvious symptom development on the original wild host of YTMMV-Cervantes, a plant of *Anthocercis littorea* (Wylie *et al.*, 2014, Li *et al.*, 2016, Wylie *et al.*, 2017). In contrast, wild-plant viruses are thought to often induce moderate to severe pathogenesis when they spillover to the simplified ecosystem of the cultivated crop (Roossinck & Garcia-Arenal, 2015), although the reasons for this are unclear. However, we found during the course of this project that this scenario did not always apply to YTMMV. We used six species or genetic variants of plants as experimental hosts for YTMMV. Four of these host species evolved on other continents and had never before encountered this virus. Of these, *S. lycopersicum* 'Tigerella' and *N. tabacum* 'Wisconsin 38', both of which evolved in the Americas, exhibited no to very slight symptoms of infection, while *C. annuum* 'Jalapeno' and *C. annuum* 'Californian Wonder' from the same continents suffered more severe pathogenesis and even systemic necrosis. YTMMV is closely related to paprika mild mottle virus (PaMMV) and obuda pepper virus (ObPV), both viruses of possible European origins and of

special concern to growers of *C. annuum* species because they induce strong symptoms and may overcome resistance genes (Genda *et al.*, 2007; Katsutochi *et al.*, 2009; Velasco *et al.*, 2002). Of the two accessions of the Australian indigenous plant species tested, *N. benthamiana* RA-4 and MtA-6, the former accession displayed severe symptoms, including death, while MtA-6 displayed only moderate symptoms. Thus, the geographical origin of the host plant was not a clear predictor of pathogenicity when exposed to YTMMV.

A major aim of this project was to assess whether YTMMV posed a risk to horticultural crops. There was good reason to ask this question. There have been a number of recent international range-expansions by very damaging tobamoviruses, including the solanaceous-infecting tomato mottle mosaic virus (ToMMV) (Webster *et al.*, 2014) and tomato brown rugose fruit virus (ToBRFV) (Ling *et al.*, 2019) and the cucurbit-infecting cucumber green mottle mosaic virus (Tesoriero *et al.*, 2016; Dombrovsky *et al.*, 2017). Humans facilitate tobamovirus movement around the world in seeds and fresh produce (Chanda *et al.*, 2021), so a virus that readily infects species of the critically-important Solanaceae should be watched carefully.

What are the chances that YTMMV will emerge from the indigenous flora of Australia and follow some of its cousins to become serious international plant pathogens? To answer this question, we undertook experiments to understand its transmission. Before we undertook these experiments, it was unknown whether YTMMV was transmitted *via* pollen to healthy plants, and if so, was it transmitted vertically to the seed and/or horizontally to the mother plant. It was unknown whether YTMMV was seed-borne as are some other tobamoviruses. There were early reports of mechanistic transmission of

tobacco mosaic virus by chewing insects and other pathways (Jones, 2018). Could this happen with YTMMV?

We found that YTMMV was indeed transmitted *via* pollen in two ways, horizontal transmission, and more commonly, by vertical transmission. TMV can be detected in pollen (Brunt *et al.* 1996), and CGMMV can be transmitted both horizontally and vertically *via* cucumber pollen under experimental conditions (Liu *et al.*, 2014)

We propose pollen transmission is probably the main means of transmission over long distances in the wild, although this has yet to be investigated experimentally. Most solanaceous plants are pollinated by arthropods, many of which can fly (Knapp, 2010). Some viruses make infected plants more attractive to arthropod vectors (Mauck *et al.*, 2010), but whether tobamovirus infection makes plants more attractive to arthropod pollinators is unknown, and a potentially fruitful line of study.

Although we showed YTMMV to be transmitted *via* pollen, we did not test this with 'real' pollinators such as bees. In our experiemtns the pollen was transmitted *via* cotton buds. Working with bees is challenging in the laboratory. In our field trial, vertical transmission by 'real' pollinators such as bees and flies etc. may have occurred, but we did not test it. It is possible that pollinators transmitted infected pollen from the virus source plants in the field to the uninfected plants, but unfortunately the seeds were not collected from the uninfected plants, germinated and tested for YTMMV. Again, future researchers should consider testing this.

Seed and pollen transmission of TMV and CGMMV occurs in some virus-plant combinations, but not in all (Mink, 1993). Pollen transmission of YTMMV could enable this tobamovirus to travel far more rapidly and widely than if it were confined to contact transmission. Like other tobamoviruses, YTMMV probably lacks specific

vectors, such as the aphids, mites and whiteflies etc. used by other viral groups. Nevertheless, it is potentially spread over long distances on flying insects. Similarly, nectar-feeding birds foraging for pollen or nectar may transmit infective pollen over kilometres between flowers, and we hypothesis that YTMMV gradually extended its range over a distance of at least 1600 km in Western Australia on the wings of arthropods and nectar-eating birds, coopted as agents of virus transmission.

It is probable that non-pollinators also spread YTMMV in another way. Larger animals such as kangaroos, emus, and even humans eat the fruit of indigenous wild solanaceous plants such as *Solanum centrale* (bush tomato) (Lee, 2012) and spread infected seed by defecating the seed elsewhere. These hypotheses have not been tested by sampling insect or bird pollinators, or fruit-eaters leaving infected plants, or by testing plants pollinated by arthropods and birds in the wild, or by testing for infected solanaceous seed in animal faeces. This is all work that could be done to more deeply understand natural transmission of wild-plant tobamoviruses.

The outcome of the pollination experiment was the discovery that vertical transmission sometimes occurred *via* the seed without horizontal transmission to the mother plant. Consequently, systemically-uninfected mother plants could bear infected seed. In other cases, pollination *via* infected pollen resulted in the mother plant's systemic infection, upon which, a proportion of subsequent seed produced would be infected *via* the maternal tissue. Thus, what we refer to as 'vertical transmission' of YTMMV could occur by two distinct pathways: one *via* the paternal gamete (pollen) and the other *via* the maternal gamete, the ovule. Every plant species/accession tested was able to transmit the virus to the next generation in the seed. In annual solanaceous plants, the

consequence of seed transmission is that all the infected host plants in the population die each year, yet the virus remains in the seed bank to re-emerge in the following year.

Foraging Eurasian honey bees (*Apis mellifera*) that carry virus-infected pollen may visit two or more flowering plant species on one flight (Woodcock *et al.*, 2013), therefore, they can potentially transmit the virus to other compatible plant species by horizontal and/or vertical transmission. Eurasian honey bees can forage up to 12 km from their hive (Ratnieks, 2007), but this species has been present in Australia only since the 1800s. There are up to 5000 species of bees indigenous to Australia (Halcroft *et al.*, 2012), all of which are potential carriers of infected pollen, but little is known about their roles in pollination of the Australian flora prior to the introduction of honey bees, and nothing is known about their roles in spreading viruses through infected pollen.

In crops, plants are usually closely spaced and leaf-to-leaf contact occurs. This is an ideal situation for contact-transmissible tobamoviruses to spread. Little work has been done to elucidate the role of root-to-root transmission. A surprising finding was that although YTMMV appeared to occur on all leaves of an infected plant, this was not always true for the root system. Some parts of the root system were infected while other parts apparently were not. We could find no information on mechanisms in root systems that might prevent systemic spread of tobamoviruses, and this is yet another fascinating area of future research. We may consider the role of RNA silencing which has been proven to be transmitted between scions and rootstocks through grafting, mostly using transgenic plants. For example, RNAi of tobacco endogenous genes such as *NtTOM1* and *NtTOM3* which were required for tobamovirus multiplication, resulted in high resistance against several tobamoviruses (Ali *et al*., 2013). Is this unique to the system we studied? Another tobamovirus, Hibiscus latent Fort Pierce virus (HLFPV) appeared to be evenly distributed in the roots (Kamenova *et al.*, 2004).

As roots grow through soil, root tips are micro-abraded against mineral particles. We wondered if these small wounds might serve as sites of entry to YTMMV that existed in the soil from previously infected plants, but we did not detect this in our experiment. Others found that transplantation of seedling cucumber plants (*Cucumis sativus*) from trays to pots filled with perlite infested with the tobamovirus cucumber fruit mottle mosaic virus (CFMMV), resulted in high disease incidence. However, when the seedlings were first removed and the root wounds allowed to heal in individual pots before transplanting into CFMMV-infected perlite, there was no infection. Newly-formed roots that penetrated into the virus-infested medium did not allow infection by the virus (Antignus *et al*., 2005). Thus, invasion of roots through microabrasion sites may not to be a common route of tobamovirus transmission.

Could YTMMV emerge from the indigenous flora and become a pathogen of international concern? We found that of the plants we tested, tomato was the host at greatest potential risk, not to the tomato crop itself, but because this host may act as an asymptomatic intermediary host from which YTMMV could emerge into other hosts. However, in Western Australia where this study was undertaken, we feel that the risk of YTMMV emerging through tomato (or capsicum) may be minimal. The reason is that in this region, tomato and capsicum/chili growers do not keep seed from the previous crop and plant it for the next crop. If this were the case, the virus could build up, and spread could occur when seed was planted elsewhere or exported. Growers in this region buy seedlings from specialist producers. We spoke to a grower in Jandabup, Perth, who grows capsicum and tomato commercially. His plants are always grown from seedlings bought from a nursery because buying seedlings saves growing time. Nursery producers grow millions of tomato seedlings in covered glasshouses from imported seed. This means of production prevents YTMMV infection that could occur

during the growing season to perpetuate the following season. Thus, the practices of commercial growers inadvertently prevent YTMMV getting a foothold in these species, even if it is emerging from the indigenous flora *via* bees and other pollinators.

Perhaps the biggest potential risk of YTMMV emergence is in a crop we did not study—potato. Potato is a vegetative-propagated crop that already is vulnerable to accumulation of a number of viruses (Wang *et al.*, 2011). Importantly for Western Australia, potato is exported from the state as both 'seed' potato to growers in Indonesia, and as ware (eating) potatoes to consumers in other international markets. If potato is infected asymptomatically, YTMMV could already have hitched a ride beyond the Australian borders, in much the same way several viruses have become internationally-distributed in 'seed' and ware garlic (Wylie *et al.*, 2015). In our opinion, this research should be undertaken urgently. If YTMMV-infected seed potatoes are being grown, the potato crop will quickly become infected and the virus will become widespread. Even if potato, like tomato, is an asymptomatic carrier of YTMMV, it will be only a matter of time before it emerges into other solanaceous species, where it could become a serious pathogen. In WA, potatoes are the third-largest vegetable crop by value and second highest by tonnage. This sector accounted for 13% of the of the state's total vegetable crop in 2015–16 by tonnage. Production was worth an estimated $42m that year. This represented 8% of total WA vegetable industry value. Fresh potatoes are the fourth biggest vegetable export from WA by value, representing just above 3% at a value of $3.3m. Major markets for WA fresh potatoes are Singapore, which was worth 44% of the value of total exports in 2017, and Malaysia and the UAE, which were worth 24% and 13% of total value respectively.

Tobamoviruses are versatile and adaptive viruses that employ multiple modes of transmission over short and long distances. They have resilient capsids and have the ability to invade pollen and ovule cells, abilities that many other plant RNA viruses lack. Although they are unable to utilize vectors *per se*, instead they use pollinators to essentially do the same job of vectors, that is to transmit the virus to new hosts. They may also transmit to roots in infested soil and perhaps even in airborne aerosols. YTMMV is a successful tobamovirus that probably evolved in Australia from the common ancestor of viruses found in Europe and Asia. It exists over a large section of south-west Australia, and possibly far further. In our estimation YTMMV certainly has the potential to spillover to exotic plants, if it hasn't already done so. Surveys of exotic wild and domesticated plants for YTMMV in Western Australia and elsewhere should be a high priority for plant virologists in Australia. An antibody-based assay would be the ideal means by which to screen large numbers of plants to test this. An estimation of the relative risks of spillover of YTMMV into local and international agriculture is presented in Figure 6.1.

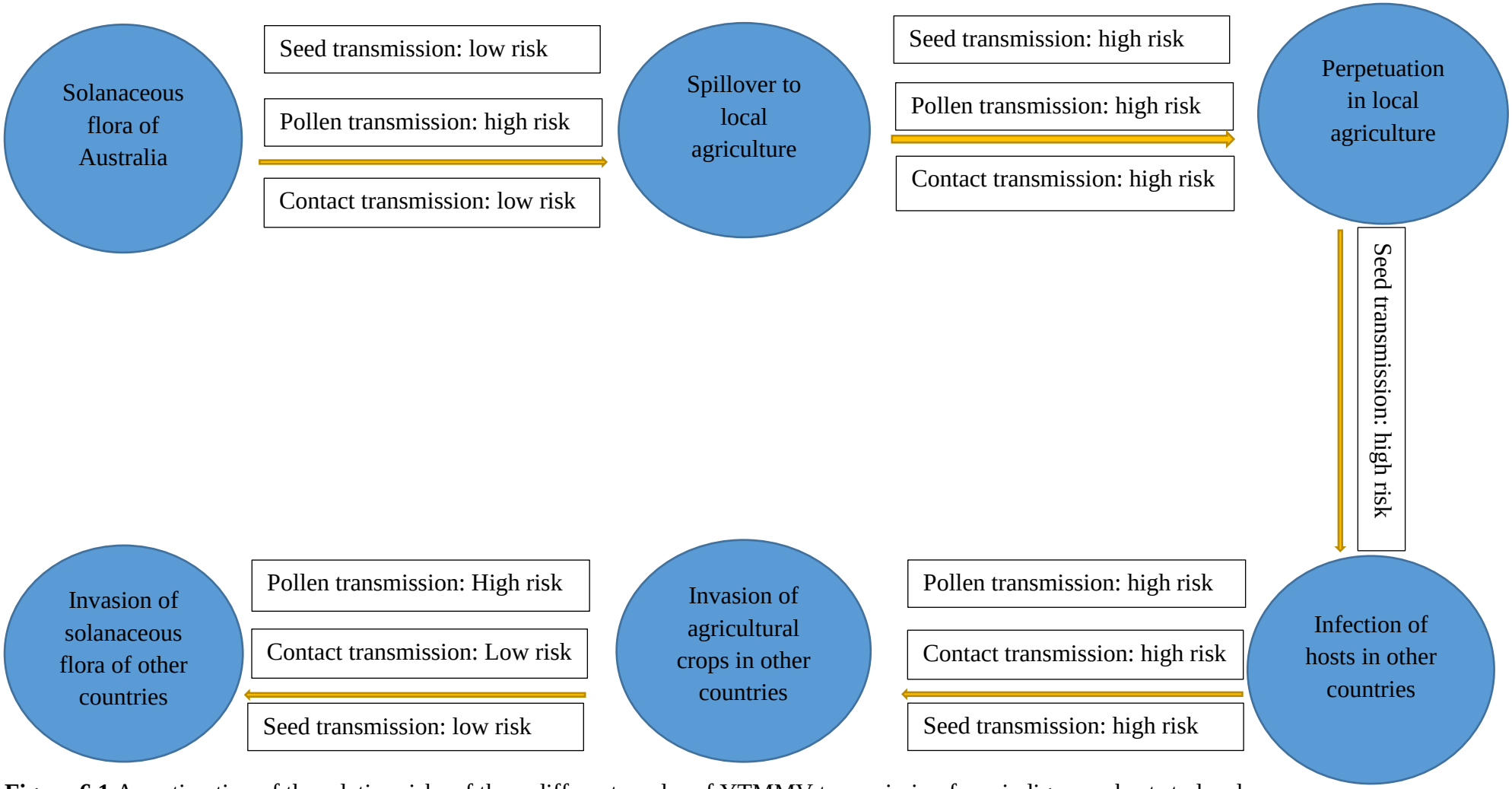

Figure 6.1 An estimation of the relative risks of three different modes of YTMMV transmission from indigenous hosts to local agricultural/horticultural crops to solanaceous plants, both domesticated and wild, in countries beyond Australia

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Appendix

1. *Solanum lycopersicum* 'Tigerella'

Table 1 Comparison of average height (H) (cm), branch numbers (NOB), fruit numbers (NOF), weight fruit (Wf) (g) of *S. lycopersicum* 'Tigerella' uninfected plants (control) between the first and the second (repeat) experiment in stage 1 of inoculation

	Repeat	Number	Mean	Std. Deviation	Std. Error Mean
H	1	13	248.23	28.865	8.006
	2	12	257.50	27.553	7.954
NOB	1	13	8.15	1.463	.406
	2	12	7.42	1.240	.358
NOF	1	13	12.38	3.305	.917
	2	12	14.92	2.193	.633
Wf	1	13	23.820	1.555	.431
	2	12	20.231	2.480	.715

Table 2 Comparison of average height (H) (cm), branch numbers (NOB), fruit numbers (NOF), weight fruit (Wf) (g) of *S. lycopersicum* 'Tigerella' uninfected plants (control) between the first and the second (repeat) experiment in stage 2 of inoculation

	Repeat	Number	Mean	Std. Deviation	Std. Error Mean
H	1	13	248.08	34.606	9.598
	2	12	236.83	36.456	10.524

NOB	1	13	8.00	1.780	.494
	2	12	6.92	.996	.288
NOF	1	13	11.54	4.352	1.207
	2	12	16.33	3.576	1.032
Wf	1	13	25.335	2.073	.574
	2	12	20.284	2.349	.678

Table 3 Comparison of average height (H) (cm), branch numbers (NOB), fruit numbers (NOF), weight fruit (Wf) (g) of *S. lycopersicum* 'Tigerella' uninfected plants (control) between the first and the second (repeat) experiment in stage 3 of inoculation

	Repeat	Number	Mean	Std. Deviation	Std. Error Mean
H	1	14	266.36	38.775	10.363
	2	12	236.33	26.276	7.585
NOB	1	14	8.43	1.158	.309
	2	12	8.33	1.073	.310
NOF	1	14	10.71	3.561	.952
	2	12	15.08	3.175	.917
Wf	1	14	26.243	1.757	.469
	2	12	20.892	2.749	.793

Table 4 Comparison of average height (H) (cm), branch numbers (NOB), fruit numbers (NOF), weight fruit (Wf) (g) of *S. lycopersicum* 'Tigerella' infected plants between the first and the second (repeat) experiment in stage 1 of inoculation

	Repeat	Number	Mean	Std. Deviation	Std. Error Mean
H	1	13	249.23	41.716	11.570
	2	12	260.50	32.380	9.347
NOB	1	13	7.23	1.301	.361
	2	12	5.58	.515	.149
NOF	1	13	11.38	3.754	1.041
	2	12	12.50	1.834	.529
Wf	1	13	23.946	1.623	.450
	2	12	17.376	1.792	.517

Table 5 Comparison of average height (H) (cm), branch numbers (NOB), fruit numbers (NOF), weight fruit (Wf) (g) of *S. lycopersicum* 'Tigerella' infected plants between the first and the second (repeat) experiment for treatment 2 of inoculation

	Repeat	Number	Mean	Std. Deviation	Std. Error Mean
H	1	13	237.69	36.865	10.225
	2	12	243.33	27.164	7.842
NOB	1	13	6.54	1.450	.402
	2	12	5.92	.669	.193
NOF	1	13	11.15	3.760	1.043

	2	12	11.83	3.157	.911
Wf	1	13	24.343	2.448	.679
	2	12	19.559	3.439	.992

Table 6 Comparison of average height (H) (cm), branch numbers (NOB), fruit numbers (NOF), weight fruit (Wf) (g) of *S. lycopersicum* 'Tigerella' infected plants between the first and the second (repeat) experiment for in stage 3 of inoculation

	Repeat	Number	Mean	Std. Deviation	Std. Error Mean
H	1	14	257.93	44.545	11.905
	2	12	236.42	33.427	9.650
NOB	1	14	6.36	1.336	.357
	2	12	5.75	1.138	.329
NOF	1	14	9.86	2.825	.755
	2	12	12.33	2.839	.820
Wf	1	14	24.531	2.263	.604
	2	12	19.910	3.280	.947

Table 7 Comparison of average height (H) (cm), branch numbers (NOB), fruit numbers (NOF), weight fruit (Wf) (g) of *S. lycopersicum* 'Tigerella' between uninfected plants (control) and infected plants in stage 1 of inoculation

	Treatment	Number	Mean	Std. Deviation	Std. Error Mean
H	Uninfected plants	25	252.68	28.052	5.610

	Infected plants	25	254.64	37.198	7.440
NOB	Uninfected plants	25	7.80	1.384	.277
	Infected plants	25	6.44	1.294	.259
NOF	Uninfected plants	25	13.60	3.055	.611
	Infected plants	25	11.92	2.985	.597
Wf	Uninfected plants	25	22.097	2.716	.543
	Infected plants	25	20.792	3.7430	.748

Table 8 Comparison of average height (H) (cm), branch numbers (NOB), fruit numbers (NOF), weight fruit (Wf) (g) of *S. lycopersicum* 'Tigerella' between uninfected plants (control) and infected plants in stage 2 of inoculation

	Treatment	Number	Mean	Std. Deviation	Std. Error Mean
H	Uninfected plants	25	242.68	35.225	7.045
	Infected plants	25	240.40	32.031	6.406
NOB	Uninfected plants	25	7.48	1.531	.306
	Infected plants	25	6.24	1.165	.233
NOF	Uninfected plants	25	13.84	4.616	.923
	Infected plants	25	11.48	3.429	.686
Wf	Uninfected plants	25	22.911	3.363	.672
	Infected plants	25	22.047	3.790	.7580

Table 9 Comparison of average height (H) (cm), branch numbers (NOB), fruit numbers (NOF), weight fruit (Wf) (g) of *S. lycopersicum* 'Tigerella' between uninfected plants (control) and infected plants in stage 3 of inoculation

	Treatment	Number	Mean	Std. Deviation	Std. Error Mean
H	Uninfected plants	26	252.50	36.312	7.121
	Infected plants	26	248.00	40.534	7.949
NOB	Uninfected plants	26	8.38	1.098	.215
	Infected plants	26	6.08	1.262	.248
NOF	Uninfected plants	26	12.73	3.996	.784
	Infected plants	26	11.00	3.046	.597
Wf	Uninfected plants	26	23.774	3.511	.688
	Infected plants	26	22.398	3.594	.704

Table 10 Comparison of average height (H) (cm), branch numbers (NOB), fruit numbers (NOF), weight fruit (Wf) (g) of *S. lycopersicum* 'Tigerella' between all uninfected plants (control) and infected plants in stage 1 of inoculation

	Treatment	Number	Mean	Std. Deviation	Std. Error Mean
H	Uninfected plants	76	249.33	33.322	3.822
	Infected plants	25	254.64	37.198	7.440
NOB	Uninfected plants	76	7.89	1.382	.158
	Infected plants	25	6.44	1.294	.259
NOF	Uninfected plants	76	13.38	3.919	.450

	Infected plants	25	11.92	2.985	.597
Wf	Uninfected plants	76	22.938	3.251	.372
	Infected plants	25	20.792	3.743	.748

Table 11 Comparison of average height (H) (cm), branch numbers (NOB), fruit numbers (NOF), weight fruit (Wf) (g) of *S. lycopersicum* 'Tigerella' between all uninfected plants (control) and infected plants in stage 2 of inoculation

	Treatment	Number	Mean	Std. Deviation	Std. Error Mean
H	Uninfected plants	76	249.33	33.322	3.822
	Infected plants	25	240.40	32.031	6.406
NOB	Uninfected plants	76	7.89	1.382	.158
	Infected plants	25	6.24	1.165	.233
NOF	Uninfected plants	76	13.38	3.919	.450
	Infected plants	25	11.48	3.429	.686
Wf	Uninfected plants	76	22.938	3.251	.372
	Infected plants	25	22.047	3.790	.758

Table 12 Comparison of average height (H) (cm), branch numbers (NOB), fruit numbers (NOF), weight fruit (Wf) (g) of *S. lycopersicum* 'Tigerella' between all uninfected plants (control) and infected plants in stage 3 of inoculation

	Treatment	Number	Mean	Std. Deviation	Std. Error Mean
H	Uninfected plants	76	249.33	33.322	3.822

	Infected plants	26	248.00	40.534	7.949
NOB	Uninfected plants	76	7.89	1.382	.158
	Infected plants	26	6.08	1.262	.248
NOF	Uninfected plants	76	13.38	3.919	.450
	Infected plants	26	11.00	3.046	.597
Wf	Uninfected plants	76	22.938	3.251	.372
	Infected plants	26	22.398	3.594	.704

Table 13 Comparison of average height (H) (cm), branch numbers (NOB), fruit numbers (NOF), weight fruit (Wf) (g) of *S. lycopersicum* 'Tigerella' between all uninfected plants (control) and infected plants of stage 1 of inoculation in first experiment

	Treatment	Number	Mean	Std. Deviation	Std. Error Mean
H	Uninfected plants	40	254.53	34.689	5.485
	Infected plants	13	249.23	41.716	11.570
NOB	Uninfected plants	40	8.20	1.454	.230
	Infected plants	13	7.23	1.301	.361
NOF	Uninfected plants	40	11.53	3.728	.589
	Infected plants	13	11.38	3.754	1.041
Wf	Uninfected plants	40	25.161	2.031	.321

	Infected plants	13	23.946	1.623	.450

Table 14 Comparison of average height (H) (cm), branch numbers (NOB), fruit numbers (NOF), weight fruit (Wf) (g) of *S. lycopersicum* 'Tigerella' between all uninfected plants (control) and infected plants of stage 2 of inoculation in first experiment

	Treatment	Number	Mean	Std. Deviation	Std. Error Mean
H	Uninfected plants	40	254.53	34.689	5.485
	Infected plants	13	237.69	36.865	10.225
NOB	Uninfected plants	40	8.20	1.454	.230
	Infected plants	13	6.54	1.450	.402
NOF	Uninfected plants	40	11.53	3.728	.589
	Infected plants	13	11.15	3.760	1.043
Wf	Uninfected plants	40	25.161	2.031	.321
	Infected plants	13	24.343	2.448	.679

Table 15 Comparison of average height (H) (cm), branch numbers (NOB), fruit numbers (NOF), weight fruit (Wf) (g) of *S. lycopersicum* 'Tigerella' between all uninfected plants (control) and infected plants of stage 3 of inoculation in first experiment

	Treatment	Number	Mean	Std. Deviation	Std. Error Mean
H	Uninfected plants	40	254.53	34.689	5.485
	Infected plants	14	257.93	44.545	11.905
NOB	Uninfected plants	40	8.20	1.454	.230
	Infected plants	14	6.36	1.336	.357
NOF	Uninfected plants	40	11.53	3.728	.589
	Infected plants	14	9.86	2.825	.755
Wf	Uninfected plants	40	25.161	2.031	.321
	Infected plants	14	24.531	2.263	.6041

Table 16 Comparison of average height (H) (cm), branch numbers (NOB), fruit numbers (NOF), weight fruit (Wf) (g) of *S. lycopersicum* 'Tigerella' between all uninfected plants (control) and infected plants of stage 1 of inoculation in second experiment (repeat)

	Treatment	Number	Mean	Std. Deviation	Std. Error Mean
H	Uninfected plants	36	243.56	31.198	5.200
	Infected plants	12	236.42	33.427	9.650
NOB	Uninfected plants	36	7.56	1.229	.205

	Infected plants	12	5.75	1.138	.329
NOF	Uninfected plants	36	15.44	3.018	.503
	Infected plants	12	12.33	2.839	.820
Wf	Uninfected plants	36	20.469	2.477	.412
	Infected plants	12	19.910	3.280	.947

Table 17 Comparison of average height (H) (cm), branch numbers (NOB), fruit numbers (NOF), weight fruit (Wf) (g) of *S. lycopersicum* 'Tigerella' between all uninfected plants (control) and infected plants of stage 2 of inoculation in second experiment (repeat)

	Treatment	Number	Mean	Std. Deviation	Std. Error Mean
H	Uninfected plants	36	243.56	31.198	5.200
	Infected plants	12	243.33	27.164	7.842
NOB	Uninfected plants	36	7.56	1.229	.205
	Infected plants	12	5.92	.669	.193
NOF	Uninfected plants	36	15.44	3.018	.503
	Infected plants	12	11.83	3.157	.911

Wf	Uninfected plants	36	20.469	2.477	.412
	Infected plants	12	19.559	3.439	.992

Table 18 Comparison of average height (H) (cm), branch numbers (NOB), fruit numbers (NOF), weight fruit (Wf) (g) of *S. lycopersicum* 'Tigerella' between all uninfected plants (control) and infected plants of stage 3 of inoculation in second experiment (repeat)

	Treatment	Number	Mean	Std. Deviation	Std. Error Mean
H	Uninfected plants	36	243.56	31.198	5.200
	Infected plants	12	260.50	32.380	9.347
NOB	Uninfected plants	36	7.56	1.229	.205
	Infected plants	12	5.58	.515	.149
NOF	Uninfected plants	36	15.44	3.018	.503
	Infected plants	12	12.50	1.834	.529
Wf	Uninfected plants	36	20.469	2.477	.412
	Infected plants	12	17.376	1.792	.517

2. *Capsicum annuum* 'Californian Wonder'

Table 19 Comparison of average height (H) (cm), branch numbers (NOB), fruit numbers (NOF), weight fruit (Wf) (g), length fruit (Lf) (cm) of *C. annuum* 'Californian Wonder'uninfected plants (control) between the first and the second (repeat) experiment in stage 1 of inoculation

	Repeat	Number	Mean	Std. Deviation	Std. Error Mean
H	1	10	57.400	5.815	1.839
	2	20	58.300	5.831	1.304
NOB	1	10	2.600	.516	.163
	2	20	2.550	.510	.114
NOF	1	10	4.200	.632	.200
	2	20	3.700	.571	.127
Wf	1	10	85.462	8.191	2.590
	2	20	78.090	14.392	3.218
Lf	1	10	7.099	.454	.1438
	2	20	6.725	.446	.099

Table 20 Comparison of average height (H) (cm), branch numbers (NOB), fruit numbers (NOF), weight fruit (Wf) (g), length fruit (Lf) (cm) of *C. annuum* 'Californian Wonder' uninfected plants (control) between the first and the second (repeat) experiment in stage 2 of inoculation

	Repeat	Number	Mean	Std. Deviation	Std. Error Mean
H	1	10	57.200	6.663	2.107
	2	20	59.150	6.466	1.446
NOB	1	10	2.400	.516	.163
	2	20	2.500	.513	.114
NOF	1	10	4.300	1.337	.423
	2	20	3.500	.7609	.170
Wf	1	10	92.371	17.690	5.594
	2	20	84.312	13.069	2.922
Lf	1	10	6.732	.473	.149
	2	20	7.437	2.759	.617

Table 21 Comparison of average height (H) (cm), branch numbers (NOB), fruit numbers (NOF), weight fruit (Wf) (g), length fruit (Lf) (cm) of *C. annuum* 'Californian Wonder' uninfected plants (control) between the first and the second (repeat) experiment in stage 3 of inoculation

	Repeat	Number	Mean	Std. Deviation	Std. Error Mean
H	1	10	60.300	6.165	1.949
	2	20	58.650	5.650	1.263
NOB	1	10	2.500	.527	.1667

	2	20	2.550	.510	.114
NOF	1	10	4.700	.948	.300
	2	20	3.400	.680	.152
Wf	1	10	90.594	13.410	4.240
	2	20	85.512	12.842	2.871
Lf	1	10	6.907	.593	.187
	2	20	6.920	.621	.138

Table 22 Comparison of average height (H) (cm), branch numbers (NOB), fruit numbers (NOF), weight fruit (Wf) (g), length fruit (Lf) (cm) of *C. annuum* 'Californian Wonder' infected plants between the first and the second (repeat) experiment in stage 1 of inoculation

	Repeat	Number	Mean	Std. Deviation	Std. Error Mean
H	1	10	54.000	11.897	3.762
	2	20	43.400	14.925	3.337
NOB	1	10	2.600	.516	.163
	2	20	2.500	.513	.114
NOF	1	10	1.900	1.449	.458
	2	20	1.600	1.046	.234
Wf	1	7	75.377	10.012	3.784

	2	17	65.694	31.243	7.577
Lf	1	7	6.177	.550	.208
	2	17	6.756	1.199	.290

Table 23 Comparison of average height (H) (cm), branch numbers (NOB), fruit numbers (NOF), weight fruit (Wf) (g), length fruit (Lf) (cm) of *C. annuum* 'Californian Wonder' infected plants between the first and the second (repeat) experiment in stage 2 of inoculation

	Repeat	Number	Mean	Std. Deviation	Std. Error Mean
H	1	10	59.900	4.148	1.311
	2	20	50.900	11.125	2.487
NOB	1	10	2.500	.527	.166
	2	20	2.400	.598	.133
NOF	1	10	3.000	1.333	.421
	2	20	1.750	.9665	.216
Wf	1	10	88.420	21.054	6.658
	2	19	82.021	42.260	9.695
Lf	1	10	6.915	.605	.191
	2	19	6.942	1.346	.308

Table 24 Comparison of average height (H) (cm), branch numbers (NOB), fruit numbers (NOF), weight fruit (Wf) (g), length fruit (Lf) (cm) of *C. annuum* 'Californian Wonder' infected plants between the first and the second (repeat) experiment in stage 3 of inoculation

	Repeat	Number	Mean	Std. Deviation	Std. Error Mean
H	1	10	60.900	5.445	1.722
	2	20	50.200	9.833	2.198
NOB	1	10	2.700	.483	.152
	2	20	2.550	.510	.114
NOF	1	10	2.400	.843	.266
	2	20	1.450	.759	.169
Wf	1	10	80.180	26.667	8.433
	2	19	90.683	34.551	7.926
Lf	1	10	7.147	1.096	.346
	2	19	7.347	1.460	.335

Table 25 Comparison of average height (H) (cm), branch numbers (NOB), fruit numbers (NOF), weight fruit (Wf) (g), length fruit (Lf) (cm) of *C. annuum* 'Californian Wonder' between uninfected plants (control) and infected plants in stage 1 of inoculation

	Treatment	Number	Mean	Std. Deviation	Std. Error Mean
H	Uninfected plants	30	58.000	5.741	1.048
	Infected plants	30	46.933	14.687	2.681

NOB	Uninfected plants	30	2.567	.504	.0920
	Infected plants	30	2.533	.507	.092
NOF	Uninfected plants	30	3.867	.628	.114
	Infected plants	30	1.700	1.178	.215
Wf	Uninfected plants	30	80.548	13.001	2.373
	Infected plants	24	68.518	26.934	5.497
Lf	Uninfected plants	30	6.850	.476	.086
	Infected plants	24	6.587	1.073	.219

Table 26 Comparison of average height (H) (cm), branch numbers (NOB), fruit numbers (NOF), weight fruit (Wf) (g), length fruit (Lf) (cm) of *C. annuum* 'Californian Wonder' between uninfected plants (control) and infected plants in stage 2 of inoculation

	Treatment	Number	Mean	Std. Deviation	Std. Error Mean
H	Uninfected plants	30	58.500	6.484	1.183
	Infected plants	30	53.900	10.249	1.871
NOB	Uninfected plants	30	2.467	.507	.092
	Infected plants	30	2.433	.568	.103
NOF	Uninfected plants	30	3.767	1.040	.189
	Infected plants	30	2.167	1.234	.225
Wf	Uninfected plants	30	86.998	14.965	2.732

	Infected plants	29	84.227	36.058	6.695
Lf	Uninfected plants	30	7.202	2.274	.415
	Infected plants	29	6.932	1.133	.210

Table 27 Comparison of average height (H) (cm), branch numbers (NOB), fruit numbers (NOF), weight fruit (Wf) (g), length fruit (Lf) (cm) of *C. annuum* 'Californian Wonder' between uninfected plants (control) and infected plants in stage 3 of inoculation

	Treatment	Number	Mean	Std. Deviation	Std. Error Mean
H	Uninfected plants	30	59.200	5.773	1.054
	Infected plants	30	53.767	9.943	1.815
NOB	Uninfected plants	30	2.533	.507	.092
	Infected plants	30	2.600	.498	.091
NOF	Uninfected plants	30	3.833	.985	.179
	Infected plants	30	1.767	.897	.163
Wf	Uninfected plants	30	87.206	13.030	2.379
	Infected plants	29	87.061	31.966	5.935
Lf	Uninfected plants	30	6.916	.601	.109
	Infected plants	29	7.278	1.329	.246

Table 28 Comparison of average height (H) (cm), branch numbers (NOB), fruit numbers (NOF), weight fruit (Wf) (g), length fruit (Lf) (cm) of *C. annuum* 'Californian Wonder' between all uninfected plants (control) and infected plants in stage 1 of inoculation

	Treatment	Number	Mean	Std. Deviation	Std. Error Mean
H	Uninfected plants	90	58.567	5.962	.628
	Infected plants	30	46.933	14.687	2.681
NOB	Uninfected plants	90	2.522	.502	.052
	Infected plants	30	2.533	.507	.092
NOF	Uninfected plants	90	3.822	.894	.094
	Infected plants	30	1.700	1.178	.215
Wf	Uninfected plants	90	84.917	13.894	1.464
	Infected plants	24	68.518	26.934	5.497
Lf	Uninfected plants	90	6.989	1.378	.145
	Infected plants	24	6.587	1.073	.219

Table 29 Comparison of average height (H) (cm), branch numbers (NOB), fruit numbers (NOF), weight fruit (Wf) (g), length fruit (cm) of *C. annuum* 'Californian Wonder' between all uninfected plants (control) and infected plants in stage 2 of inoculation

	Treatment	Number	Mean	Std. Deviation	Std. Error Mean
H	Uninfected plants	90	58.567	5.962	.628
	Infected plants	31	52.871	11.592	2.082
NOB	Uninfected plants	90	2.522	.502	.052
	Infected plants	31	2.419	.564	.101

NOF	Uninfected plants	90	3.822	.894	.094
	Infected plants	31	2.129	1.231	.221
Wf	Uninfected plants	90	84.917	13.894	1.464
	Infected plants	30	82.190	37.147	6.782
Lf	Uninfected plants	90	6.989	1.378	.1453
	Infected plants	30	6.885	1.143	.208

Table 30 Comparison of average height (H) (cm), branch numbers (NOB), fruit numbers (NOF), weight fruit (Wf) (g), length fruit (Lf) (cm) of *C. annuum* 'Californian Wonder' between all uninfected plants (control) and infected plants in stage 3 of inoculation

	Treatment	Number	Mean	Std. Deviation	Std. Error Mean
H	Uninfected plants	90	58.567	5.962	.628
	Infected plants	30	53.767	9.943	1.815
NOB	Uninfected plants	90	2.522	.502	.052
	Infected plants	30	2.600	.498	.091
NOF	Uninfected plants	90	3.822	.894	.094
	Infected plants	30	1.767	.897	.163
Wf	Uninfected plants	90	84.917	13.894	1.464
	Infected plants	29	87.061	31.966	5.935
Lf	Uninfected plants	90	6.989	1.378	.145

	Infected plants	29	7.278	1.329	.246

Table 31 Comparison of average height (H) (cm), branch numbers (NOB), fruit numbers (NOF), weight fruit (Wf) (g), length fruit (Lf) (cm) of *C. annuum* 'Californian Wonder' between all uninfected plants (control) and infected plants of stage 1 of inoculation in first experiment

	Treatment	Number	Mean	Std. Deviation	Std. Error Mean
H	Uninfected plants	30	58.300	6.176	1.127
	Infected plants	10	54.000	11.897	3.762
NOB	Uninfected plants	30	2.500	.508	.092
	Infected plants	10	2.600	.5164	.163
NOF	Uninfected plants	30	4.400	1.003	.183
	Infected plants	7	2.714	.7559	.2857
Wf	Uninfected plants	30	89.476	13.514	2.467
	Infected plants	7	75.377	10.012	3.784
Lf	Uninfected plants	30	6.913	.516	.094

	Infected plants	7	6.177	.550	.208

Table 32 Comparison of average height (H) (cm), branch numbers (NOB), fruit numbers (NOF), weight fruit (Wf) (g), length fruit (Lf) (cm) of *C. annuum* 'Californian Wonder' between all uninfected plants (control) and infected plants of stage 2 of inoculation in first experiment

	Treatment	Number	Mean	Std. Deviation	Std. Error Mean
H	Uninfected plants	30	58.300	6.176	1.127
	Infected plants	10	59.900	4.148	1.311
NOB	Uninfected plants	30	2.500	.508	.092
	Infected plants	10	2.500	.527	.166
NOF	Uninfected plants	30	4.400	1.003	.183
	Infected plants	10	3.000	1.333	.421
Wf	Uninfected plants	30	89.476	13.514	2.467
	Infected plants	10	88.420	21.054	6.658
Lf	Uninfected plants	30	6.913	.516	.094

	Infected plants	10	6.915	.605	.191

Table 33 Comparison of average height (H) (cm), branch numbers (NOB), fruit numbers (NOF), weight fruit (Wf) (g), length fruit (Lf) (cm) of *C. annuum* 'Californian Wonder' between all uninfected plants (control) and infected plants of stage 3 of inoculation in first experiment

	Treatment	Number	Mean	Std. Deviation	Std. Error Mean
H	Uninfected plants	30	58.300	6.176	1.127
	Infected plants	10	60.900	5.445	1.722
NOB	Uninfected plants	30	2.500	.508	.092
	Infected plants	10	2.700	.483	.152
NOF	Uninfected plants	30	4.400	1.003	.183
	Infected plants	10	2.400	.843	.266
Wf	Uninfected plants	30	89.476	13.514	2.467
	Infected plants	10	80.180	26.667	8.433
Lf	Uninfected plants	30	6.913	.516	.094

	Infected plants	10	7.147	1.096	.346

Table 34 Comparison of average height (H) (cm), branch numbers (NOB), fruit numbers (NOF), weight fruit (Wf) (g), length fruit (Lf) (cm) of *C. annuum* 'Californian Wonder' between all uninfected plants (control) and infected plants of stage 1 of inoculation in second experiment (repeat)

	Treatment	Number	Mean	Std. Deviation	Std. Error Mean
H	Uninfected plants	60	58.700	5.901	.761
	Infected plants	20	43.400	14.925	3.337
NOB	Uninfected plants	60	2.533	.503	.064
	Infected plants	20	2.500	.513	.114
NOF	Uninfected plants	60	3.533	.675	.087
	Infected plants	17	1.882	.857	.208
Wf	Uninfected plants	60	82.638	13.623	1.758
	Infected plants	17	65.694	31.243	7.577
Lf	Uninfected plants	60	7.027	1.652	.213
	Infected plants	17	6.756	1.199	.290

Table 35 Comparison of average height (H) (cm), branch numbers (NOB), fruit numbers (NOF), weight fruit (Wf) (g), length fruit (Lf) (cm) of *C. annuum* 'Californian Wonder' between all uninfected plants (control) and infected plants of stage 2 of inoculation in second experiment (repeat)

	Treatment	Number	Mean	Std. Deviation	Std. Error Mean
H	Uninfected plants	60	58.700	5.901	.761
	Infected plants	20	50.900	11.125	2.487
NOB	Uninfected plants	60	2.533	.503	.064
	Infected plants	20	2.400	.598	.133
NOF	Uninfected plants	60	3.533	.675	.0872
	Infected plants	19	1.842	.898	.206
Wf	Uninfected plants	60	82.638	13.623	1.758
	Infected plants	19	82.021	42.260	9.695
Lf	Uninfected plants	60	7.027	1.652	.213
	Infected plants	19	6.942	1.346	.308

Table 36 Comparison of average height (H) (cm), branch numbers (NOB), fruit numbers (NOF), weight fruit (Wf) (g), length fruit (Lf) (cm) of *C. annuum* 'Californian Wonder' between all uninfected plants (control) and infected plants of stage 3 of inoculation in second experiment (repeat)

	Treatment	Number	Mean	Std. Deviation	Std. Error Mean
H	Uninfected plants	60	58.700	5.901	.761
	Infected plants	20	50.200	9.833	2.198
NOB	Uninfected plants	60	2.533	.503	.064
	Infected plants	20	2.550	.510	.114
NOF	Uninfected plants	60	3.533	.675	.0872
	Infected plants	19	1.526	.696	.15
Wf	Uninfected plants	60	82.638	13.623	1.758
	Infected plants	19	90.683	34.551	7.926
Lf	Uninfected plants	60	7.027	1.652	.213
	Infected plants	19	7.347	1.460	.335

3. *Capsicum annuum* 'Jalapeno'

Table 37 Comparison of average height (H) (cm), branch numbers (NOB), fruit numbers (NOF), weight fruit (Wf) (g), length fruit (Lf) (cm) of *C. annuum* 'Jalapeno' of uninfected plants (control) between the first and the second (repeat) experiment in stage 1 of inoculation

	Repeat	Number	Mean	Std. Deviation	Std. Error Mean
H	1	10	90.400	9.732	3.077
	2	16	98.588	11.607	2.901
NOB	1	10	6.30	1.059	.335
	2	16	6.69	.704	.176
NOF	1	10	10.00	2.211	.699
	2	16	10.38	1.746	.437
Wf	1	10	28.839	5.115	1.617
	2	16	23.579	3.180	.795
Lf	1	10	8.889	1.641	.519
	2	16	8.103	.888	.222

Table 38 Comparison of average height (H) (cm), branch numbers (NOB), fruit numbers (NOF), weight fruit (Wf) (g), length fruit (Lf) (cm) of *C. annuum* 'Jalapeno' of uninfected plants (control) between the first and the second (repeat) experiment in stage 2 of inoculation

	Repeat	Number	Mean	Std. Deviation	Std. Error Mean
H	1	10	92.100	8.949	2.830
	2	16	97.625	25.329	6.332
NOB	1	10	6.50	1.080	.342
	2	16	6.44	.964	.241
NOF	1	10	9.90	2.807	.888
	2	16	8.38	1.746	.437
Wf	1	10	24.784	6.293	1.990
	2	16	24.193	4.995	1.248
Lf	1	10	8.078	2.069	.654
	2	16	8.989	1.342	.335

Table 39 Comparison of average height (H) (cm), branch numbers (NOB), fruit numbers (NOF), weight fruit (Wf) (g), length fruit (Lf) (cm) of *C. annuum* 'Jalapeno' of uninfected plants (control) between the first and the second (repeat) experiment in stage 3 of inoculation

	Repeat	Number	Mean	Std. Deviation	Std. Error Mean
H	1	10	92.100	10.397	3.287

	2	16	101.875	12.274	3.068
NOB	1	10	7.00	1.155	.365
	2	16	6.69	.946	.237
NOF	1	10	10.60	2.675	.846
	2	16	7.63	2.527	.632
Wf	1	10	24.707	4.735	1.497
	2	16	28.782	9.434	2.358
Lf	1	10	8.981	1.720	.544
	2	16	9.222	1.832	.458

Table 40 Comparison of average height (H) (cm), branch numbers (NOB), fruit numbers (NOF), weight fruit (Wf) (g), length fruit (Lf) (cm) of *C. annuum* 'Jalapeno' of infected plants between the first and the second (repeat) experiment in stage 3 of inoculation

	Repeat	Number	Mean	Std. Deviation	Std. Error Mean
H	1	7	89.143	9.805	3.706
	2	3	93.333	8.736	5.044
NOB	1	7	6.43	1.718	.649
	2	3	6.00	2.000	1.155
NOF	1	7	6.14	3.891	1.471
	2	3	5.00	1.732	1.000

Wf	1	6	22.578	4.447	1.815
	2	3	27.803	14.906	8.606
Lf	1	6	8.977	1.827	.746
	2	3	10.072	3.704	2.139

Table 40 Comparison of average height (H) (cm), branch numbers (NOB), fruit numbers (NOF), weight fruit (Wf) (g), length fruit (Lf) (cm) of *C. annuum* 'Jalapeno' of stage 1 inoculation between uninfected plants (control) and infected plants (only two infected plants survived from both two experiments and no fruit produced)

	Treatment	Number	Mean	Std. Deviation	Std. Error Mean
H	Uninfected plants	26	95.438	11.464	2.248
	Infected plants	2	57.000	32.526	23.000
NOB	Uninfected plants	26	6.54	.859	.169
	Infected plants	2	5.00	4.243	3.000
NOF	Uninfected plants	26	10.23	1.904	.373
	Infected plants	0	.	.	.
Wf	Uninfected plants	26	25.602	4.722	.926
	Infected plants	0	.	.	.
Lf	Uninfected plants	26	8.405	1.263	.247
	Infected plants	0	.	.	.

Table 41 Comparison of average height (H) (cm), branch numbers (NOB), fruit numbers (NOF), weight fruit (Wf) (g), length fruit (Lf) (cm) of *C. annuum* 'Jalapeno' of stage 3 inoculation between uninfected plants (control) and infected plants. There were total 10 infected plants survived including seven plants of experiment 1 and three plants of second experiment (repeat) when they were inoculated in stage 3 (before flowering)

	Treatment	Number	Mean	Std. Deviation	Std. Error Mean
H	Uninfected plants	26	98.115	12.362	2.424
	Infected plants	10	90.400	9.228	2.918
NOB	Uninfected plants	26	6.81	1.021	.200
	Infected plants	10	6.30	1.703	.539
NOF	Uninfected plants	26	8.77	2.930	.575
	Infected plants	10	5.80	3.327	1.052
Wf	Uninfected plants	26	27.215	8.096	1.587
	Infected plants	9	24.319	8.645	2.881
Lf	Uninfected plants	26	9.129	1.759	.345
	Infected plants	9	9.342	2.412	.804

Table 42 Comparison of average height (H) (cm), branch numbers (NOB), fruit numbers (NOF), weight fruit (Wf) (g), length fruit (Lf) (cm) of *C. annuum* 'Jalapeno' between all uninfected plants (control) and infected plants of stage 1 inoculation

	Treatment	Number	Mean	Std. Deviation	Std. Error Mean
H	Uninfected plants	78	96.351	15.1873	1.7196

	Infected plants	2	57.000	32.526	23.000
NOB	Uninfected plants	78	6.60	.958	.108
	Infected plants	2	5.00	4.243	3.000
NOF	Uninfected plants	78	9.32	2.468	.279
	Infected plants	0	.	.	.
Wf	Uninfected plants	78	25.746	6.274	.710
	Infected plants	0	.	.	.
Lf	Uninfected plants	78	8.724	1.591	.180
	Infected plants	0	.	.	.

Table 43 Comparison of average height (H) (cm), branch numbers (NOB), fruit numbers (NOF), weight fruit (Wf) (g), length fruit (Lf) (cm) of *C. annuum* 'Jalapeno' between all uninfected plants (control) and infected plants of stage 3 inoculation

	Treatment	Number	Mean	Std. Deviation	Std. Error Mean
H	Uninfected plants	78	96.351	15.187	1.719
	Infected plants	10	90.400	9.228	2.918
NOB	Uninfected plants	78	6.60	.958	.108
	Infected plants	10	6.30	1.703	.539
NOF	Uninfected plants	78	9.32	2.468	.279
	Infected plants	10	5.80	3.327	1.052

Wf	Uninfected plants	78	25.746	6.274	.710
	Infected plants	9	24.319	8.645	2.881
Lf	Uninfected plants	78	8.724	1.591	.180
	Infected plants	9	9.342	2.412	.804

Table 44 Comparison of average height (H) (cm), branch numbers (NOB), fruit numbers (NOF), weight fruit (Wf) (g), length fruit (Lf) (cm) of *C. annuum* 'Jalapeno' of experiment 1 between all uninfected plants (control) and infected plants of inoculation stage 1 (only two plants survived but no fruit produced)

	Treatment	Number	Mean	Std. Deviation	Std. Error Mean
H	Uninfected plants	30	91.533	9.405	1.717
	Infected plants	2	57.000	32.526	23.000
NOB	Uninfected plants	30	6.60	1.102	.201
	Infected plants	2	5.00	4.243	3.000

Table 45 Comparison of average height (H) (cm), branch numbers (NOB), fruit numbers (NOF), weight fruit (Wf) (g), length fruit (Lf) (cm) of *C. annuum* 'Jalapeno' of experiment 1 between all uninfected plants (control) and infected plants of inoculation stage 3 (only six infected plants produced fruits of seven plants survived)

	Treatment	Number	Mean	Std. Deviation	Std. Error Mean
H	Uninfected plants	30	91.533	9.405	1.717
	Infected plants	7	89.143	9.805	3.706
NOB	Uninfected plants	30	6.60	1.102	.201
	Infected plants	7	6.43	1.718	.649
NOF	Uninfected plants	30	10.30	2.535	.463
	Infected plants	6	7.17	3.061	1.249
Wf	Uninfected plants	30	26.074	5.775	1.054
	Infected plants	6	23.042	4.557	1.860
Lf	Uninfected plants	30	8.641	1.807	.330
	Infected plants	6	9.152	1.811	.739

Table 46 Comparison of average height (H) (cm), branch numbers (NOB), fruit numbers (NOF), weight fruit (Wf) (g), length fruit (Lf) (cm) of *C. annuum* 'Jalapeno' of experiment 2 (repeat) between all uninfected plants (control) and infected plants of inoculation stage 3 (only four infected plants survived and produced fruits)

	Treatment	Number	Mean	Std. Deviation	Std. Error Mean
H	Uninfected plants	48	99.363	17.297	2.496
	Infected plants	4	91.750	7.804	3.902
NOB	Uninfected plants	48	6.60	.869	.125
	Infected plants	4	6.25	1.708	.854
NOF	Uninfected plants	48	8.81	2.385	.344
	Infected plants	4	5.50	1.732	.866
Wf	Uninfected plants	48	25.335	7.199	1.039
	Infected plants	4	31.109	13.851	6.925
Lf	Uninfected plants	48	8.882	1.476	.213
	Infected plants	4	10.536	3.164	1.582