Low root-to-root transmission of a tobamovirus, yellow tailflower mild mottle virus, and resilience of its virions

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Low root-to-root transmission of a tobamovirus, yellow tailflower mild mottle virus, and resilience of its virions ~~under various treatments~~

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Abstract

Tobamoviruses are serious pathogens because they have extremely stable virions, they are transmitted by contact, and they often induce severe disease in crops. Knowledge of the routes of transmission and resilience of tobamovirus virions is essential in understanding the epidemiology of this group of viruses. We used an isolate of the tobamovirus yellow tailflower mild mottle virus (YTMMV) to examine root-to-root transmission in soil and in a hydroponic growth environment. Root-to-root transmission occurred rarely, and when it occurred plants did not exhibit systemic movement of the virus from the roots to the shoots over a 30-day period. The ~~famous~~ resilience of YTMMV ~~tobamovirus~~ virions was tested in dried leaf tissue over time periods from one hour to one year under temperatures ranging from -80ºC to 160ºC. Infectivity was maintained for at least a year when incubated at -80ºC, 22ºC or at fluctuating ambient temperatures of 0.8ºC to 44.4ºC, but incubation under dry conditions at 160ºC for >4 days eliminated infectivity. Exposure of virions to 0.1 M sodium hydroxide or 20% w/v skim milk solution for 30 min, treatments recommended for tobamovirus inactivation, did not abolish infectivity of YTMMV.

Key words

Root transmission, virion stability, tobamovirus control, viruses of Solanaceae, Yellow tailflower mild mottle virus

Introduction

Tobamoviruses are amongst the most destructive viruses in horticulture because they can cause severe disease, are easily transmitted by contact, and virions are famously resilient. In 2009, isolates of the type species of genus *Tobamovirus* (family *Virgaviridae*), *Tobacco mosaic virus* (TMV) reduced the tobacco crop in China by 168,000 tonnes (Shen et al., 2013). TMV can be transmitted to new host plants through virions in soil debris, dead plant tissue, irrigation water, on farm machinery, on the hands and clothing of workers, and in processed tobacco (LeClair, 1967, Balique et al., 2012).

TMV can also be transmitted *via* contact with pollen or seed. The pollen and seed of *Capsicum annuum* plants infected with TMV carried infective virions on their surface, but not internally. Seedlings grown from unwashed seeds became infected, but no infection of seedlings occurred after treatment of the seeds with NaOH or Na_3PO_4, indicating that seedlings became infected from virions adhering to the outside of the seed coat (Salamon and Kaszta, 2000).

The internationally distributed tobamovirus c~~C~~ucumber green mottle mosaic virus (CGMMV) ~~i~~was reported to be vertically transmitted, but the mode of infection was uncertain (Vani & Varma, 1993; Liu et al., 2014). Surface sterilisation of seed reduced transmission of CGMMV from seed to seedlings, but it did not always eliminate it. A recent study showed that virions were present inside some seeds, indicative that virions externally adhered to seed as well ~~as virion~~ass within the seed contribute to transmission between plant generations (Reingold et al., 2015).

~~Another commercially devastating tobamovirus with international distribution is cucumber green mottle mosaic virus (CGMMV). CGMMV is transmitted horizontally in many of the same ways described for TMV, but it is also transmitted vertically *via* the seed of some cucurbits (Vani & Varma, 1993).~~

Yellow tailflower mild mottle virus (YTMMV) was discovered in 2014 infecting a wild indigenous solanaceous plant of the genus *Anthocercis* in Western Australia (Wylie et al., 2014). No extensive surveys of natural YTMMV distribution and host range have been done, but under experimental conditions the virus is also capable of infecting species of four other solanaceous genera, including *Solanum lycopersicum* (tomato), *S. betaceum* (tamarillo), *S. melongena* (aubergine), *S. nigrum* (black nightshade), *Capsicum annuum* (bell pepper, chilli),

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19 species of *Nicotiana*, and three species of *Physalis* (Li et al., 2015a, Wylie et al., 2015). Across this range of experimental hosts, symptoms ranged from mild leaf mosaic in tomato to death of whole plants in *N. benthamiana* accession RA-4 and a cultivar of *C. annuum* (Li et al., 2015a, Wylie et al., 2015).

Knowledge of transmission and virion resilience under various conditions is important to understand epidemiology of viruses. Tobamoviruses such as CGMMV, tomato mosaic virus (ToMV) and TMV have been found to be infectious in water and are able to infect plants *via* roots (Vani & Varma, 1993, Jacobi & Castello, 1991, Beijerinck, 1898, Paludan, 1985). These tobamoviruses are responsible for causing economic losses (Shen et al., 2013, Coates & McCarthy, 2015). Here, we aimed to determine whether YTMMV is similarly transmitted *via* water and by root to root contact, and the viability of virions over time and under decontamination treatments. The laboratory accession of the model virus host plant *Nicotiana benthamiana*, a species indigenous to the region where YTMMV was discovered, was used to assess transmission because it exhibits a rapid systemic hypersensitive response to YTMMV ~~infection to assess its level of risk to the agriculture industry in Australia~~. ~~We used YTMMV as a model from which to study how tobamoviruses might respond in high density plantings where roots are in contact with one another, such as in hydroponics-based horticulture. An assessment of virion viability was done after treatment with two recommended decontamination solutions, and after a range of time vs temperature treatments.~~

Materials and Methods

Viruses and plants

Four-week-old *N. benthamiana* RA-4 plants (Wylie et al., 2015) were used as indicator plants for YTMMV infection because young infected seedlings responded with systemic necrosis at early onset, 14 to 35 days post inoculation (dpi) (Wylie et al., 2015). Approximately 500 mg of YTMMV, isolate Cervantes, infected *N. benthamiana* leaf tissue (Wylie et al., 2014) was

macerated with approximately 10 ml of 0.1 M phosphate buffer (pH 7.0) and 0.5 g diatomaceous earth (Sigma Corp), and the mixture was mechanically applied to every leaf on 4-week old *N. benthamiana* seedlings. Control plants were mock-inoculated as above but without virus inoculum. All plants were housed in a temperature-controlled (22ºC day and 17ºC night), insect-proofed glasshouse under natural light.

To prevent cross-contamination of YTMMV, steam-sterilised pots were used, plants were spaced apart from one another with no leaf contact within and between different treatments, and strict hygiene measures were imposed, including hand washing before and after entering the facility.

Plants were tested for the presence of YTMMV using reverse transcription PCR (RT-PCR). RNA was extracted from 100 mg of leaf tissue, as described by Morris & Dodds (1979), resuspended in 20 µl of RNAse-free water and stored at -20°C. Extracted RNA was used as templates for RT-PCR or RT-qPCR. Samples were reverse transcribed using GoScript™ reverse transcriptase (Promega) with a random primer (5'-CGTACAGTTAGCAGGCNNNNNNNNNNN-3', where N represents any nucleotide). PCR primers were YT-CPF (5'-AGCGAATTGATGAGGTTAAGGA-3') and YT-CPR (5'-TGGAGGGAAAAACACTACGC-3') (Koh et al., 2017), that amplified a 574 nucleotide (nt) fragment of the coat protein gene. PCR was done using GoTaq® Green mastermix (Promega) at 95ºC for 3 min, 30 cycles of 95ºC for 30 s, 55ºC for 30 s, 72ºC for 90 s and a final extension of 72ºC for 10 min.

Measuring infectivity and developing a symptom severity index

A control experiment was done to observe the infectivity, viral titres and inoculum potential of YTMMV by inoculating with different amounts (1, 10, 20, 50 and 100 mg) of fresh

YTMMV-infectious leaf materials to healthy *N. benthamiana* plants as described above. Six replicates were done on each treatment, and experiments were carried out twice.

A symptom severity index was developed to record symptoms at 20 dpi or at a time otherwise stated. Symptom severity was reflective of the speed of symptom development as manifest at 20 dpi. All infected *N. benthamiana* RA-4 plants died by 35 dpi. The symptom severity index followed an ordinal scale of increasing severity as follows: 0: No visible disease symptoms, 1: Barely visible leaf mosaic, slight (10-20%) plant stunting, slight down-curling of leaves, 2: Moderate leaf mosaic, moderate 20-30% plant stunting, down-curling of some leaves, 3: Severe (>40%) plant stunting, severe leaf mosaic/chlorosis, most leaves curled, necrosis of leaf veins sometimes visible, and 4: Plant death. Two assessors did scoring independently, and the median of the two scores was recorded.

Quantitative PCR (qPCR)

The copy numbers of YTMMV coat protein (CP) molecules were measured using reverse-transcription quantitative-PCR (RT-qPCR) at 20 dpi. It was assumed that the copy number of CP molecules was proportional to the number of virions in the plant. RT-qPCR were carried out in a Qiagen (Corbett) RotorGene™ 3000 using 0.5 µl dsRNA-enriched sample as template with YTMMV-CP primers (YT-qCPF: 5'-CTCAGAATGCCAGAACAACTG-3' and YT-qCPR: 5'-CGAATTTAACACCGACGTGA-3') and reference gene cytochrome oxidase (COX) primers (COX-F: 5'-CGTCGCATTCCAGATTATCCA-3' and COX-R: 5'-CAACTACGGATATATAAGAGCCAAAACTG-3') using SensiFAST™ SYBR® No-Rox One-Step Kit (Bioline) according to the manufacturer's protocol with annealing temperature of 55ºC. Each reaction was done in a 10 µl volume and replicated three times. A melting curve was generated from 72°C to 95°C to detect primer dimers and confirm reaction specificity at 82°C. Quantification cycle (C_q) values were generated using RotorGene Q

Series software (v6.1.93). Positive controls were from infected YTMMV leaf materials, while negative controls were from mock-inoculated leaf materials.

Viral RNA load was estimated by RT-qPCR in reference to a standard curve. Five dilution standards were carried out (0x, 50x, 100x, 500x and 1000x dilution) using a 100 mg positive control sample. The copy number of the viral RNA load was estimated using the absolute quantification method with reference to the trend line of a linear regression.

Transmission of YTMMV *via* roots

To investigate if virus transmission occurred *via* roots, two healthy *N. benthamiana* plants were grown together in a 20 cm round pot. One plant was inoculated with YTMMV. Leaf contact was prevented by a clear polycarbonate screen placed between the plants. Plants were grown for 30 dpi and symptoms recorded on both plants. After 30 dpi, leaf and root samples were taken from the non-inoculated plant from each pot and tested for the presence of YTMMV by RT-PCR assay as described above. To determine if root contact led to transmission of the virus between plants, roots from the non-inoculated plant that were not in direct contact with roots from infected plants, and those that were in contact were carefully removed separately from the plant and tested for YTMMV using RT-PCR. Roots were thoroughly rinsed with distilled water to rid them of soil prior to RNA extraction and RT-PCR was done as described previously. Eighteen replicates of this experiment were done.

YTMMV transmission *via* water

A hydroponics system consisted of two 100 cm by 50 cm tanks connected at the base by tubing so that water could pass between the two tanks (Figure 1) was constructed. The system was used to investigate viral transmission from one virus-infected plant to uninfected plants whose roots were immersed in the same water source. Plants were grown in 1 L pots that had drainage holes in their bases. Plants in pots were placed in the tanks in 2 cm water. In the first

158 tank, a YTMMV-infected *N. benthamiana* plant and six uninfected *N. benthamiana* plants
159 were placed, and in the second tank, eight uninfected *N. benthamiana* plants were placed
160 (Figure 1). There was no physical contact between any parts of the plants. Three treatments
161 were carried out to mimic means by which virions might enter the liquid medium shared by
162 all the plants in the tank, and provide opportunities for infection to occur. In the first
163 treatment, water was poured over the virus-infected plant so that virions in the water drained
164 through the pot and into the tank. In the second treatment, a leaf on the virus-infected *N.*
165 *benthamiana* plant was wounded every other day by rubbing it with diatomaceous earth
166 before applying water to the wound site to allow virions to be carried by water into the tank.
167 In the third treatment, 500 mg of viruliferous leaf material was macerated in inoculation
168 buffer and added to the tank water every other day. This was done to mimic a situation where
169 an infected plant might be immersed in the liquid, or dies and disintegrate in the liquid
170 medium. When the source plant died from symptoms of YTMMV infection, it was replaced
171 by another infected plant. Plants were maintained in tanks for 45 d and symptom severity
172 indices recorded regularly. After 45 d, leaves and roots of the non-inoculated plants were
173 tested for the presence of YTMMV in leaves and roots by RT-PCR assay as described above.
174 Taking care not to touch surfaces exposed to virions, roots were collected near the stem base
175 of the plants (not from roots extending from the pots into the water) and washed thoroughly
176 with warm water and detergent before RNA extraction. This was done to remove as many
177 virions adhering to the surface as possible. Between experiments, tanks were sterilised by
178 wiping with 70% ethanol and washing twice with detergent. Experiments were carried out at
179 three times.

180 Virion stability: lyophilisation

181 Samples (100 mg each) of YTMMV-infected leaves were collected in a 1.5 ml eppendorf
182 tube and lyophilised in a vacuum freeze-dryer. After lyophilising, samples were either used

for RNA extraction as previously described, or inoculated onto six *N. benthamiana* seedlings. Symptoms on inoculated plants were assessed 20 dpi.

Virion stability: temperature and time

YTMMV-infected leaves were collected individually into paper envelopes and lyophilised before incubating under treatments that varied in temperature and time. Envelopes were kept in zip-lock bags with desiccant beads to eliminate moisture. Six bags of leaves per treatment were stored under temperature regimes ranging from -80ºC to 160ºC for different time periods (Table 1). At the end of each incubation period, the lyophilised samples (100 mg) were soaked in inoculation buffer for one minute before maceration and application to leaves of *N. benthamiana* seedlings as described above. Experiments were done with six replicate plants and repeated twice.

Virion stability: inactivation treatment

Sodium hydroxide (NaOH), milk and detergents are reported as inactivators of tobamoviruses (Nitzany, 1960, Hu et al., 1994, Lewandowski et al., 2010, Li et al., 2015c). To investigate the effects of these inactivation agents, 500 mg of viruliferous *N. benthamiana*-RA-4 leaves were ground and incubated with either 0.1 M NaOH or 20% w/v skim milk for 1, 2, 5, 10, 15 and 30 min (Table 1). After incubation, the mixture was drained, and approximately 5 ml of inoculation buffer was added to the vessel. The mixture was applied to leaves of *N. benthamiana*-RA-4 seedlings as described above. There were six replicates for each treatment. Negative controls were incubation of the mortar and pestle in the same inactivation solution but without using infected YTMMV leaves, and the positive control was 500 mg infected *N. benthamiana* leaf ground in inoculation buffer and applied as above. Experiments were repeated in triplicate.

Statistical analysis

Ordinal regression models were fitted to investigate if temperature, incubation time, different inactivation agents, and time of exposure to inactivation agents for YTMMV were significant factors in explaining symptom severity index at 20 dpi with YTMMV. All statistical analyses were performed using the R statistical programming language (R Development Core Team, 2016).

Results

Inoculum strength

Healthy *N. benthamiana* seedlings inoculated with 1, 10, 20, 50 and 100 mg of infectious leaf materials developed symptoms in proportion to the amount of inoculum at 20 dpi. Mean symptom severity indices at 20 dpi for the specified levels of inoculum were 2.5 (±0.55), 2.67 (±0.82), 3.0 (±0.63), 3.17 (±0.41) and 3.33 (±0.52), respectively. This experiment showed that 1 mg of infectious leaf material was capable of establishing infection.

RT-qPCR analysis

A standard curve was established to quantify the YTMMV CP molecules. The detection limit of the RT-qPCR was in the range of 1.06×10^{8} to 1.06×10^{11} in 100 mg of infectious fresh leaf materials. The threshold to denote the C_q values was generated in the RotorGene Q Series software (v6.1.93) and was calculated to be 0.117. A standard curve was generated with a R^2 value of 0.991, a reaction efficiency of 1.040 and given by $y = -3.230x + 20.727$, where y denotes the C_q of the unknown sample and x denotes the quantity of the unknown sample.

Virus transmission *via* roots

Two *N. benthamiana* plants grown in the same pot, one leaf-inoculated with YTMMV and the other uninoculated. They were scored separately for symptom development. All

uninoculated plants had a symptom severity index of 0 (no apparent symptoms). Roots from uninoculated plants that were in contact with inoculated plants tested positive for YTMMV, but the shoots of the same plants did not. The roots of uninoculated plants that were in contacted with roots from inoculated plants tested positive for YTMMV.

Virus transmission *via* water

Transmission was not observed in the first two treatments where water was used to wash virions from unwounded and wounded plants (Table 2). When macerated infectious leaf material was added to the water, the virus was detected from roots of 42.9% of the uninfected plants. No virus was detected in the shoots of uninoculated plants. Roots of plants in both tanks became infected, showing that transmission in water occurred up to 1.9 m.

Virion stability

Plants were inoculated with 100 mg of lyophilised or fresh infected leaf tissue. Both established infections in *N. benthamiana* plants with symptom severity indices of 2.83 (± 0.41) and 3.33 (±0.52), respectively. RT-qPCR showed that infections derived from lyophilised tissue had 6.65 x 10^{10} ng/µl CP per 100 mg fresh leaf weight and those from fresh inoculum had 1.11 x 10^{11} ng/µl per 100 mg.

The effect of temperature on YTMMV infectivity

Infected leaf materials remained infectious when incubated at -80ºC, 22ºC and ambient temperatures (ranging from 0.8ºC to 44.4ºC) for one year (Figure 2a). Mean symptom severity indices corresponding to these temperatures were 2.83 (± 0.58), 2.75 (± 0.62) and 2.17 (± 1.38), respectively.

Lyophilised infected leaf material stored at 55ºC for 30 d, 90 d, 180 d and 365 d became less virulent over time. Mean symptom severity indices were 3.42 (± 0.51), 1.64 (± 1.69), 0.50 (±1.17) and 0 after storage for 30 d, 90 d, 180 d and 365 d, respectively (Figure 2a).

Ordinal regression of symptom severity index was done for temperature (-80ºC, 22ºC, ambient and 55ºC) and incubation time for plants inoculated with lyophilized plant tissues. Analysis showed that the only significant difference in the symptom severity indices between treatment temperatures was between -80ºC and 55ºC (Figure 2b). However, the effect of incubation time was highly significant and had a slightly negative coefficient value, indicative of reduced symptom severity with increased incubation time, notably when inoculum was incubated at -80ºC. Non-significant main effects for most inoculum incubation temperatures suggest no significant differences in symptom severity index at baseline. Non-significant time-temperature interaction effects at 22ºC and at ambient temperature suggest effects of incubation time for these temperatures are not dissimilar to those observed for -80ºC. However, the highly significant and negative time-temperature interaction effect corresponding to 55ºC (Figure 2b) indicates significantly and increasingly lower symptom severity index with greater inoculum incubation period.

A comparative analysis of YTMMV titre of plants infected with inoculum incubated for a year at various temperatures was done. CP copy number in plants inoculated with inoculum stored for one year at -80°C, 22°C and ambient temperature was 5.01×10^{10} ($\pm 4.95 \times 10^{9}$), 2.13×10^{10} ($\pm 3.91 \times 10^{9}$) and 6.90×10^{9} ($\pm 2.81 \times 10^{9}$) ng/ µl per 100 mg of fresh leaf weight, respectively. Plants inoculated with inoculum incubated at 55ºC for a year did not become infected, indicating that this treatment inactivated virions.

Effect of storage at 160ºC on infectivity

Incubations of lyophilized inoculum were done at 160ºC for 1, 3, 6, 24, 48, 72, 96, 120 and 144 h. Infectivity was not significantly different from positive controls (fresh inoculum) after incubation for 24 h, and the mean symptom severity index decreased with incubation time to 144 h, where the symptom severity indices were 0 (Figure 3a).

An ordinal regression analysis of symptom severity index at 20 dpi on hours of incubation of inoculum at 160ºC showed that the symptom severity indices declined rapidly over time, as shown by the highly significant and negative coefficient for incubation time (Figure 3b).

Milk and NaOH treatments

Inoculum remained infectious for up to 30 min after treatment by immersion in either 20% w/v skim milk solution or 0.1 M NaOH, although infectivity decreased over the period tested (Figure 4a).

An ordinal regression of symptom severity index suggests there was no significant difference between milk and NaOH treatments with regard to symptom severity index at baseline, based on the non-significant main effect corresponding to NaOH (Figure 4b). However, as incubation time increased, symptom severity index decreased slightly for both milk and NaOH treatments, as evidenced by the statistically significant main effect for incubation time and non-significant interaction effect. The fairly low p-value corresponding to the interaction effect (p-value = 0.0621) may be indicative of a slightly greater efficacy of NaOH in reducing infectivity with greater incubation time. When compared to the positive control (untreated inoculum), both skim milk and NaOH treatments were significantly more effective, as seen by the positive control's significantly higher symptom severity index at baseline (p-value < 0.001 and highly positive coefficient) and similar trajectory based on incubation time (p-value of 0.7347) (Figure 4b).

Discussion

Like other tobamoviruses, YTMMV is readily transmitted between plants ~~by leaf contact~~. It was surprising to discover it was not efficiently transmitted through root contact in either water or in soil. Although care was taken to avoid detection of viruses adhering to the

external surfaces of roots (washing and choice of root material), it is possible that contamination by externally-adhering virions occurred. In water it was transmitted between plants only when the amount of virus in the water was deliberately increased by periodically adding large amounts of inoculum in the form of macerated fresh leaves to it. Park et al (1999) found that transmission of TMV to tobacco, tomato and capsicum growing in a hydroponics system occurred only when roots of the inoculum source self-grafted to those of healthy plants. Li et al. (2015b), studied the spread of CGMMV between watermelons growing in soil under flow and drip irrigation. They found that irrigation did aid spread of the virus, but they did not check whether root grafting was a pre-requisite for transmission. In the present study, the roots of the inoculum source were not allowed to self-graft with roots of healthy plants, and the virus was not transmitted under conditions where virions were presumably washed from wounded and unwounded leaves. However, when inoculum concentration in the water was deliberately increased, infection of healthy plants occurred. The influence of increased virus concentration affecting efficiency of transmission was also shown by Mehle et al (2014) using a potyvirus, a potexvirus and a viroid. Thus, it seems unlikely that YTMMV will cause epidemics if it occurred in commercial hydroponics systems through infection of roots of healthy plants *via* water-borne virions, unless they are present in high concentrations. The risk of transmission could be lessened by removing visibly infected plants before they die and leach particles into the solution.

In soil, roots of plants growing closely together often make contact with one another, and the abrasive action of the soil on roots as they push through it may provide opportunities for virus particles to enter cells. However, transmission of YTMMV between plants by this route occurred uncommonly under our experimental conditions. Surprisingly, in the few cases where the virus was transmitted *via* the roots in soil or water media, the shoots of the plants did not become visibly symptomatic for the 45 days in which plants were observed. These

results should be taken with caution because symptoms may appear over a longer time period and be differentially expressed in other host species. In situations where seedlings are transplanted, root damage inevitably occurs, and these damaged roots may be more vulnerable to virus infection. TMV was shown not to move through the xylem in roots to that of shoots in tobacco and tomato (Caldwell, 1931, Caldwell, 1934) even when it was introduced directly to the xylem (Caldwell, 1931). In red spruce (*Picea rubens*), the tobamovirus tomato mosaic virus (ToMV) was efficiently transmitted by contact with underground roots to 80% of seedlings, but the virus was detected in the shoots of ~~only~~ 7% of root-infected plants (Bachand and Castello, 1998). Thus, there seems to be a barrier to efficient movement of some tobamoviruses from roots to shoots in at least some host species. It would also be useful in the future to assay for soil contamination in roots by comparing washed root samples with unwashed root samples and measure the virions present.

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Virion viability experiments revealed that like other tobamoviruses, YTMMV virions are highly resilient. Dried leaf tissue harboured infectious virions for at least one year at ambient temperatures, -80 ºC and 22ºC. Treatment with milk solution and alkaline NaOH solution, both compounds previously described as being effective for decontaminating tools of tobamoviruses (Nitzany, 1960, Hu et al., 1994, Kamenova & Adkins, 2004, Lewandowski et al., 2010, Li et al., 2015c), were largely ineffective for YTMMV. Dry heating of infected leaf materials was an effective phytosanitary measure, but only after exposure to 160ºC for five days.

In most cases, all inoculated *N. benthamiana* plants died from symptoms of infection, but symptom expression was delayed by some treatments, presumably because the titre and infectivity of inoculum was affected by the treatment. Furthermore, symptom progression was not always predicted by RT-qPCR results, indicating the presence of unviable virions

might nevertheless provide templates for RT-qPCR. The same observation was made by Lewandowski et al (2010) where inoculation with different concentrations of TMV inoculum resulted in different host responses in *Petunia* plants. Such differences may also reflect the relative efficiencies of manual inoculation between plants. Local lesion host response may provide a measure of inoculum strength (Koh et al., 2017).

The stability of YTMMV was demonstrated across a range of storage temperatures for up to a year. Indeed, it seems probable that YTMMV virions are able to survive for far longer than a year in dried plant material, although this was not tested. TMV remained infectious in compost for six months and in vegetables for nine months (van Dorst, 1969). ToMV RNA collected from Greenland's glacial ice cores was approximately 140,000 years old, although the researchers did not confirm infectivity (Castello et al., 1999). Other researchers found that different strains of TMV exhibited differential resilience over time, with some strains predicted to remain infectious for 30 years in lyophilised leaves (Yordanova et al., 2005). They incubated viruliferous lyophilised leaf material containing TMV-B (which was the most robust strain tested) at 28ºC, 37ºC and 45ºC and found that it remained infectious for > 120 d, ~95 d and ~ 30 d for each respective temperature (Yordanova et al., 2005). In comparison, the isolate of YTMMV tested here was more resilient than TMV-B because it remained infectious at 55ºC for 180 d.

Both 0.1 M NaOH and 20% w/v skim milk were ineffective at decontaminating YTMMV-infected tools when incubating for 30 min. Milk treatment was reported to completely inactivate Hibiscus latent Fort Pierce virus (HLFPV) (Kamenova & Adkins, 2004), TMV (Lewandowski et al., 2010, Li et al., 2015c) and ToMV (Li et al., 2015c), but was ineffective in some cases with TMV (Crowley, 1958, Denby & Wilks, 1963). Milk contains casein and whey, proteins that inactivate virions (Hagborg & Chelack, 1960). Milk proteins may inhibit

viral enzymes (Ng et al., 2001) or aggregate with virions (Hu et al., 1994). Milk may inhibit binding of viral proteins to target receptors in the host. In human virus studies, lactoferrin in milk inhibited the binding of human immunodeficiency virus to the CD4 receptor (Newburg et al., 1992). Lactoferrin completely inhibited replication of tomato yellow leaf curl virus (a begomovirus), but the mechanism for this is unknown (Abdelbacki et al., 2010).

Sodium hydroxide was slightly more effective than milk. As an alkaline solution, sodium hydroxide denatures nucleic acids (Lehninger, 1975, Ma et al., 1994). Incubation of inoculum in 0.1 M NaOH for at least 30 min did not completely inactivate YTMMV, possibly because virions and/or plant cell walls protected the genome from exposure to the denaturant. NaOH treatment inactivated Odontoglossum ringspot tobamovirus (Hu et al., 1994) and TMV (Nitzany, 1960, Milinkó, 1966), while others reported that it was ineffective for TMV (Choi et al., 1999) and HLFPV (Kamenova & Adkins, 2004).

Dried leaves were used here to test the stability of virions over time. In agriculture and in nature, tobamoviruses may be harboured in the dried dead leaves of infected plants, from which they may become foci of infection. Dried leaves may also act as vehicles in which tobamoviruses are spread over distance by air currents (Sarra et al., 2004).

An understanding and knowledge of the infectivity and transmission of YTMMV will be beneficial in the development of control strategies against YTMMV, should the virus ever 'emerge' to become a pathogen of solanaceous crops. ~~Because of its apparently greater resilience to commonly adopted sanitisation procedures used against other tobamoviruses, YTMMV may serve as a 'worst-case scenario' against which to test tobamovirus sanitisation procedures.~~

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401

402 **Conflict of Interest** The authors do not have any conflict of interest to disclose.

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Figure 1 Layout of hydroponics experiment showing the position of the pots (white circles) in the hydroponics tanks connected by tubing.

Figure 2 (a) Graph of symptom severity indices over time (in days) for inoculum incubated at -80ºC, 22ºC, 55ºC and ambient temperature. Grey symbols in the background represent raw data indicative of the range of severity indices observed for a particular incubation temperature. (b) Ordinal regression of symptom severity indices on temperature (-80ºC, ambient, 22ºC, 55ºC) and incubation time (in days) for plants inoculated with YTMMV viruliferous plant tissues.

Figure 3 (a) Graph of symptom severity indices over time (hours) for inoculum incubated at 160ºC. Grey symbols in the background represent raw data. (b) Ordinal regression of symptom severity indices at 20 dpi on incubation time (in hours) at 160ºC for plants inoculated with YTMMV viruliferous plant tissues.

Figure 4 (a) Graph of symptom severity indices of plants infected with inoculum incubated in inoculation buffer (positive control), 0.1 M sodium hydroxide and 20% w/v skim milk treatments over time (in min). (b) Ordinal regression of symptom severity indices at 20 dpi on plants incubating in inoculation buffer (positive control) and inactivation agents (0.1 M NaOH, 20% w/v milk) and incubation time for plants inoculated with YTMMV viruliferous plant tissues.

Table 1 Description of the treatments used to investigate virion resilience.

Table 2 Results of the YTMMV water transmission experiments. Presence (+) or the absence (-) of the viruses in the leaves and roots of the plants were recorded.

~~**Table 3** Mean symptom severity index of treated *N. benthamiana* RA 4 plants with known decontaminants.~~

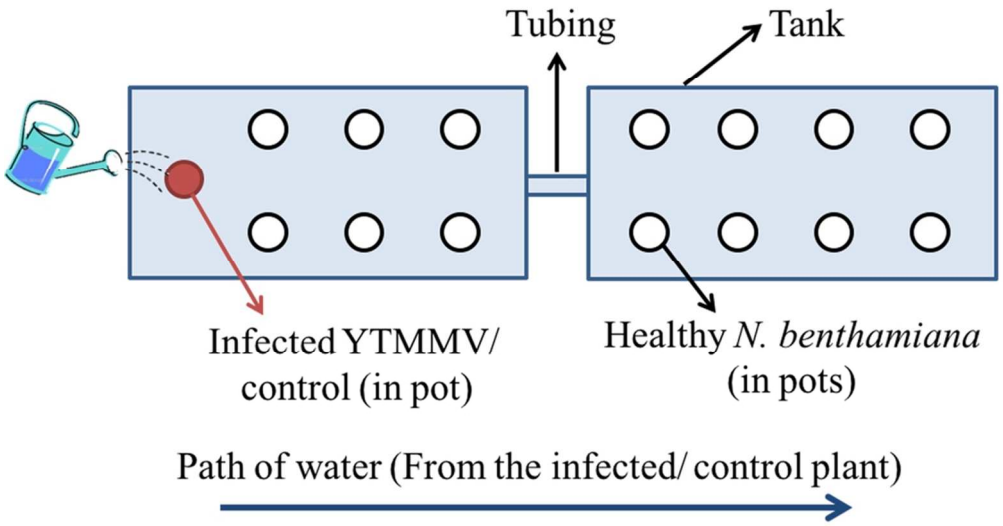
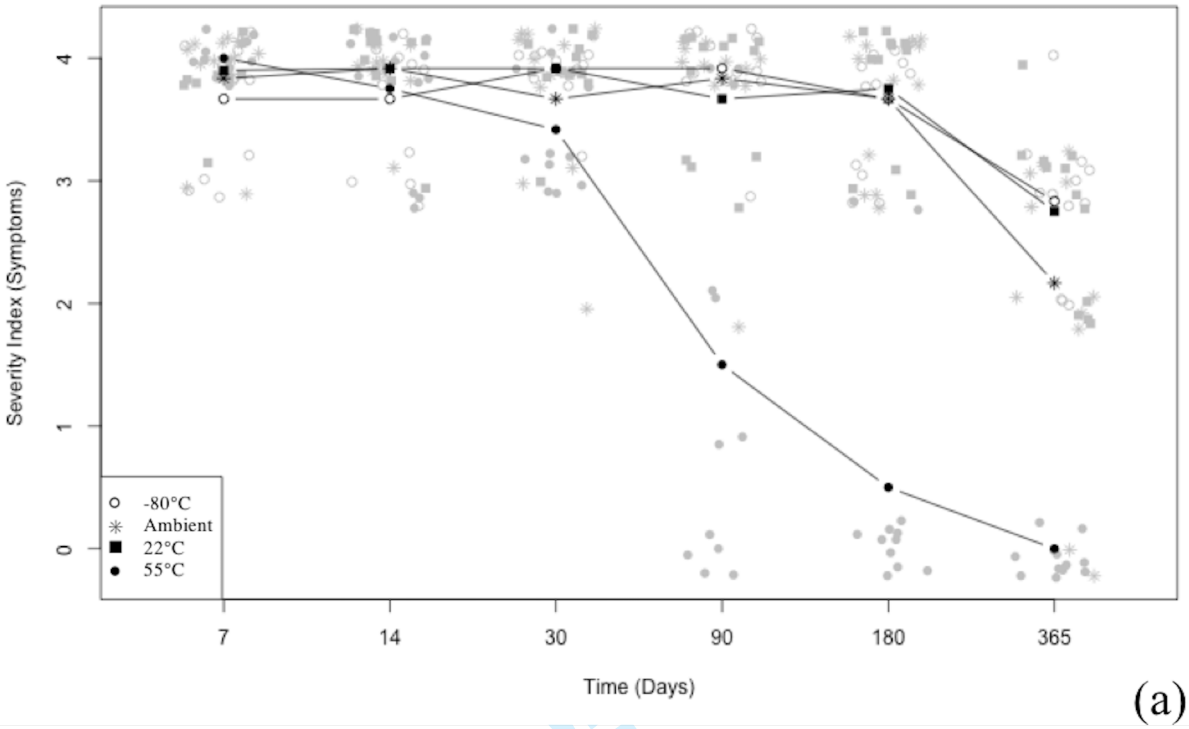


Figure 1 Layout of hydroponics experiment showing the position of the pots (white circles) in the hydroponics tanks connected by tubing.

91x50mm (300 x 300 DPI)

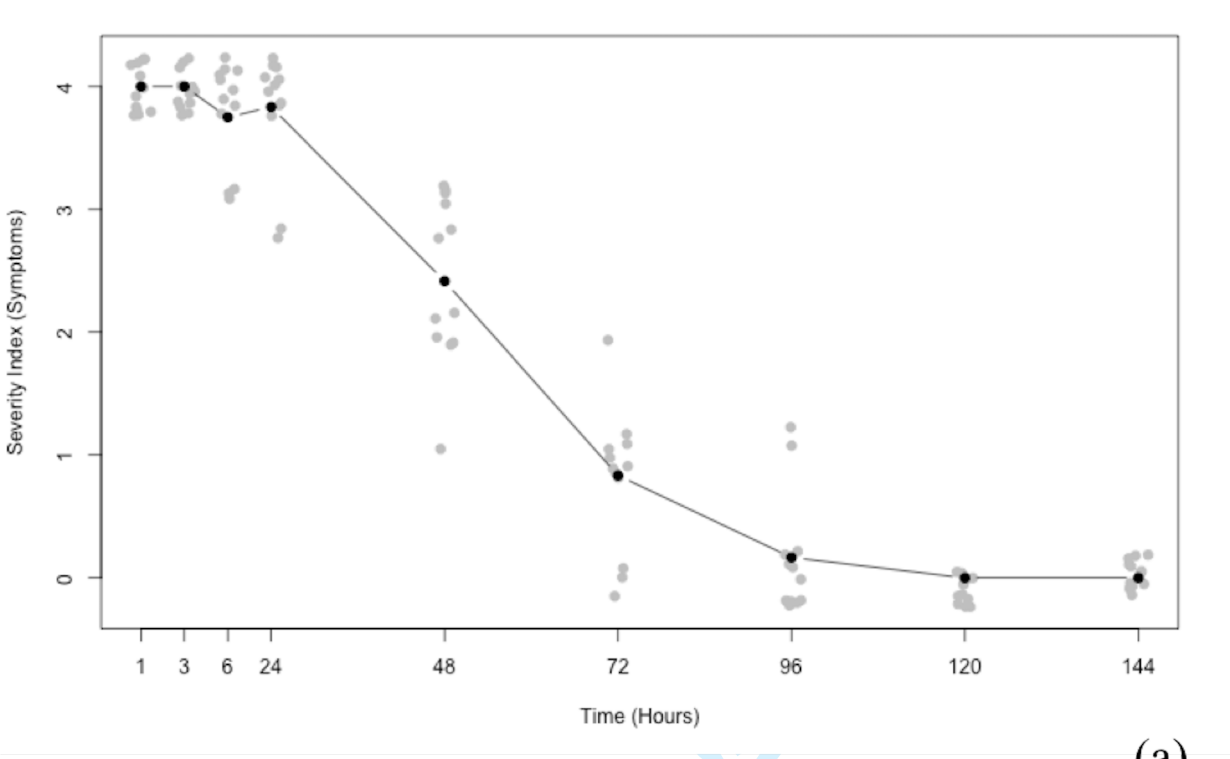


(a)

Coefficients	Estimate	Std. Error	Z value	Pr (>|z|)
Temperature (Ambient)	0.6838	0.6100	1.121	0.262
Temperature (22ºC)	0.7595	0.6249	1.215	0.224
Temperature (55ºC)	0.0387	0.5855	0.066	0.947
Time	-0.0081	0.0020	-4.105	4.05e-05 ***
Time * Temperature (Ambient)	-0.0046	0.0028	-1.630	0.103
Time * Temperature (22ºC)	-0.0032	0.0029	-1.114	0.265
Time * Temperature (55ºC)	-0.0410	0.0081	-5.065	4.09e-07 ***

Note: Temperature was treated as a dummy variable with coefficients interpretable with reference to a baseline temperature of -80ºC
Significance codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

(b)

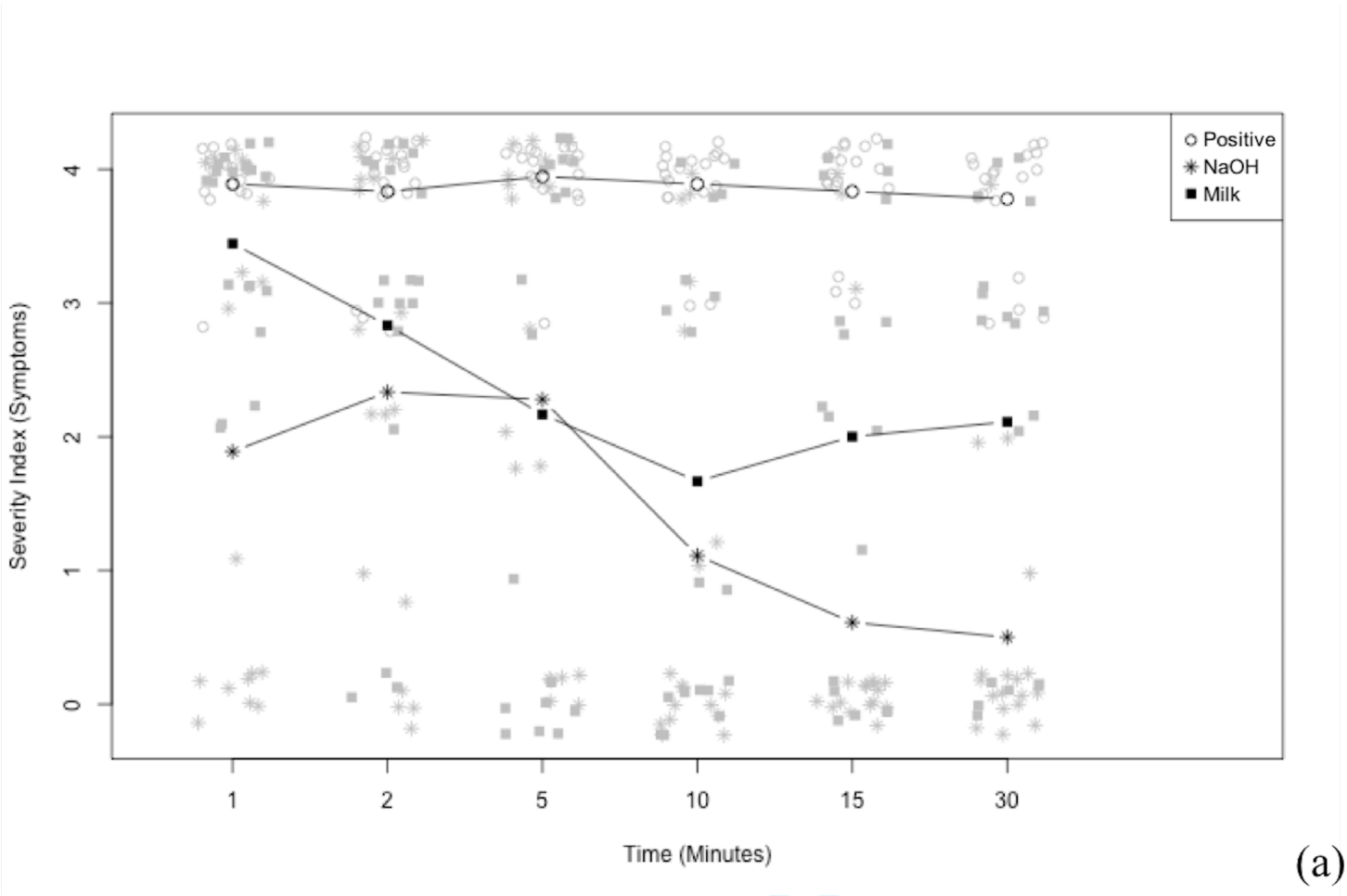


(a)

Coefficient	Estimate	Std. Error	Z value	Pr (>|z|)
Time	-3.5322	0.5187	-6.809	9.82e-12 ***

Significance codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

(b)



(a)

Coefficients	Estimate	Std. Error	Z value	Pr (>|z|)
Inactivation agent (NaOH)	-0.5099	0.3750	-1.360	0.1738
Positive control	2.3659	0.5002	4.730	2.25e-06***
Time	-0.0372	0.017	-2.139	0.0325 *
Time * Inactivation agent (NaOH)	-0.0553	0.0297	-1.865	0.0621 .
Time * Positive control	0.0105	0.0309	0.339	0.7347

Note: Inactivation agent was treated as a dummy variable with coefficients interpretable with reference to 20% w/v milk.
Significance codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

(b)

Table 1 Description of the treatments used to investigate virion resilience.

Experiment	Treatment of infected leaf material/ contaminated mortar	Incubation duration	Analysed by
Effect of temperature on YTMMV infectivity	(i) Lyophilised and subjected to -80ºC (± 2ºC), 22ºC (± 3ºC), 55ºC (± 2ºC) and ambient temperature (0.8ºC to 44.4ºC)	1, 2, 4, 12, 26 and 52 weeks	Symptom severity index and RT-qPCR (20 dpi)
	(ii) Lyophilised and subjected to 160ºC (± 2ºC)	1, 3, 6 and 24 hours, 2, 3, 4, 5 and 6 d	
Effect of reported virus inactivators on YTMMV infectivity	(i) 0.1 M sodium hydroxide (NaOH)	1, 2, 5, 10, 15 and 30 min	Symptom severity index (20 dpi)
	(ii) 20% w/v skim milk		
	(iii) Diluted Dettol hand wash (with water)	1, 5 and 10 min	Symptom severity index and RT-PCR (20 dpi)

Table 2 Results of the YTMMV water transmission experiments. Presence (+) or the absence (-) of the viruses in the leaves and roots of the plants were recorded.

Experiment	Viruses in leaves	Viruses in Roots
Watering on infected plant	-	-
Watering on wounded infected plant	-	-
Applying 500 mg of macerated infectious leaf materials in watering water every other day	-	+ (42.9%)

Table 3 Mean symptom severity index of treated *N. benthamiana* RA-4 plants with known decontaminants.

Inoculum treatment	Virus-positive	Mean severity index (± s.d)
Incubating in 0.1 M sodium hydroxide (NaOH)		
- 1 min	+	1.89 (± 1.88)
- 2 min	+	2.33 (± 1.64)
- 5 min	+	2.28 (± 1.81)
- 10 min	+	1.11 (± 1.64)
- 15 min	+	0.61 (± 1.42)
- 30 min	+	0.5 (± 1.10)
Incubating in 20% w/v skim milk		
- 1 min	+	3.44 (± 0.78)
- 2 min	+	2.83 (± 1.42)
- 5 min	+	2.17 (± 1.92)
- 10 min	+	1.67 (± 1.75)
- 15 min	+	2.0 (± 1.68)
- 30 min	+	2.11 (± 1.641)