THE UNIVERSITY OF NOTTINGHAM

Development and Deployment of Genotype-Specific LAMP Assays for Monitoring *Pepino mosaic virus* (PepMV) in Tomato

Thesis submitted to The University of Nottingham for the degree of Masters of Research

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Abstract

Tomato (*Solanum lycopersicum*) is regarded as one of the most important vegetable crops worldwide, being cultivated across the globe with a total yield of approximately 162 million tonnes in 2012. *Pepino mosaic virus* (PepMV) is currently the most threatening pathogen to global commercial tomato production. The *Potex-virus* was first identified on pepino plants in Peru in 1974. PepMV was initially discovered in Europe in 1999 on greenhouse tomato plants in the Netherlands and has since spread worldwide. The virus induces a wide range of symptoms in tomato plants including leaf mosaics, yellow rectangular leaf spots, leaf necrosis, fruit marbling and fruit flaming. The fruit symptoms exhibited by PepMV-infected crops often lead to reductions in the marketability of the fruit. PepMV infection can also be symptomless, making disease diagnosis difficult. Currently, six PepMV genotypes have been characterised; the European (EU) strain, the original Peruvian (LP) isolates, the North American (US1/CH1) strain, the Chilean-2 (CH2) strain and the PES strain recently discovered in wild tomatoes in Peru. The CH2 genotype is the dominant genotype found in PepMV-infected tomato crops in Europe. Mixed-infections with multiple PepMV genotypes can also be observed. Mixed-genotype PepMV infections often induce more severe disease symptoms.

PepMV is highly contagious, being spread easily via mechanical means, and is able to survive on glasshouse surfaces, tools, hands and clothes. This means that the hands-on practices required for tomato crop production can result in the rapid dissemination of the virus around the glasshouse environment and to other glasshouses to infect other crops. It is essential that tomato growers are able to identify PepMV infection in their crops to allow for the implementation of strict hygiene protocols to prevent the spread of PepMV to uninfected crops.

Loop-mediated isothermal amplification (LAMP) can be used for plant pathogen detection. This study aimed to validate genotype-specific RT-LAMP primers designed by Ling *et al*. (2013) for the detection of the CH2, EU and US1 genotypes of PepMV. After successful validation, the primers were used to test tomato leaf and fruit samples collected from six crops from four UK tomato sites in order to determine the distribution and occurrence of mixed-genotype PepMV infection in the UK. RT-LAMP tests revealed that PepMV infection was widespread in the crops assessed, with PepMV being detected on all sites. The CH2 genotype was found in single infection in over 60% of the samples tested and mixed-genotype infection was detected in approximately 20% of plants assessed. The

symptoms observed were variable between the crops and symptomless infection also existed.

The second half of this study aimed to identify sources of PepMV inocula within the glasshouse. Firstly, glasshouse surfaces and equipment from three UK sites were swabbed before and after end-of-season glasshouse cleanups. The pre and post-cleanup swabs were tested using RT-LAMP for the presence of PepMV in order to assess the efficacy of end-of-season cleanups at eliminating PepMV. Seventy six %, 86% and 98% of the pre-clean swabs tested positive for PepMV. Twenty %, 44% and 68% still remained PepMV-positive at Sites 1, 2 and 3, respectively, after end-of-season cleanups. Despite positive results for the post-cleanup swabs being obtained with RT-LAMP, sap inoculation studies revealed that the PepMV detected by RT-LAMP was not viable, indicating that the end-of-season cleanups conducted were successful at eliminating viable PepMV.

Secondly, the survival of PepMV in composted tomato waste was monitored. A PepMV-infected tomato crop was chipped and subjected to composting. Compost samples were collected at monthly and then weekly intervals over an approximate sixteen week composting period and tested for the presence of PepMV using RT-LAMP. Samples taken from the chipped crop and stored in a 24°C incubator for the duration of the investigation were used as control samples. The control samples were sampled and tested with RT-LAMP at the same time as the compost samples in order to determine the effect of composting on PepMV. The RT-LAMP amplification times were used as an indication of the level of PepMV within the samples. Results showed that the level of PepMV within the samples did decrease over time and the average level of PepMV in the control samples was greater than that in the compost samples. However, the rate of PepMV degradation did not differ significantly between the compost samples and controls, suggesting that PepMV degrades overtime, irrespective of whether or not the virus is subjected to composting.

Finally, water samples from three UK tomato nurseries were collected and concentrated, using a method developed at the National Institute of Biology (NIB) Slovenia, in order to allow for the detection of PepMV using RT-LAMP. PepMV was detected in 50% of the water samples collected, showing that the virus can survive and be transported in water/nutrient solutions. Glasshouse irrigation systems may therefore aid the dissemination of PepMV between crops, particularly if the water is recirculated and is used to irrigate multiple glasshouses.

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Chapter 1: Introduction

1.1 Tomatoes: History, Origin and Domestication

Tomatoes (*Solanum lycopersicum*) are a member of the Solanaceae family, which represents the third most economically important plant family in terms of vegetable crops. The Solanaceae family contains over three thousand plant species, including other important vegetable species such as eggplant (*S. melongena*) and potato (*S. tuberosum*) (Wu and Tanksley, 2010). The *Lycopersicon* genus is comprised of the domesticated tomato (*S. lycopersicum*) and its twelve most closely related wild relatives (Bauchet and Causse, 2012). Vegetables that belong to the Solanaceae family are consumed worldwide due to their richness in compounds that are beneficial to health. Lycopene, for example, is a red pigment that increases in concentration in tomatoes as they ripen. This pigment has been found to have anticarcinogenic properties in vitro and in vivo by acting as a free radical scavenger, helping to protect against oxidative damage (Friedman, 2013).

Tomato and its wild relatives originated along the coast and in the high Andes in South America, spanning from central Ecuador to Peru, Bolivia and northern Chile (Peralta and Spooner, 2006). The ancestor of the big-fruited cultivated tomato is thought to be the cherry tomato (*S. lycopersicum* var. *cerasiforme*), which is widespread through warm regions of the world and was likely domesticated from the wild species *S. pimpinellifolium* (Ranc *et al.*, 2008). The origin of tomato domestication is unclear. One hypothesis supports Peru as the country of first domestication; however, the majority of evidence suggests Mexico as the most likely origin (Bauchet and Causse, 2012). After domestication, the cultivated forms of tomato spread across the globe.

Tomatoes were introduced into Europe in the sixteenth century (Jenkins, 1948), at first only as ornamental garden plants as they were thought to be inedible or poisonous. In the late sixteenth/early seventeenth century, tomatoes were accepted for use as food (Peralta and Spooner, 2006). The migration and domestication of tomatoes, involving adaptation to the Northern hemisphere and human selection for desirable crop characteristics, resulted in decreased genetic variability of the tomato genome. Such selection and introgression to allow for crop improvement, therefore, ultimately led to the restriction of further crop improvement through conventional breeding (Lin *et al.*, 2014).

1.2 Tomato Production and Economic Importance

Tomato is regarded as the world's leading vegetable crop, being cultivated worldwide with a global yield of 162 million tonnes in 2012 (FAOSTAT, 2015). Asia is the greatest contributing region to tomato production, with the average production from this region constituting 60.7% of global tomato production in 2013 (Figure 1.1). China dominates globally for tomato production, with approximately 5.7 million tonnes of tomatoes produced in 2013; however, European countries such as the United Kingdom are actually global leaders in terms of yield. The total tomato yield in the UK was over 4 million Hg/Ha compared to just over 500,000 Hg/Ha in China in 2013 (Figure 1.2, FAOSTAT 2015).

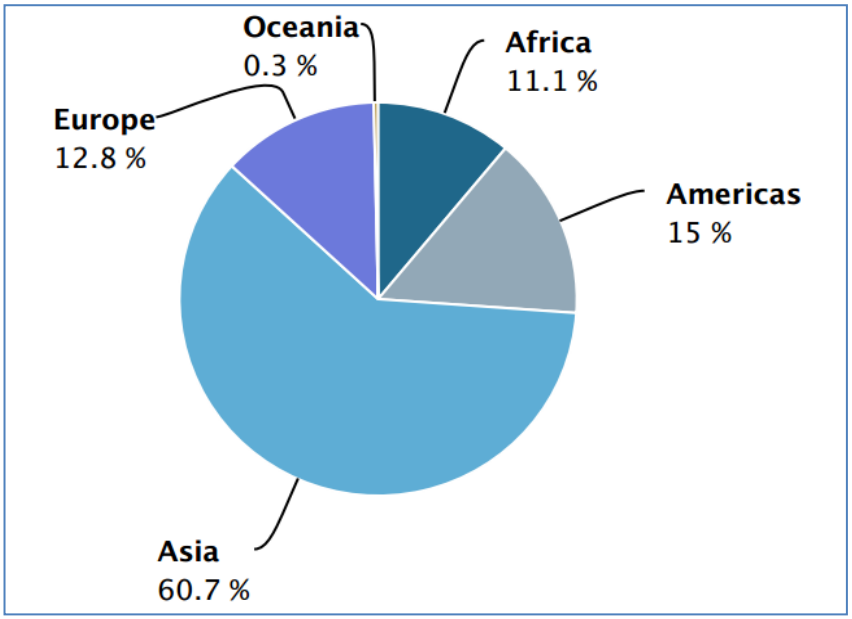

Figure 1.1- The proportion of worldwide tomato production per region (FAOSTAT, 2015).

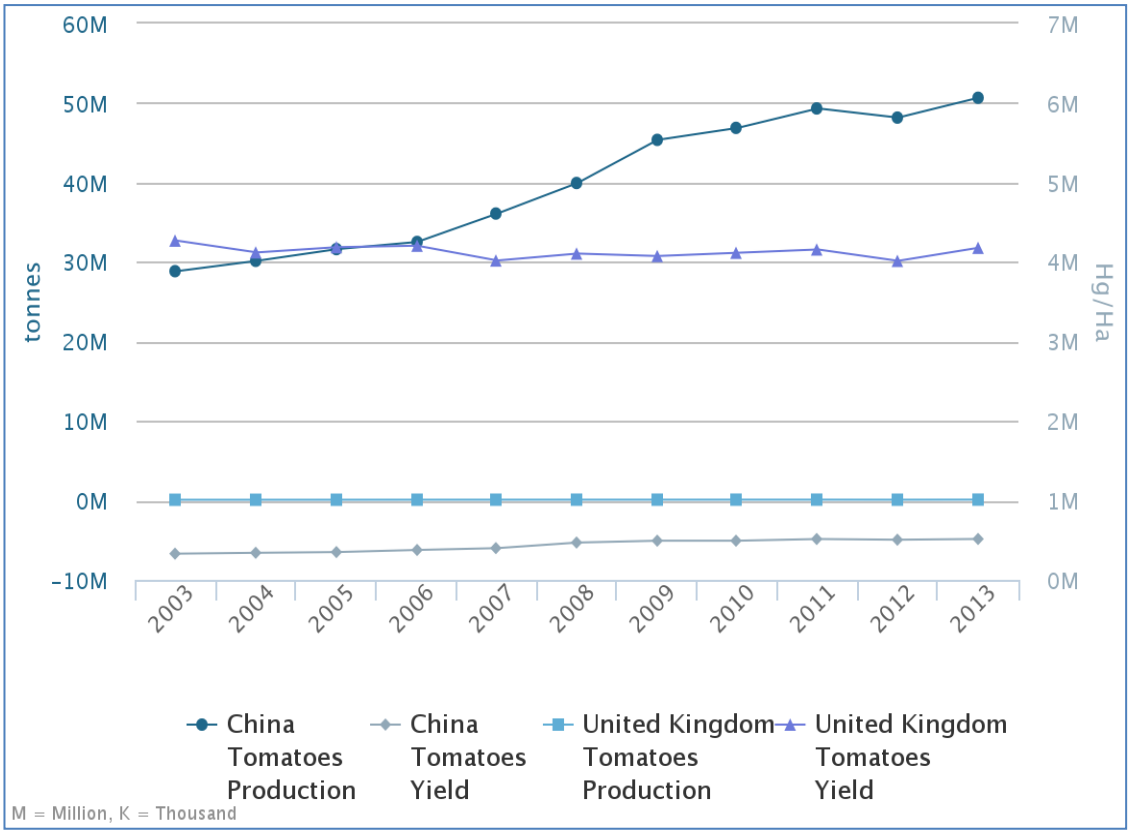

Figure 1.2- Comparison of tomato production and tomato yield from 2003 to 2013 in China and the United Kingdom (FAOSTAT, 2015).

1.2.1 Tomato Production in the UK

Tomatoes can be grown in a variety of ways including in the field and under protection in plastic tunnels/greenhouses or glasshouses. Growing tomatoes in glasshouses allows for the manipulation of the climate to favour crop production and the type of protected cultivation used, combined with technical expertise, can often explain yield differences such as that seen between China and the UK in Figure 1.2 (Peet and Welles, 2005). Growth in glasshouses means that tomato production does not have to be restricted to regions where conditions, such as light intensity, are optimal for tomato growth.

Almost all tomatoes in Britain are grown in glasshouses, with the British tomato glasshouse industry presently covering an area of 200 hectares. The largest tomato glasshouse in the UK covers 10.7 hectares and is currently being

extended to a size of 18 hectares. The retail value of British tomato production is approximately £175 million, with premium tomatoes, such as those on the vine, accounting for half of the UK production area. The UK also imports tomatoes from Spain and the Canary Islands, Holland, Morocco, Poland, Italy, Belgium and Israel (The British Tomato Growers' Association, 2015).

Glasshouse production of a crop is more expensive than growing the crop in the field due to degradation of structure and equipment and labour and energy costs (Peet and Welles, 2005). For this reason, commercial tomato growers have had to increase their production intensity in order to remain competitive in the market. One method adopted to maximise output is soilless crop production, also known as hydroponics, which has become increasingly popular in recent decades. Hydroponics replaces soil with inert, porous materials such as rockwool, perlite and pumice (Savvas, 2003). The main incentive for the shift from soil to soilless production was the elimination of soil-borne pathogens which pose a problem in intensively cultivated glasshouses (Raviv and Lieth, 2008). Furthermore, hydroponics supplies plants with a nutrient solution that can be specifically tailored to enable optimum growth of individual plants. This aids in maximising output and improving the quality of the crop.

One example of a hydroponic technique is the nutrient film technique (NFT), which was developed in the UK and is utilised by a number of tomato growers (The British Tomato Growers' Association, 2015). NFT uses a support medium (such as rockwool) to anchor the plants which are then "fed by a moving film of nutrient solution" (Martin *et al*., 1994). NFT reduces the application of pesticides and other chemicals required for soil-grown crops. Additionally, the nutrient solution in NFT is recirculated, eliminating the release of nitrate and phosphate into environmental waters (Savvas, 2003).

1.3 The Origins of *Pepino Mosaic Virus* (PepMV)

Pepino mosaic virus (PepMV) is currently one of the most threatening pathogens to tomato production worldwide (Minicka *et al.*, 2015). PepMV was first discovered in 1974 in Peru on pepino plants (*S. muricatum*) which were displaying yellow leaf mosaics. The emergence of PepMV in Europe was first found to be on greenhouse tomato crops (*S. lycopersicum*) in the Netherlands in 1999 (Van der Vlugt *et al.*, 2000). Since then, the virus has spread throughout

Europe, being reported in many European countries including Spain, Italy, France, the UK, Poland and Belgium (Roggero *et al.*, 2001; Pospieszny and Borodynko, 2006; Hanssen *et al.*, 2009). Outside of Europe, PepMV has also been described in the USA, Canada (French *et al.*, 2001), Chile and China (Zhang *et al.*, 2003). More recently, PepMV has been found in Mexico (Ling and Zhang, 2011), Greece (Efthimiou *et al.*, 2011) and South Africa (Charmichael *et al.*, 2011). This global distribution explains why PepMV is poses such a threat to commercial tomato production worldwide.

1.4 Genome Organisation of PepMV

PepMV is a member of the genus *Potex-virus*, in the family *Alphaflexiviridae*. PepMV is a single-stranded, positive-sense RNA virus composed of five open reading frames (ORFs, Figure 1.3), constituting a total length of 6410 nucleotides. ORF1 encodes an RNA dependent RNA polymerase (RdRp), an enzyme responsible for the replication of the RNA genome into messenger RNA. ORF2-4 are partially overlapping and constitute the PepMV triple gene block (TGB). The TGB codes for three proteins required for intercellular viral movement within plants (Morozov and Solovyev, 2003). ORF5 encodes a coat protein (CP) which plays a structural role and has some involvement in cell-to-cell movement within plants (Cruz *et al.,* 1998). The CP has also been found to participate in protein-protein interactions with plant host factors such as the tomato heat shock protein cognate 70 (Matthaios *et al.*, 2012). Intergenic regions (IR) are present between ORF1 and ORF2 (IR2) and between ORF4 and ORF5 (IR2). The sequence of IR2 exhibits high variability between different PepMV strains. At the 5’ end of the virus there is an untranslated region that is 85 nucleotides in length and begins with the pentanucleotide GAAAA which is characteristic of potexviruses (Van der Vlugt, 2009). There is also a poly(A) tail located at the 3’ end of the RNA strand (Cotillon and Ducouret, 2002).

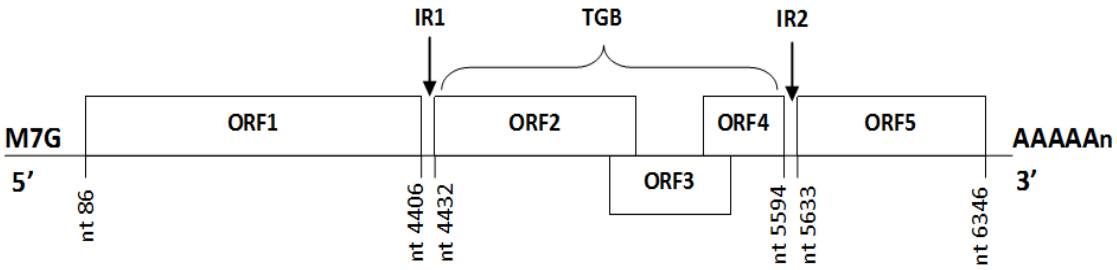

Figure 1.3- Schematic representation of the PepMV RNA genome showing the 5' cap (M7G), the five open reading frames (ORF1-5), the triple gene block (TGB), the intergenic regions (IR1 and IR2) and the polyA tail ($AAAAA_n$) (adapted from Van der Vlugt, 2009).

RNA viruses are known to have high mutation rates due to the lack of proofreading activity by the RdRp that replicates the RNA genomes, resulting in an estimated mutation rate of between 0.4 and 1.1 nucleotides mutated per genome per round of replication (Hasiów-Jaroszewska *et al.*, 2010). The high mutation rate creates populations of RNA viruses that are genetically heterogeneous. Such viruses are referred to as a quasispecies, which describes a virus population that infects a single host as an assortment of phylogenetically related, yet genetically different variants (Hasiów-Jaroszewska *et al.*, 2010). The presence of genetically different variants within the virus population confers a strong adaptive potential in response to environmental changes, where the variant most suitably adapted to a particular environment or host would be selected for.

1.5 PepMV Genotypes and Mixed-Genotype Infections

Currently, PepMV isolates have been characterised into six genotypes; the European (EU) isolates, the original Peruvian (LP) isolates, the North American (US1/CH1) isolates, the Chilean-2 (CH2) isolates and the PES strain recently discovered in wild tomatoes in Peru (Blystad *et al.*, 2015; Davino *et al.*, 2016). The initial isolates of PepMV which had spread throughout Europe were found to be highly genetically similar (99%) and so were termed the European (EU) strain. The original PepMV isolate found in Peru on pepino, named SM.74, among

other isolates found in Peru, comprise the LP strain to which the EU isolates show 96% genetic similarity (Moreno-Pérez *et al.*, 2014). Maroon-Lango *et al.* (2005) reported the discovery of two unique isolates in the United States (US1 and US2), showing only 80% sequence similarity with the EU and LP strains. Ling (2007) completed the genome sequencing of two other variants of PepMV (CH1 and CH2) isolated from a tomato seed lot produced in Chile. Further analyses of the US and Chilean isolates revealed that there was possible recombination between the isolates (Hanssen *et al.*, 2008), with regions of the US2 sequence resembling the CH2 sequence and the US1 sequence (Van der Vlugt, 2009).

In Europe, the once dominant EU genotype appears to have been overtaken by the CH2 genotype in commercial tomato production. This genotype shift was first discovered in Belgium by Hanssen *et al.* (2008), who found that the CH2 genotype was present in 85% of the greenhouses containing tomato crops involved in the study. Before the Hanssen *et al.* (2008) study, the CH2 genotype had not been detected in commercial tomato production in Europe. The appearance of the shift to the CH2 genotype has now been reported in Poland and France (Hanssen *et al.*, 2009), and outside of Europe in North America (Ling *et al.*, 2013). The shift from the EU to CH2 genotype suggests that the CH2 genotype may have a biological advantage over the EU genotype.

Co-infection with more than one genotype of PepMV can often be observed. In their study on Belgian greenhouse tomatoes, Hanssen *et al.* (2008) found that mixed-genotype infection by the CH2 and EU genotypes was present in 15 out of 48 greenhouses. Those tomato crops that were burdened with mixed-genotype infections exhibited more severe disease symptoms compared to tomato crops where only a single genotype was present. Hanssen *et al.* (2008) also discovered that recombination between the CH2 and EU genotypes was common, and that the recombinant virus could transmit successfully between plants. Although the CH2 genotype has overtaken the EU genotype in Europe, Gómez *et al.* (2009) highlighted that the EU genotype has not been completely displaced, but instead persists in mixed infection with the CH2 genotype. Using molecular probes that were able to hybridize to and discriminate between the CH2 and EU genotypes, Gómez *et al.* (2009) found that 75.5% of the infected tomato plants in the study contained the CH2 genotype, 22.7% contained mixed infections of the CH2 and EU genotypes and only 1.8% of the samples were infected with the EU genotype alone.

1.6 Host Range of PepMV

Since its discovery on pepino plants in Peru, the main hosts of PepMV have been found to be members of the Solanaceae family, particularly *Lycopersicon* and *Solanum* species. A survey by Soler *et al.* (2002) revealed that PepMV naturally infects wild and cultivated species of *Lycopersicon*, including *L. chilense, L. chmielewskii, L. parviflorum, L. peruvianum* and *L. esculentum*. As well as infecting tomatoes (*S. lycopersicum*) naturally, it has been found that PepMV can experimentally infect other solanaceous crops such as *S. melongena* (eggplant) and *S. tuberosum* (potato) (Blystad *et al.*, 2015).

Research has shown that weed hosts exist, such as *Datura stramonium*, *Nicandra physaloides* and *Physalis peruvianum* (Jones *et al.*, 1980). Furthermore, Jordá *et al.* (2001) identified other natural weed hosts of PepMV by collecting weed samples from around greenhouses burdened with PepMV infection in different areas in Spain and testing them for the presence of PepMV. Despite the fact that all samples assessed were symptomless, results showed that *Malva parviflora, Nicotiana glauca, S. nigrum* and *Sonchus olerceus* were reservoir hosts for the virus.

1.7 Symptomatology of PepMV infection

PepMV has been found to induce a range of symptoms. In tomato, symptoms exhibited by PepMV infection include leaf mosaics, yellow rectangular leaf spots, leaf distortions, leaf bubbling, fruit marbling, fruit flaming, fruit dwarfing and nettle-heads. Some of these symptoms can be seen in Figure 1.4. As new PepMV genotypes began to emerge, more damaging symptoms were observed, such as fruit splitting (Figure 1.5), open fruit and leaf senescence/necrosis (Hanssen *et al.*, 2009). Despite the wide range of symptoms observed, it is also possible for PepMV infection to be symptomless.

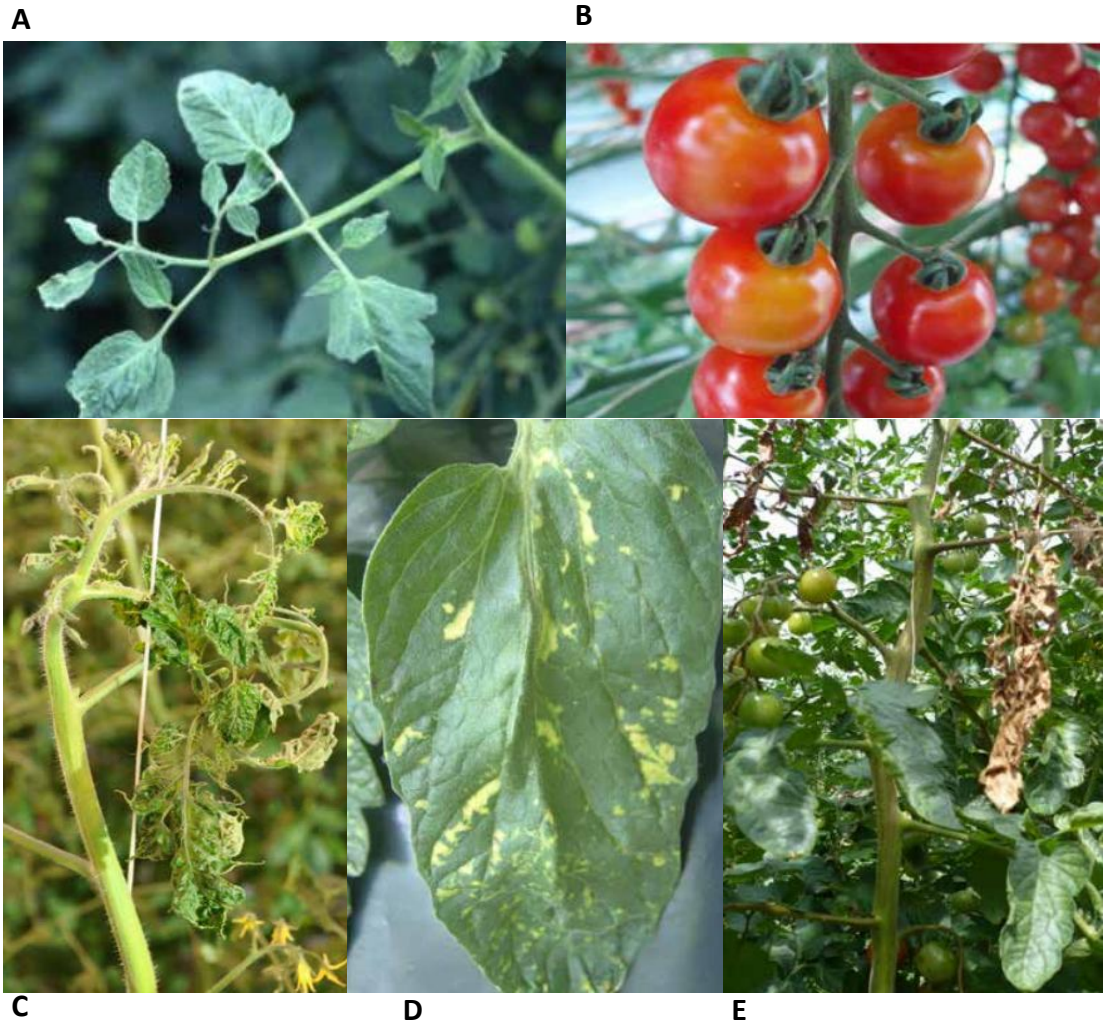

Figure 1.4- Symptoms seen in PepMV-infected tomato plants **A)** Transient leaf distortion and nettle head symptoms **B)** Fruit marbling **C)** Severe leaf distortion in the plant head **D)** Yellow rectangular leaf spots **E)** Leaf necrosis (O'Neill, 2014).

Figure 1.5- Tomato fruit splitting caused by PepMV infection

There are a number of factors that affect the symptoms displayed by a PepMV-infected tomato crop. Firstly, infection by the various genotypes of PepMV, and indeed the different isolates of the same genotype, induce differential symptoms with varying degrees of severity. For example, Hanssen *et al.* (2009) investigated the symptoms expressed when greenhouse tomato plants were inoculated with four different isolates of PepMV. Their results indicated that the infecting viral isolate largely impacts the symptoms that will ensue; the mild EU and mild CH2 isolates used in the investigation caused only mild symptoms, whereas the aggressive CH2 isolate and the mixed infection isolate (EU and CH2) induced more severe symptoms such as open fruit.

Single nucleotide substitutions in the PepMV genome have been found to influence the emergence of different disease symptoms in tomatoes, with one nucleotide substitution converting a mild isolate into an aggressive/necrotic

isolate. The first necrotic CH2 genotype was discovered in Poland in 2007 by Hasiów-Jaroszewska *et al.* (2009). This new necrotic isolate induced severe necrosis in tomato plants which had not been previously observed. Before the emergence of the new isolate, isolates of PepMV in Poland caused milder symptoms in tomatoes such as leaf mosaics and fruit marbling.

Hasiów-Jaroszewska *et al.* (2009) inoculated tomato plants with mild and necrotic CH2 isolates in order to investigate the difference in symptoms produced by each PepMV isolate. Inoculation with the mild isolate, representing those PepMV isolates found in Poland between 2002 and 2007, resulted in symptomless infection or mild mosaics and fruit marbling. However, the necrotic isolates induced leaf necrosis in the tomato plants, followed by death in some cases. Following their research in 2009, Hasiów-Jaroszewska *et al.* (2011) were able to identify, using site-directed mutagenesis, a single point mutation (K67E) in the TGB3 protein. This mutation caused a change in the amino acid present, which resulted in the conversion of the mild PepMV strain to the necrotic strain. They concluded that this single amino acid change is needed to induce necrotic symptoms in tomato plants and may "act as a virulence factor in host-pathogen interactions." Similarly, Hasiów-Jaroszewska and Borodynko (2012) found an alteration to the same amino acid to be responsible for the necrotic pathotype of the EU PepMV genotype, and so this necrotic mutation is not restricted to one genotype.

Furthermore, Hasiów-Jaroszewska *et al.* (2013) isolated PepMV from tomato crops from different European countries and were able to identify point mutations in the PepMV coat protein (CP) gene which were responsible for leaf yellowing symptoms. Through the use of cloning and sequence analyses, it was found that a point mutation at position 155 of the CP gene, resulting in a change of codon from GAA to AAA and so a change in amino acid from glutamic acid to lysine, was able to induce the leaf yellowing phenotype. Additionally, a point mutation at position 166, causing a codon alteration from GAT to GGT and an amino acid change from aspartic acid to glycine, was able to cause the yellowing symptom. Both mutations modify the chemical properties of the CP protein, causing changes in the protein-protein interactions between the virus and the host plant and subsequently altering the host response to viral infection. Such research shows that minor alterations at the genetic level can result in significant differences in virus pathotype.

Aside from the specific PepMV isolate and genetic alterations, environmental factors are also able to influence the symptoms that develop from PepMV infection. Studies, such as those carried out by Jones and Lammers (2005), have shown that lower levels of light intensity induce more severe symptoms. Similarly, Jordá *et al.* (2001) found that leaf symptoms on tomato plants in Spain attenuate as temperature increases. The time at which a crop becomes infected with PepMV is also believed to influence the severity of symptoms, with infection late in the growing season proving to be more detrimental through the induction of more severe symptoms (Spence *et al.*, 2006). Furthermore, symptoms have been seen to fluctuate throughout the growing season in response to altered growing conditions (Van der Vlugt, 2009).

1.8 Transmission of PepMV

As tomato is one of the most important crops in Europe, it is essential to know how PepMV is spread in order to prevent its transmission from infected to un-infected tomato plants. PepMV can be transmitted in a number of different ways, including through contact (mechanically), via vectors such as bumblebees and in water/nutrient solutions, as discussed below.

1.8.1 Mechanical Transmission

The dominant route of PepMV transmission is mechanical, meaning the virus can be easily disseminated from diseased to healthy plants through plant-to-plant contact, with an infected plant being capable of infecting six healthy plants in a row through contact during cultivation (Córdoba-Sellés *et al.*, 2007). Due to the highly contagious nature of the virus, PepMV can contaminate surfaces, tools, shoes, clothing and hands and is able to survive and remain viable for several weeks on contaminated surfaces and in plant debris (Van der Vlugt, 2009). Plant sap is able to remain infective for at least three months at 20°C (Jones *et al.*, 1980). It is not always clear where PepMV infection is present as infection can often be symptomless. This generates the potential for rapid viral spread throughout all stages of tomato production such as planting, pruning, fruit picking and movement in trade (Werkman and Sansford, 2010). Hygiene protocols and crop handling practices can limit the mechanical transmission of PepMV between greenhouses during tomato production.

Another route for mechanical transmission is via contaminated seeds. Infected tomato seeds are suspected to be the cause of the introduction of PepMV to a new area and for transmission over long distances. PepMV has been found to be capable of accumulating in the seed coat of immature and mature tomato seeds; however, it has not been detected in the embryo (Ling, 2008). Research has been conducted on the rate of transmission via infected seeds. Results have been conflicting and inconclusive and show variation in transmission rates depending on the tomato cultivar and seedling sample size. For example, Salomone and Roggero (2002) investigated seed transmission of an Italian isolate of PepMV in the tomato cultivar Camone. Seeds obtained from infected fruits were planted. One month after emergence of the seedlings, leaves were taken from 52 plants and tested for the presence of PepMV infection. All leaves tested negative for the virus.

Contrastingly, using a larger sample size, Córdoba-Sellés *et al.* (2007) investigated seed transmission in seeds collected from naturally PepMV-infected symptomatic tomato fruits. The seeds were planted and, once they had reached the cotyledon and transplant stage, seedlings were tested for the presence of PepMV infection. Out of 168 seedlings grown from seeds from symptomatic plants, three tested positive for PepMV*,* giving a seed-to-seedling transmission rate of 1.84%. The authors concluded that seed transmission is likely a contributing factor, along with contaminated transport trays, tools and human contact, to the introduction of PepMV to new areas and its spread throughout tomato greenhouses worldwide.

Due to the discrepancy between the results of different studies, Hanssen *et al.* (2010) sought to discover a "statistically sound" estimation of the rate of PepMV seed transmission in tomato. They obtained more than 100,000 seeds from tomato plants artificially inoculated with the CH2 and EU genotypes of PepMV. PepMV infection was confirmed by testing tomato plant leaf samples using ELISA at 8, 12 and 15 weeks post-inoculation (WPI). At each time point, tomatoes were harvested and cleaned to industrial standards. The seeds from the tomatoes were then extracted and subjected to treatment, including being soaked in citric acid and pectinase. After treatment, the seeds were analysed by ELISA to determine presence of PepMV infections and infected seeds from each time point were used in seed-transmission grow-out trials. In total there were 8,776 stonewool blocks each consisting of ten seedlings. Each seedling was irrigated separately to prevent contamination between plants. ELISA tests

revealed that 23 of these seedlings tested positive for PepMV and these results were confirmed by additional testing with RT-PCR. From these results, a minimum PepMV seed transmission rate of 0.026% was calculated. Interestingly, the seed transmission rate varied between the seed batches collected at the different time points, with the seed batch collected at eight WPI giving a transmission rate of 0.0053% compared to the seed batch collected at 15 WPI giving a transmission rate of 0.0567%. This in-depth study confirmed that contaminated seeds are capable of transmitting PepMV to the subsequent generation, most likely due to "contact between the germinating seedling and the virus-contaminated seed coat" (Hanssen *et al.*, 2010).

1.8.2 Vector Transmission

Research into PepMV transmission has also revealed that vectors may contribute to the spread of the virus between plants. Such vectors include bumblebees, which are often utilised in greenhouses to enhance pollination. As the bumblebees pollinate infected plants, it is possible for infected pollen and other plant extracts to attach to the legs and abdomen of the bees. This infected material could subsequently be passed onto healthy plants with which the bees come into contact. Lacasa *et al.* (2003) discovered that healthy tomato plants became infected with PepMV after transplantation into a PepMV infected greenhouse containing bumblebees. However, there was no control greenhouse used in this study where bumblebees were not present, making it impossible to conclude that the virus was not disseminated by other means, such as contaminated tools.

Shipp *et al.* (2008) conducted the first replicated and statistically valid investigation into the capability of bumblebees (*Bombus impatiens*) to acquire and transmit PepMV between tomato plants. In order to investigate this, two identical greenhouses were established, each containing 32 tomato plants inoculated with PepMV at one end and 18 PepMV-negative plants at the other end. The inoculated and healthy plants were two metres away from each other and separated by a mesh barrier to prevent contact/contamination. A hive containing 25-30 PepMV-negative bumblebees was placed in the treatment greenhouse and the bees were free to pollinate between the infected and healthy plants. After a two week acquisition period, the bees were analysed using RT-PCR for the presence of PepMV. Additionally, leaves, flowers and a fruit were sampled at 14, 28 and 42 days after bumblebee release from each of the

originally healthy target plants from both the treatment and control greenhouses. These samples were tested using ELISA. The experiment was repeated three times.

Their analyses revealed that, after two weeks, 83.3 ± 16.7% of the bees sampled from the flowers and 61.0 ± 19.5% of the bees taken from the hive tested positive for PepMV. In the control greenhouse where bumblebees were absent, after two weeks only 3.7 ± 3.7% of the healthy target plants became infected with PepMV, this increased to 5.6 ± 5.6% after six weeks. This increase was likely due to contamination during plant maintenance. In comparison, in the treatment greenhouse where bumblebees were present, the percentage of PepMV-positive leaves from the target plants reached 52.80 ± 2.80% and the percentage of positive fruit from the target plants reached 80.55 ± 8.35% six weeks after bee release. Therefore, this study clearly highlights the ability of bumblebees to carry and transmit PepMV from infected to healthy tomato plants during pollination.

As well as transfer between tomato plants, research has shown that bumblebees are able to transmit PepMV from infected tomato plants to other plant types. Stobbs and Greig (2014) demonstrated the ability of bumblebees to carry PepMV from infected tomato plants to perennial climbing nightshade (*Solanum dulcamara L*). Previous research by Stobbs *et al.* (2009) found that a variety of nightshade species were susceptible to PepMV infection, either through artificial inoculation, or through vector transmission by bumblebees. These nightshade species included perennial climbing nightshade (*S. dulcamara* L.), black nightshade (*S.nigrum*), eastern black nightshade (*S. ptycanthum* Dunal) and black nightshade (*S. sarrachoides* Sendtn). Such weed species that surround tomato greenhouses where bumblebees are able to move freely between the tomato crops and the outdoors could represent potential PepMV reservoirs.

Greenhouse whiteflies (*Trialeurodes vaporariorum*) have been found to be capable of transmitting a number of plant viruses including *Potato yellow vein virus, Tomato chlorosis virus* and *Tomato infectious chlorosis virus* (Jones, 2003; Bragard *et al.*, 2013). Noël *et al.* (2013) conducted two experiments in order to determine if PepMV could be transmitted between tomato plants by *T. vaporariorum*. In the first experiment (a 1:1 set-up), which was repeated ten times, one PepMV-infected tomato plant was placed in a cage with one healthy tomato plant and approximately 469 whiteflies. In the second experiment (a 1:4

set-up), which was repeated eight times, one Pep-MV infected plant was surrounded by four healthy plants and approximately 601 whiteflies. The whiteflies were left in the cages for 48 hours to allow for their movement between plants. Control cages were also set up, which contained a PepMV-infected tomato plant surrounded by one or four healthy test plants but with no whiteflies present. In all cages, contact between the infected and healthy plants was prevented to ensure no mechanical transmission could occur.

Six weeks later, RNA extractions were carried out on the leaves of the test plants and RT-PCR was used to test the extractions to determine whether or not the test plants had become infected with PepMV. In the first experiment, three out of ten test plants became infected with PepMV and five out of 32 plants in the second experiment were found to be positive for PepMV infection. No transmission was observed in the control cage where whiteflies were not present. In addition, the whiteflies were captured so that viral particles present on the flies could be counted. From the 55 whiteflies, an average of 1.33 virus particles were recovered. Despite the fact that the presence of viral particles on the whiteflies was low, Noël *et al.* (2013) concluded that greenhouse whiteflies can act as an unconventional mode of PepMV transmission between tomato plants.

1.8.3 Transmission in Water

As mentioned in section 1.2.1, in recent decades, the use of hydroponic systems (where plants are grown in a soilless nutrient solution) has become increasingly important in horticulture, particularly in tomato crop production (Savvas, 2003). In these systems, it is possible for the nutrient solution to be recirculated around the crop. Initially this method of growing crops was designed to avoid the issues associated with soil-grown crops, such as soil-borne diseases and poor quality soil for plant growth (Savvos, 2003). However, the fact that some hydroponic methods require the recirculation of nutrient media creates problems of its own, as it is providing a medium in which root-infecting or water-transmissible pathogens can spread throughout a whole crop. As hydroponic systems are commonly used in tomato production, PepMV has the potential to spread in the nutrient solution from plant to plant via the plant roots.

PepMV has been found to be able to survive in an aqueous environment. Mehle *et al.* (2014) investigated the survival of PepMV in water by macerating and

incubating PepMV-infected leaves of the EU and CH2 genotypes in water. The water was cleared of the plant debris, stored in a quarantined greenhouse at 20 ± 4°C and tested weekly using reverse transcription quantitative-PCR (RT-qPCR) to confirm whether or not PepMV was present. This experiment was able to confirm that, at this temperature, the CH2 and EU genotypes of PepMV could survive and remain infectious in water for up to three weeks.

In addition, Mehle *et al.* (2014) examined the spread of the CH2 genotype of PepMV in water. In order to do this, an experimental hydroponic system was set up and a transmission experiment was carried out twice. Healthy (bait) tomato plants were placed into a tank and artificially inoculated tomato plants were placed into a separate tank. The nutrient solution used to irrigate the inoculated plants was used for irrigation of the healthy bait plants by pumping the solution through from the inoculated tank to the healthy tank using a manual pump and plastic tubing. In order to more realistically represent the conditions of a hydroponic system, from time to time the roots of the inoculated plants and the bait plants were stirred with a glass rod to create injuries to the roots. This experiment was conducted for four months, and at certain times during this experimental period the leaves, fruits and roots of the inoculated and bait plants were analysed for the presence of PepMV using RT-qPCR. Additionally, mechanical inoculation of healthy test plants was used to assess the infectivity of the nutrient solution.

Results from this experiment affirmed that PepMV-CH2 is able to be distributed through a hydroponic system to infect other healthy plants. PepMV was detected in the roots of the bait plants after the first month of the experiment and was detected in the fruit and leaves of the bait plants after three to four months of experiment initiation. Of the healthy test plants, 12% became PepMV-positive after mechanical inoculation with the nutrient solution used in the experiment. This investigation confirmed that PepMV can be released from the roots of infected tomato plants and transmitted through the nutrient solution of hydroponic systems to infect the roots of healthy plants and ultimately spread to the leaves and fruit.

The findings published by Mehle *et al.* (2014) supported an earlier study conducted by Schwarz *et al.* (2010), who found that PepMV could be successfully disseminated between tomato plants via a PepMV-infested nutrient solution. In their experiment, Schwarz *et al.* (2010) set up a climate chamber in which they

placed nine tomato seedlings (cv. Castle Rock). Each plant was grown in 300 ml of nutrient solution. Two weeks subsequent to leaf emergence, two leaves each of four of the seedlings were mechanically inoculated with PepMV. One week later, after PepMV infection had been confirmed, each 300 ml volume of nutrient solution was taken weekly from the inoculated plants and used to irrigate four healthy plants. At one, three, five and seven weeks after application of the nutrient solution to the healthy plant roots, DAS-ELISA was used to analyse different parts of the test plants. This experiment was then repeated once more.

Their analyses showed that PepMV was detectable in the test plant roots 7-35 days subsequent to application of the nutrient solution to the roots. Despite the fact that there were no observable symptoms on the test plants, in the first experiment PepMV could be detected in all four test plants three weeks after the application of the first nutrient solution. In the repeat experiment, PepMV was detected in one plant seven days after the first inoculation and in two more plants four to five weeks post inoculation with the contaminated nutrient solution. The authors therefore concluded that PepMV particles can be released from infected plant roots into the nutrient solution and such nutrient solutions in hydroponic systems can act as a significant medium for PepMV transmission between plants.

Some research conducted into the spread of PepMV in water/nutrient solutions has also included investigations on the virus' interaction with other water-borne pathogens. Alfaro-Fernández *et al.* (2010) studied the transmission of PepMV in irrigation water by the fungal vector *Olpidium virulentus*. Their study found that test plants only became infected with PepMV when *O. virulentus* was also present in the irrigation water, suggesting that the fungus operates as a vector to transmit PepMV in water to infect healthy plants. Test plants irrigated with PepMV-infested irrigation water in the absence of *O. virulentus* did not become infected with PepMV. This finding is in contrast with the results published by Mehle *et al.* (2014) and Schwarz *et al.* (2010) as discussed above. Alfaro-Fernández *et al.* (2010) concluded that the interaction between PepMV and *O. virulentus* induced 'tomato collapse' in the test plants, which is a syndrome that exhibits severe wilting and was also observed by Córdoba *et al.* (2004).

1.9 The Economic Impact of PepMV Infection on Commercial Tomato Production

Economic loss as a result of PepMV infection has only been reported in tomato production. This loss is a result of fruit symptoms caused by PepMV infection, such as blemishes, fruit marbling/flaming or reduction in fruit size which make the tomatoes unmarketable. The majority of tomato retailers in the UK require the fruit to be unblemished and of a particular diameter (which varies with the tomato variety). Tomatoes with these characteristics are regarded as class 1. If fruits have a slightly smaller diameter than that which is desired, they can still be sold as class 1 as long as they are unblemished. In the UK, it is difficult to sell tomatoes that are lower than class 1; however, this is not the case in all European countries (Spence *et al.*, 2006).

A number of publications on yield loss in tomato crops caused my PepMV infections exist; however, the extent of loss appears to differ from country to country. For example, Verhoeven *et al.* (2003) reported low yield losses soon after the PepMV outbreak in Europe, with hardly any yield losses seen in Germany and the Netherlands, and yield losses of up to 15% observed in the rest of Europe. Contrastingly, Soler-Aleixandre *et al.* (2005) described the association between PepMV infection and the collapse of up to 90% of tomato plants in areas along the Spanish Mediterranean coast.

Spence *et al.* (2005) conducted replicated trials to determine the effect of PepMV on tomato production and quality in the UK. Two classic tomato cultivars were used in the experiment (Espero and Encore) which, at the time, represented 59% of UK tomato production. The study used three main treatment groups; early inoculation, late inoculation and uninoculated controls. PepMV-infected leaves were macerated in 1% potassium phosphate buffer and this was used to inoculate the test plants. Inoculated plants and uninoculated controls were kept under the same environmental conditions throughout the experiment. Fruits were harvested at regular intervals from each treatment group and were commercially graded for fruit quality and size. Fruit quality was assessed based on the severity of fruit symptoms including marbling, uneven ripening, blotchy ripening, dark patches and shape distortion.

Spence *et al.* (2005) found that there was no significant reduction in monthly yield; however, their study found that PepMV infection led to a substantial

reduction in the percentage of fruits graded as class 1 from both early and late inoculation treatments. For the Espero cultivar, an average of 33.4% and 44% of fruits were downgraded from class 1 in the early inoculated treatment and the late inoculated treatment, respectively. These losses were also similar for the Encore cultivar. Such downgrading meant that, overall, only 55% of tomatoes from PepMV-infected plants were given a class 1 grading, compared to 88.7% of the tomatoes from uninoculated control plants.

Although PepMV did not consistently decrease fruit size, increased blotchy ripening was the cause of the majority of fruit downgrading. Even though fruit disorders such as blotchy ripening were seen in the uninoculated control plants, these were much less severe than in the PepMV-infected plants and so the authors concluded that PepMV may interact with adverse environmental factors to enhance the levels of stress experienced by the plant. Such downgrading of tomatoes from class 1 has the potential to have detrimental impacts on commercial tomato production. In 2005, the average tomato production per season was 53 kg m^{-2} at £0·80 kg^{-1} and so the level of fruit downgrading observed in this study would represent a loss of over £16 m^{-2} (Spence *et al.*, 2005). With 224 hectares of land used for tomato production in 2013 (FAOSTAT, 2015), this level of loss would have severe adverse effects on the tomato industry.

Research has indicated that PepMV infection may have different effects on yield depending on the tomato cultivar. The investigation conducted by Schwarz *et al*. (2010), mentioned in Chapter 1.8.5, looked at the effects of PepMV infection on tomato growth and yield. Mechanical inoculation of tomato plants led to a significant reduction in total yield (23.3%) of the Castle Rock cultivar compared to uninoculated control plants. The total yield of the Hildares cultivar was not affected; however, there was a reduction of 7% in fruit weight in this cultivar compared to uninoculated controls. This research also found that young seedlings were more susceptible to PepMV infection than older plants, as seedlings inoculated early in the second leaf stage exhibited the highest yield losses. This finding is in contrast to other reports, which state that PepMV infection late in the season is much more detrimental to fruit quality than early infections (Spence *et al.*, 2006).

1.10 Control and Management of PepMV

The fact that PepMV is so contagious means that it can spread rapidly, particularly in the glasshouse environment where a large amount of hands-on activities are involved in tomato crop production. Currently, the predominant preventative action taken against the spread of PepMV is the adoption of strict hygiene protocols within the glasshouse. Additionally, research has demonstrated that cross-protection using a mild strain of PepMV may limit the detrimental effects caused by subsequent infections by necrotic PepMV strains.

1.10.1 Hygiene Procedures in the Glasshouse

As hands, clothes and tools are believed to be the dominant means of spread within the glasshouse (O'Neill *et al.*, 2003), hygiene regulations must be followed to minimise PepMV transmission during tomato production. In order to prevent the spread of the virus between glasshouses, members of staff are advised to change into new overalls, gloves and overshoes before moving from one glasshouse to another. Additionally, each glasshouse should have its own set of equipment that is used only in the one glasshouse.

O'Neill *et al.* (2003) demonstrated that PepMV is able to survive on surfaces within the glasshouse, such as concrete pathways, containers, trolleys and aluminium stanchions. In order to eliminate PepMV from glasshouse surfaces, a variety of disinfectants are used. Different disinfectants have differing degrees of efficiency at reducing PepMV infectivity. Li *et al.* (2015) evaluated the ability of 16 disinfectants to prevent mechanical transmission of glasshouse viruses and viroids. Replicate experiments were conducted to determine the efficacy of each disinfectant, where viral inocula were exposed to each disinfectant for 0-10, 30 and 60 seconds before being used to mechanically inoculate healthy test plants. ELISA was used to determine if the plants were positive for PepMV infection. Results indicated that two disinfectants (2% Virkon and 10% Clorow regular bleach) were effective at stopping PepMV infectivity; however, no significant differences were observed between the exposure times. The findings of Li *et al.* (2015) supported the earlier findings of O'Neill *et al.* (2003), who discovered that Virkon was effective at disinfecting surfaces purposefully contaminated with PepMV-infected leaf sap and fruit juice after 1-30 minutes exposure with the disinfectant.

1.10.2 Disinfection of Seed Coats

As discussed in Chapter 1.8.2, it is suspected that PepMV may be introduced into a new area by contaminated tomato seeds. Although research has found the rate of seed transmission to be low, infected seeds still represent a primary source of inocula for PepMV infections. In 2004, the European Committee stated that there were to be strict control measures adopted to monitor the entry and circulation of PepMV-infected seeds within the EU. Therefore inspections to detect whether PepMV was present in nurseries and seed production establishments, using ELISA-based seed tests, were necessary. If PepMV infection is found, seeds can be treated to eliminate the virus.

Córdoba-Sellés *et al.* (2007) evaluated the efficacy of different physical and chemical seed treatments in their ability to remove PepMV and their effect on seed germination. The seed treatments tested included high-temperature heat treatment and treatment with trisodium phosphate (TP), a pectinase solution (P), pectinase supplemented with 2% HCl (PH) and pectinase supplemented with 2% HCl and 30% commercial bleach (PHB). Their results indicated that heat-treatment was ineffective at eradicating PepMV. All chemical treatments, apart from the pectinase solution, were able to eliminate PepMV from the seeds. Treatment with 10% TP for three hours was able to prevent transmission to seedlings and favoured germination. The authors concluded that seed testing and treatment protocols are beneficial in helping to prevent the spread of PepMV; however, further research is required to evaluate the efficacy of treatments on a wider range of tomato cultivars and against different PepMV isolates.

1.10.3 Mild-Strain Cross-Protection

Mild-strain cross-protection can be used to protect crops from subsequent infection by more damaging viral isolates. McKinney (1929) first described the effectiveness of viral cross-protection on tobacco plants which were infected by a mild strain of *Tobacco mosaic virus* (TMV). The mild-strain TMV prevented the plants from becoming infected with a severe TMV strain which usually induces yellow mosaic symptoms. Cross-protection has since been used as a control method for a variety of vegetable pathogens, such as the use of the MII-16 protector isolate of TMV in greenhouse tomato production (Rast, 1972) and the

use of the mild WK protector isolate of *Zucchini yellow mosaic virus* (ZYMV) to protect against severe infection in squash production (Lecoq and Lemaire, 1991).

The use of cross-protection in control of PepMV is controversial. As mentioned in Chapter 1.7, it is thought that early infection by PepMV is less detrimental than infections later in the growing season. PepMV isolates of the LP genotype induce only mild symptoms in tomato (López *et al*., 2005) and so may be suitable candidates for cross-protection and have been utilised for cross-protection in the Netherlands (Brakeboer, 2007). Hanssen *et al.* (2010) investigated the efficacy of a mild LP isolate in protecting tomato crops against an aggressive CH2 isolate. After inoculation with the mild LP isolate and subsequent inoculation with the aggressive CH2 isolate, it was observed that co-infection with both isolates actually resulted in enhanced symptom severity. The fruit marbling symptom was especially more profound in the test plants.

Similar additional tests were carried out by Hanssen *et al.* (2010), using a mild-EU and a mild-CH2 isolate to protect against infection by the aggressive CH2 isolate. Enhanced symptom severity was seen in the test plants pre-inoculated with the mild-EU isolate. This supports earlier findings by Hanssen *et al.* (2009), who showed that plants co-infected with EU and CH2 isolates exhibited severe symptoms such as open fruit. Contrastingly, pre-inoculation with the mild-CH2 isolate achieved successful cross-protection by suppressing fruit flaming and marbling symptoms. It is thought that the mild-CH2 was more effective than the LP and EU isolates at inducing cross-protection against the aggressive CH2 isolate due to higher genetic similarity. The sequence homology between the mild and aggressive CH2 strains was 99.4%, whereas the homology of the LP and EU with the aggressive CH2 was approximately 79%. The influence of sequence homology on cross-protection was more recently explored by Hasiów-Jaroszewska *et al.* (2014). Pre-inoculation with a mild CH2 strain sharing 99.9% sequence homology with the challenging CH2 strain reduced symptom severity compared to non-protected plants. However, this protection was not sustained as necrotic symptoms began to emerge two months after infection. This led the authors to conclude that sequence homology is not the only factor influencing the success of viral cross-protection.

Despite discrepancies between the results of different studies, the European Food Safety Authority (EFSA) has recently approved the use of a wild-type avirulent Belgian CH2 isolate (1906) for the use in cross-protection in

greenhouse tomato plants in some EU countries. The approval has followed ten years of research and five years of commercial testing on over 2500 hectares of tomato crops in Belgium, the Netherlands and other countries. The DCM Corporation has formulated the 1906 isolate into 'PMV-01,' a plant protection product which can be applied to tomato seedlings via low-volume (4-8L PMV-01/ha) spraying (EFSA, 2015). This treatment protects against damage, such as fruit marbling, caused by infection by severe strains of PepMV. In recent months, the use of PMV-01 has been approved in the UK (ADAS, personal communication).

1.11 PepMV Detection Methods

As discussed in Chapter 1.9, infection by plant pathogens, such as PepMV, can lead to yield loss and crop damage. Pathogen infections have been found to contribute to yield losses, along with animals and weeds, of 20-40% worldwide per year (Savary *et al.*, 2012). In order to minimise the devastating effects of plant pathogens on crop production, it is vital to utilise effective disease detection methods to maximise crop productivity and sustainability (Fang and Ramasamy, 2015). Early detection is fundamental in minimising the spread of the disease. The advantages and disadvantages of the main techniques used for PepMV detection are summarised in table 1.1.

Table 1.1- Advantages and disadvantages of PepMV detection methods.

Detection Methods	Advantages	Disadvantages
LFD	Virus specific	Cannot detect viruses present at low concentrations
	Rapid detection (3-5 minutes)	
	Low cost	Not genotype specific
	Can be used in the field	
ELISA	Virus specific	Cannot detect viruses present at low concentrations
	Gives partial quantification	
	Relatively low cost	Not genotype specific
	Simple visual detection via colour change	Cannot be used in the field
TaqMan RT-PCR	Gives a quantitative result	Cannot be used in the field
	Virus specific and can be made genotype	Expensive due to equipment needed

	specific through primer design	Risk of false positives from contamination
	Highly sensitive	
RT-LAMP	Virus and genotype specific through primer design	Can be less sensitive than RT-PCR methods (such as RT-qPCR)
	Rapid amplification	
	Highly sensitive	
	Can be used in the field	
	Relatively low cost as complex equipment is not needed	
	Quantitative results can be obtained	

1.11.1 Serological Detection Methods

Serological detection methods represented the first breakthrough in molecular detection of plant pathogens. Serological methods, such as the enzyme-linked immunosorbent assay (ELISA), utilise a diagnostic antibody to capture a target pathogenic antigen, such as a viral coat protein. The first report of plant virus detection by ELISA was published in 1977 by Clark and Adams. The double antibody sandwich ELISA (DAS-ELISA), is a form of ELISA that is the preferred and recommended diagnostic technique for analysing large quantities of suspected PepMV-infected samples (European and Mediterranean Plant Protection Organization, 2013). DAS-ELISA is often performed as confirmation of a PepMV-positive sample after a biological assay has been conducted by inoculating test plants such as *Nicotiana benthamiana*. In order to carry out DAS-ELISA, the wells of an ELISA plate must be coated with the capture/coating antibody. The plant extract is then added to the wells of the ELISA plate and any pathogenic antigens present in the plant extract will be bound by the capture antibody. A second antibody, conjugated with an enzyme, is required to detect if any pathogenic antigens have been captured. After the addition of the conjugate, the substrate specific to the enzyme attached to the detecting antibody is added to the plate. Binding of the substrate to the enzyme induces an observable colour change which confirms the presence of the plant pathogen within the plant extracts and the speed at which the colour change occurs indicates the concentration of the virus present, giving a quantitative result.

One of the disadvantages of ELISA is that it is not suitable for on-site diagnostics. This drawback led to the development of on-site methods that utilise

antibodies in the same way as ELISA tests, such as lateral flow devices (LFD). LFDs utilise latex beads that are coated in specific monoclonal and polyclonal antibodies. A plant extract is applied to the release pad of the LFD which contains the specific antibody-coated latex beads. If the pathogenic antigen is present within the plant extract, the antibody binds to the antigen and the complex migrates laterally along the membrane of the device. The antibody-antigen complexes are then trapped on a test strip made up of target-specific antibody, causing the complexes to accumulate in a visible line, indicating a positive result. The membrane also contains a control strip composed of an antibody specific to the antibody coating the beads. If no pathogenic antigens are present, the coated beads will accumulate on the control strip and not on the test strip.

1.11.2 PCR-based Detection

In certain cases, concentrations of a pathogen present in a sample may be below the limit of detection for serological tests. For this reason, it is essential to have sensitive detection techniques that can amplify nucleic acids present at low concentrations within a sample. A variety of nucleic acid-based detection methods have the ability to do this and have been adapted for the detection and identification of RNA viruses such as PepMV. These methods include RT-PCR, real-time RT-PCR, immunocapture RT-PCR and RT-PCR with restriction fragment length polymorphism (RT-PCR RFLP) (European and Mediterranean Plant Protection Organization, 2013). The broad array of viral sequence data that is now available has made it possible to design PCR primers that not only detect a specific pathogen, but can also discriminate between different genotypes of the same pathogen. The specificity of a RT-PCR reaction is dependent upon primer design, as the primers are used to amplify a specific region of the pathogen genome and, therefore, are unique to the target organism (Schaad and Frederick, 2002).

Ling *et al.* (2007) used sequence alignment of 11 PepMV isolates to identify a conserved sequence region of the PepMV *TGB2* gene in order to design primers specific for this region. This primer set was then used in a real-time RT-PCR assay for the broad spectrum detection of PepMV. This real time RT-PCR assay was able to detect the US1, US2, CH1, CH2 and additional field isolates of PepMV collected in the USA and Canada. The development of real-time PCR technology has eliminated the need for post-PCR processing of PCR products, such as

analysis with gel electrophoresis, simplifying the process and providing quantitative results. The real-time RT-PCR assay developed by Ling *et al.* (2007) utilised a TaqMan probe, which is labelled with a fluorochrome at the 5' end and a quencher fluorochrome at the 3' end. As the primer is extended along the complementary target sequence during the RT-PCR reaction by the *Taq* DNA polymerase, the 5'-3' exonuclease activity of the DNA polymerase degrades the TaqMan probe, separating the fluorochrome from the quencher and resulting in a fluorescent signal. The level of fluorescence is proportional to the amount of PCR product being generated.

1.11.3 Loop-mediated Isothermal Amplification (LAMP)

Despite the fact that molecular detection methods like TaqMan RT-PCR are accurate in identifying PepMV infection, they require specialized laboratory equipment and so are not suitable for on-site diagnosis. As mentioned in Chapter 1.8, PepMV is highly contagious and easily mechanically transmitted. It is essential for tomato growers to be aware if their crop is infected with the virus as soon as possible so that the correct control procedures can be implemented and further spread is prevented. As it is suspected that PepMV can be disseminated by contaminated seeds, it is also important that the seeds can be tested for the presence of the virus before they are planted or distributed. Having to send samples to a laboratory for RT-PCR-testing is time consuming and costly and so there was a requirement for a rapid, on-site diagnostic tool to identify PepMV infected crops.

Notomi *et al.* (2000) developed a technique called loop-mediated isothermal amplification (LAMP). The main advantage of LAMP over PCR is its capability of amplifying DNA under isothermal conditions, eliminating the requirement for a thermal cycler. LAMP is able to amplify very low copy numbers (as few as six copies) of DNA to 10^9 copies in under 60 minutes. LAMP utilises a DNA polymerase and a set of four or six specific primers; a forward inner primer (FIP), a backwards inner primer (BIP), a forward primer (F3) and a backwards primer (B3). Forward and backwards loop primers (FL and BL) can be used for acceleration of the LAMP reaction (Nagamine *et al.*, 2002). The set of four (or six) primers recognise six (or eight) regions within the target DNA sequence (Figure 1.6), making LAMP highly specific.

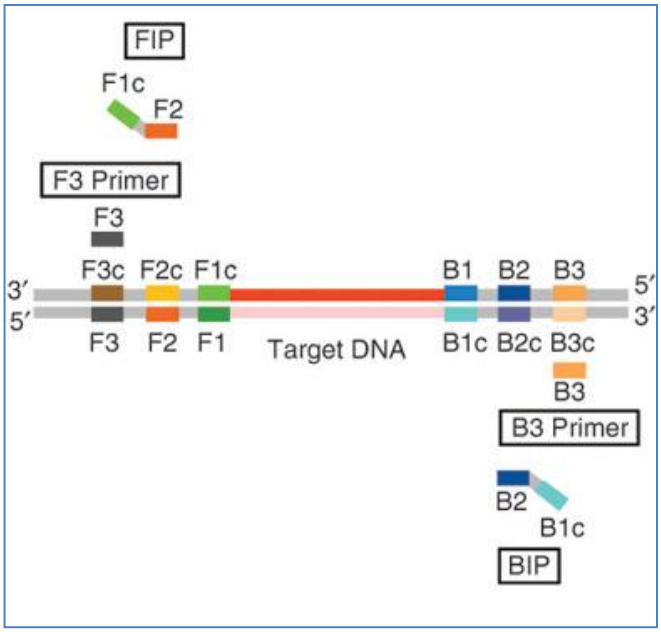

Figure 1.6- RT-LAMP primers (FIP, BIP, F3 and B3) and their complementary regions (F3, F2, F1, B1c, B2c and B3c) within the target DNA sequence (Tomita *et al.,* 2008).

The FIP initiates the reaction by annealing to the complementary region on the target DNA and triggering elongation (Figure 1.7a). The F3 primer then anneals to the complementary sequence in the target DNA and causes strand displacement of the DNA strand elongated from FIP to occur (Figure 1.7b and c). The 5′ end of the released strand forms a loop structure (Figure 1.7d) and this single-stranded DNA forms the template as synthesis continues with the BIP and B3 primers (in the same way as with the FIP and F3 primers). The BIP and B3 primers create a dumbbell-like structure with a loop at both ends (Figure 1.7e). Cycling amplification then occurs with the dumbbell-like structure as a template (Tomita *et al.*, 2008). The inner primers anneal to complementary sequences within the loop structure, triggering strand displacement synthesis and creating more stem-loop DNA strands. This cycling reaction continues, creating stem-loop DNA products with inverted repeats of the target DNA (Notomi *et al.*, 2000). The addition of loop primers to the reaction accelerates the process; the primers hybridise to the stem-loops that are not hybridised by the inner primers and initiate strand displacement, resulting in a 50% decrease in amplification time compared to when the loop primers are not employed (Nagamine *et al.*, 2002).

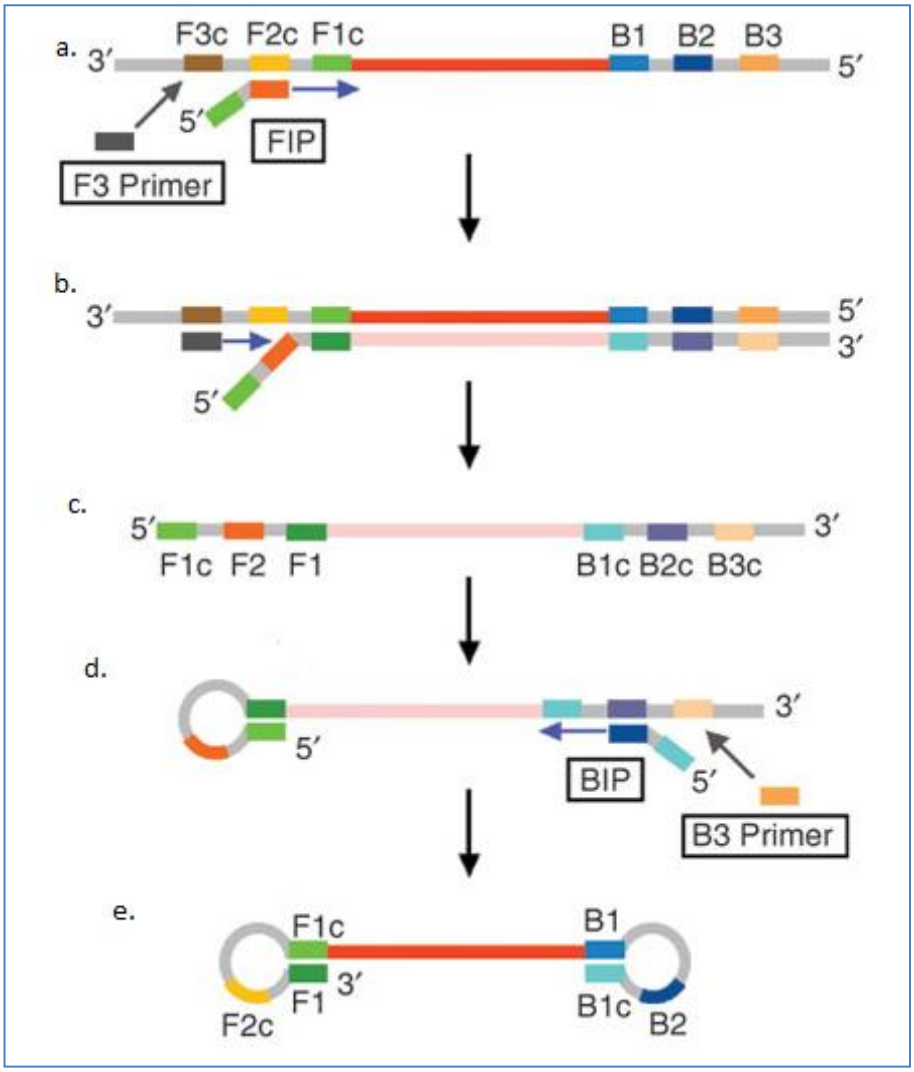

Figure 1.7- The LAMP method **a)** Initiation of DNA synthesis by FIP **b)** and **c)** Strand displacement by primer F3 **d)** Formation of loop structure at 5′ end of displaced strand which acts as template for subsequent DNA synthesis **e)** Formation of dumbbell-like structure by BIP and primer B3 (Tomita *et al.,* 2008).

The LAMP protocol has been successfully applied to detect a variety of plant pathogens, such as *Peach latent mosaic viroid* (Boubourakas et al., 2009), *Tomato yellow leaf curl virus* (Fukuta *et al.*, 2003) and *Tomato spotted wilt virus* (Fukuta et al., 2004). It has also been utilised in the detection of human pathogens, with the aim to be used in developing countries where resources are limited and many devastating diseases are endemic (Mori and Notomi, 2009). As

with PCR, the LAMP reaction can be adapted to include a reverse transcription step (RT-LAMP), through the use of reverse transcriptase, to allow for the amplification of RNA viruses such as PepMV.

Ling *et al.* (2013) developed a rapid RT-LAMP assay for the detection of the CH2, EU and US1 PepMV genotypes. Through sequence alignment of multiple PepMV sequences, Ling *et al.* (2013) were able to identify conserved sequence regions within each genotype. These conserved regions were used to design a set of six primers specific to the CH2, EU and US1 genotypes. Each primer was used in a RT-LAMP assay to test 50 field samples collected in North America for the presence of PepMV. The results from the RT-LAMP tests identified 34 samples with CH2 infection. Thirteen of the CH2-infected samples were also infected with the EU genotype and five with the US1-genotype. Thirteen of the samples were infected with the US1 genotype only. The RT-LAMP assays were validated for specificity with genotype-specific RT-PCR and cloning of RT-LAMP products. Forty-four out of fifty of the results from RT-LAMP and RT-PCR matched, and through further investigation it was found that the RT-LAMP assay was more sensitive than RT-PCR in detecting low virus titres. Ling *et al.* (2013) were able to use their RT-LAMP assay to monitor the distribution of PepMV in North America and the genetic diversity present within the viral population.

Portable instruments have made the LAMP protocol quick and simple to conduct out of the laboratory environment. Optigene Limited designed Genie II and Genie III, portable instruments which are able to run isothermal amplification methods and detect fluorescent readouts which are displayed on the built-in, user-friendly touch screen. Genie II and III contain two and one heating blocks, respectively, which can be heated to the desired temperature. Each block can hold eight LAMP reactions at a time. As the LAMP reaction proceeds, the time at which the target DNA amplifies (when a fluorescent signal is detected) is depicted as an amplification curve on the screen. These instruments have made the use of LAMP for diagnostics extremely simple, and their portability makes the Genie machines ideal for the use in on-site diagnostics.

1.12 Aims

This project aims to increase the understanding of PepMV symptom severity and persistence on nurseries in the UK. In order to monitor and determine the presence and genetic diversity of PepMV in the UK, an RT-LAMP assay utilising the genotype-specific primers published by Ling *et al.* (2013) will be validated and used to test a variety of tomato leaf/fruit samples from different tomato crops with suspected PepMV infection in the UK. This will enable the verification of distribution of mixed-genotype PepMV infection in UK tomato crops.

Additionally, the survival of PepMV and sources of PepMV inocula which can ultimately lead to viral dissemination will be identified. The efficiency of end-of-season glasshouse cleanups will be evaluated by monitoring greenhouse structures for the presence of PepMV before and after crop removal. As it has been found that PepMV can survive in tomato debris (Van der Vlugt, 2009), tomato crops that have been subjected to composting will be tested for the presence of PepMV at different composting stages to determine the survival of PepMV throughout the composting process. Water samples used to irrigate tomato crops will be collected from UK nurseries to be tested for the presence of PepMV, establishing whether or not PepMV has the potential to be spread throughout crops via water at these sites. The findings will be communicated to tomato growers, allowing them to assess their current crop-hygiene protocols and to allow for any changes to be implemented in order to limit/prevent PepMV infection and dissemination.

Chapter 2: General Methods

2.1 Site Identification and Symptom Analysis

All communication with tomato nurseries and all sampling was conducted by Sarah Mayne, of ADAS, under the instruction of Tim O'Neill.

2.1.1 Symptom Analysis

Six greenhouse tomato crops in the UK with a history of PepMV infection, suspicious symptoms or confirmed PepMV infection were identified. Visits were made to each crop, and at each site records were taken. Information collected included tomato variety, rootstock, growing medium, irrigation (closed or run-to-waste), date that suspect PepMV symptoms were first observed, nature of symptoms and location from where samples were taken (greenhouse number and row number). Where possible, one of the visits was assessed for incidence and severity of PepMV infection during the autumn months, where light intensity was low and symptoms were likely to be more severe, or during the spring months where light levels were higher. Two sampling visits were conducted at four of the crops within the same season, with approximately one month between each sampling visit. The remaining two crops were visited once each. At each visit, 100 plants were selected at random and each plant was assessed on one stem using a scale adapted from the virus symptom rating scale published by Hanssen *et al.* (2009), as can be seen in Table 2.1. Symptom recognition was aided by photographs in O'Neill (2014), as can be seen in Chapter 1, Figure 1.4.

Table 2.1- PepMV symptom rating scale for tomato plants (adapted from Hanssen *et al.*, 2009).

Part of Plant	Symptom	Score	Symptom Description
Head	Leaf bubbling, nettle heads, mosaic/yellowing of heads	0	No symptom
		1	Bubbling of the leaf
		2	Nettle head where leaf surface is reduced with a jagged leaf margin
		3	Total mosaic/yellowing of head/very severe nettle head
Foliage	Chlorotic spots	0	No symptom
		1	One chlorotic spot
		2	Two chlorotic spots
		3	More than two chlorotic spots
Foliage	Necrotic spots	0	No symptom
		1	One necrotic spot
		2	Two or more necrotic spots
		3	Leaf death
Fruit	Marbling	0	No symptom
		1	One marbled fruit
		2	Two marbled fruits
		3	More than two marbled fruits
Fruit	Flaming	0	No symptom
		1	One flamed fruit
		2	Two flamed fruits
		3	More than two flamed fruits
Fruit	Open fruit	0	No symptom
		1	One open fruit
		2	Two open fruits
		3	More than two open fruits
Petiole and stem	Twisted/distorted tissue, necrotic spots and streaks	0	No symptom
		1	Twisted/distorted tissue
		2	Severe distortion
		3	Necrotic spots and streaks

2.2 Sampling

2.2.1 Sampling of Tomato Leaves and Fruit

At each symptom analysis visit to the six different crops, 30 samples were collected from each crop for testing in order to determine the presence of PepMV in the crop. One leaf from the plant head of each of ten plants showing no obvious symptoms was sampled and placed in separate labelled bags. Also, ten plants with the most severe symptoms in the crop were selected and the third leaf down from the plant head was sampled. Each sample was placed in a separate labelled bag. Finally, ten separate samples of tissue showing severe

symptoms, for example marbled fruit or necrotic-spotted leaves, were collected and again placed in separate labelled bags. It was important to collect the symptomless samples first (or using new gloves) in order to minimise the possibility of mechanical PepMV transmission between samples. This gave a total of 30 samples from each visit to each crop. The samples were posted by next day delivery to Sutton Bonington campus of the University of Nottingham for RNA extraction (Chapter 2.3) and RT-LAMP testing (Chapter 2.4) to determine the presence/absence of each PepMV genotype (CH2, EU and US1) in the samples.

2.2.2 Swab Sampling

Swab collection was carried out at four nurseries with confirmed PepMV infection. Swabs were collected from the same glasshouse surfaces on two occasions at each site; firstly within two weeks of crop pull-out and secondly within two weeks of the end of the glasshouse/equipment disinfection and cleaning processes. This was to allow for the evaluation of the effectiveness of such cleaning procedures to eliminate PepMV from the glasshouse environment. On each visit to each site, a set of 50 swabs was taken from the glasshouse area from surfaces that were likely to have been in contact with tomato crop, fruit or sap. Some examples included concrete pathways, drip pegs, picking crates, metal heating pipes and door handles.

Each surface was swabbed with a cotton bud, which was moistened in 1-2 ml of phosphate buffer (pH 7.2, Gibco), and then placed in a Sterilin tube labelled with the site, swab number and date. A table was made listing the swab numbers and recording the precise details of each swab location, for example the surface type, appearance, location of the surface and disinfection product used for cleaning. For the first visits to two of the sites, two sets of 50 swabs (two swabs per surface) were collected so that one set could be sent to Sutton Bonington for RT-LAMP testing and one set could be sent to Fera Science Ltd for ELISA testing. For the remaining visits to each site, just one set of 50 swabs was collected and posted to Sutton Bonington by next day delivery. On arrival at Sutton Bonington, the phosphate buffer was expelled from the swabs and 1.5 µl of the buffer was tested in an RT-LAMP assay with the CH2 primer, as described in Chapter 2.4 below, to determine the presence of PepMV in the sample. Selections of positive post-cleanup swabs from the sites were sent to Fera Science Ltd for the use in sap inoculation tests on tomato seedlings.

2.2.3 Sampling of Compost Material

A site with known PepMV-infected Piccolo crop that was being chipped and composted was selected for this objective. Samples were collected (at monthly and then weekly intervals) from the time at which the crop was chipped (T0) until the end of the composting process (dry phase, T9). Ten pieces of chipped crop or compost were collected on each sampling visit. Five pieces were taken from different positions in the compost heap at a depth of 15 cm and five pieces taken from different positions in the heap at a depth of 30 cm. Each piece of chipped crop/compost was placed in a separate Sterilin tube. Each sample was labelled with sample occasion (T0-T9), sample number (1-10), sample depth (15 cm or 30 cm) and the date. The temperature of the stack on each occasion at each depth (15 cm and 30 cm) was recorded. In order to be able to compare the survival of PepMV during the composting process to its survival when it is not subjected to composting, samples were taken of the chipped crop at T0 and taken to the ADAS Boxworth pathology laboratory where they were kept in an incubator at 24°C for the duration of the experiment to act as a control treatment. These controls were sampled and tested at the same time points as the site samples.

The T0 samples and controls were sent to Fera Science Ltd for ELISA tests and sap inoculation onto tomato seedlings and *N. benthamiana* to determine the presence and viability of PepMV. On the remaining nine occasions (T1-T9), after collection, the compost samples and controls were posted to Sutton Bonington by next day delivery. On arrival at Sutton Bonington, RNA extractions (Chapter 2.3) were carried out on each sample and each control and then the extraction was tested in RT-LAMP assays with the CH2 primer (Chapter 2.4). Samples that tested positive with RT-LAMP were sent to Fera Science Ltd where ELISA tests were carried out and sap inoculations onto tomato seedlings and *N. benthamiana* plants were conducted to determine the viability of the virus detected.

2.3 RNA Extraction

In preparation for extraction, screw-cap micro-Eppendorf tubes were filled with approximately 30 2 mm and approximately five 5 mm glass beads and autoclaved. On arrival of the samples at Sutton Bonington, 30 mg of each sample was weighed out and placed into one of the autoclaved tubes. All samples contained within the micro-Eppendorf tubes were then placed in liquid nitrogen for five minutes. Subsequently, the micro-Eppendorf tubes were placed in a Fastprep FP120 (Thermo-Savant) and processed at 6.5 ms^{-1} for 45 seconds in order to homogenise the samples. Following homogenisation, RNA extraction from the samples was carried out using the Promega SV Total RNA Isolation kit, following the manufacturer's instructions with one modification; for the final elution step, 35µl of nuclease-free water was used instead of 100 µl. The extractions were stored at -80°C until ready for use.

2.4 The RT-LAMP Assay

Primer sets thought to be genotype-specific for the CH2, EU and US1 genotypes (as can be seen in Table 2.2) of PepMV, published by Ling *et al.* (2013), were purchased. Each primer set for each genotype was made up into a primer mix containing 152 µl of distilled water, 4 µl of 100 µM B3 primer, 4 µl of 100 µM F3 primer, 20 µl of 100 µM FIP, 20 µl of 100 µM BIP, 20 µl of 100 µM LF primer and 20 µl of 100 µM LB primer. For the RT-LAMP reaction mixture, each reaction consisted of 3.2 µl of distilled water, 3 µl primer mix (as above), 15 µl of Isothermal Mastermix (Optigene, containing a DNA polymerase, optimised reaction buffer, Mg_2SO_4 and dNTPs), 0.5 µl of RNAse inhibitor (Applied Biosystems), 1.3 µl of M-MLV reverse transcriptase (200 units/µl, Sigma-Aldrich) and 1.5 µl of RNA sample (from RNA extraction), giving a total reaction volume of 24.5 µl. The reaction mixtures were then placed in a Genie II or Genie III platform and heated at 50°C for 10 minutes (reverse-transcription step), 65°C for 30 minutes (amplification step), followed by an anneal step ranging from 98-80°C. The Optigene Isothermal Mastermix allowed for fluorescence detection of any product made during the RT-LAMP reaction.

Table 2.2- Primer sequences for the CH2, EU and US1 genotypes of PepMV (Ling *et al.*, 2013).

PepMV Genotype	Primer Type	Primer Sequence
CH2	F3	5′-CGATGAAGCTGAACAACATTTCC-3′
	FIP	5′-CTTAATGGGTTGATCTTGGTGGAAGCTGTGAGAAAGCTTCACAAAC-3′
	BIP	5′-GGGTTAAGTTTTCCCCAGTTTGAAAATTCCTTCAGTGTTAATCTTGTG-3′
	B3	5′-TCCAGCAATTCCGTGCACAACAA-3′
	Loop F	5′-GGCCTCGCCTTGATGGA-3′
	Loop B	5′-TGGAAAGATCAACTTTGATCAATT-3′
EU	F3	5′-ACCAAGAAGATACAAAATTTGC-3′
	FIP	5′-TRAGACCATCAGCAGGCTGCTGCATTTGACTTCTTCGATG-3′
	BIP	5′-TCAGGCARCCAAATGAGAAAGAAACCTGTGGAGATCTTTTGC-3′
	B3	5′-TGACTTCTCCAAGTGTGG-3′
	Loop F	5′-TGGCAGGGTTGGTGACTC-3
	Loop B	5′-CTAGCTGCTCACTCCGTAGCTAA-3′
US1	F3	5′-GCATTCATACCAAATGGGAG-3′
	FIP	5′-TGCGAACAGCCAAGAAATGT-ATAAATTGCATGAATACCTTACTCC-3′
	BIP	5′-TTGCACAAACTCCACCAAGGACTTAACCCGTCAATGTGTT-3′
	B3	5′-CCATTTCGAACAGGGGAA-3′
	Loop F	5′-TGCTCAGCTTCATCA-3′
	Loop B	5′-TGAAGCCATGAGACTT-3′

Chapter 3: Validation of Genotype-Specific PepMV RT-LAMP Assays and the Occurrence of Mixed-Genotype PepMV Infection in UK Tomato Crops

3.1 Introduction

As previously mentioned (Chapter 1.3), after the first emergence of PepMV in Europe in 1999, in the Netherlands, the virus has become widespread in many European and non-European countries. PepMV represents the most economically threatening pathogen to tomato production in the UK, inducing a wide range of symptoms, some of which can result in reduction of fruit quality and yield loss. Fruit symptoms, such as fruit marbling, are the main cause of fruit quality degradation; however, PepMV does not always induce symptoms and so infection can often be symptomless. Symptomless infection coupled with the highly contagious nature of the virus makes avoidance of the transmission of PepMV extremely difficult.

The availability of rapid on-site diagnostic tests for PepMV will enable growers to identify both symptomatic and symptomless PepMV infection in their crops. Efficient diagnosis of PepMV-infected crops will enable growers to take the appropriate action, such as the implementation of hygiene procedures, to prevent PepMV dissemination to other crops on the site and to tomato crops elsewhere. As discussed in Chapter 1.11.3, the RT-LAMP method represents a promising diagnostic tool for on-site PepMV identification. Ling *et al*. (2013) designed RT-LAMP primers capable of discriminating between the CH2, EU and US1 genotypes of PepMV. Genotype-specific primers, such as these, allow for the identification of mixed-genotype infections in samples which may induce more severe symptoms than crops infected by a single genotype (Hanssen *et al.*, 2009).

In this study, the Ling *et al.* (2013) primers were tested on a variety of leaf and fruit samples from tomato crops from different sites in the UK. New primers were designed to compare their efficiency and specificity to the Ling *et al*. (2013) primers. A genotype-specific RT-LAMP assay would provide tomato growers with a tool to determine the occurrence of mixed-genotype infection in their crops and would allow insight to be gained on the PepMV genotypes which are currently present in UK tomato crops. Symptom assessment of PepMV-infected tomato crops at different UK sites and subsequent RT-LAMP testing could provide

a potential explanation of why different crops exhibit differing symptoms and different degrees of symptom severity.

3.2 Methods

3.2.1 Validation of Previously Published RT-LAMP Primers

3.2.1.1 Testing the Ling *et al.* (2013) Primers in RT-LAMP Assays

The Ling *et al.* (2013) primers (Chapter 2, Table 2.2) were purchased and used in RT-LAMP reactions. Initially, tomato leaf and fruit samples were collected from Sutton Bonington glasshouses and RNA extractions (Chapter 2.3) were carried out on these samples which were then tested in RT-LAMP reactions (Chapter 2.4) with each of the primer sets. In order to validate each of the genotype-specific primers, samples infected with one known genotype (CH2, EU or US1) of PepMV needed to be tested with each of the primers to ensure that there was no cross-reactivity between the primer sets. To allow for this, purified PepMV RNA samples were obtained from Fera Science Ltd; six of which were the EU genotype and six of which were the US1 genotype. All of the samples obtained from Fera Science Ltd were tested with the CH2, EU and US1 primers. More tomato fruit and leaf samples for testing were provided by the various tomato growers involved in the project.

3.2.1.2 Designing Alternative RT-LAMP Primers

Novel primers for each PepMV genotype were created and their efficiency at detecting PepMV was evaluated and compared to the Ling *et al.* (2013) primers. Sequence alignment was used to identify regions that differed between the sequences of CH2, EU and US1 isolates in order to create three sets of primers that would each be specific to one genotype. The sequence information was imported into LAMP Designer 1.10 (Premier Biosoft) and the primers (Table 3.1) were purchased and used to test PepMV RNA samples in RT-LAMP assays. In total, two alternative CH2 primer sets were designed, and one alternative primer set each for the EU and US1 genotypes.

Table 3.1- Alternative PepMV RT-LAMP Primers.

PepMV Primer set	Primer Type	Primer Sequence
CH2 Alternative 1	F3	5′- TTGCTTCACATGCCTTACA –3′
	FIP	5′–ACCTTGGGTGCTGTTAAACCTTGAGTGCTATGTTAGACATTGGA–3′
	BIP	5′-TCCTTGATAACCACACGCAACAATGAAGTGGATGGATCAACTG-3′
	B3	5′-TTCTGAATTGTTGGGACCAG-3′
	Loop F	5′-CAACCAGCATAGGTCATGGA-3′
	Loop B	5′-GTACACCTGTCTGTCTCGC-3′
CH2 Alternative 2	F3	5′-GCTGCGACCATATACAAAGA-3′
	FIP	5′-GTGAGTGTACTCCACTTGCTGGTCAAACAAGGCGTAGTAACAT-3′
	BIP	5′-AACCCCACCGGAGCTATGAGGAACTTTCAGGTACATCCTC-3′
	B3	5′-CATGTTCAGCAGATGACATTG-3′
	Loop F	5′-TCCGCTTCTTTGAAATCAGCTA-3′
	Loop B	5′-CCATCAACAATCTTGGCATTGA-3′
EU Alternative	F3	5′-TAGGTCTTTCTGCACTTGATG-3′
	FIP	5′-TCATGAATGGCCGCAGCAACAGTCATATACACTGCAATGA-3′
	BIP	5′-TAGCTCCTCAACTCCCAGCAGGATGAAGTGTCTGAAGCAT-3′
	B3	5′-TTCTCAATCTCACGGTTCTTC-3′
	Loop F	5′-TGTCGTCATCAGTCATTGCTAA-3′
	Loop B	5′-TGCCACACAAGACATGGG-3′
US1 Alternative	F3	5′-CTGGTCAAGCAAGGAGTG-3′
	FIP	5′-AGTCACACAATCAACTGGTGGTTTTCAATCTGAAGAGCCCAAA-3′
	BIP	5′-AAGAACCTTGGCCTGTCTGAACGAACAGAGTTTCTAGCGTATGA-3'
	B3	5′-TTGTCAGCATCAGTCATAGC-3′
	Loop F	5′-CTCGCTCGAATTCGGTGTA-3′
	Loop B	5′-AGTGGATCTGCCAGAATGC-3′

3.2.1.3 cDNA Synthesis and PCR

Cloning was carried out using two of the EU samples and two of the US1 samples from Fera Science Ltd. cDNA synthesis was conducted on each of the four RNA samples using the F3 and B3 primers (10 µM) of the Ling *et al.* (2013) EU primer set. The appropriate negative controls were also used. cDNA synthesis was conducted using 1 µl of 10 nM dNTPs, 1 µl of F3 (10 µM) primer, 1 µl of B3 (10 µM) primer and 7 µl of RNA sample per reaction. The mixture was added to a PCR tube and incubated at 70°C for ten minutes, followed by 5 minutes of incubation on ice. During incubation, 6.5 µl of nuclease-free water, 2 µl of M-MLV reverse transcriptase buffer (Sigma-Aldrich), 0.5 µl of RNase inhibitor (Applied Biosystems) and 1 µl of M-MLV reverse transcriptase (200 units/µl, Sigma-Aldrich) was added to an Eppendorf. This 10 µl mixture was then added to the PCR tube containing the RNA sample mixture. The 20 µl mixture was then incubated at 25°C for 10 minutes, 37°C for 50 minutes and 80°C for 10 minutes.

PCR was then conducted on each cDNA sample. 8.5 µl of distilled water, 12.5 µl of 2xMangomix (Bioline), 1 µl of F3 primer and 1 µl of B3 primer (10 µM) from the Ling *et al.* (2013) RT-LAMP EU primer set and 2 µl of cDNA sample were added into a PCR tube. All PCR reactions were processed in a BioRad® S1000 thermal cycler programmed with the following steps: 94°C for 5 minutes followed by 35 cycles of; 94°C for 1 minute, 50°C for 1 minute and 72°C for 2 minutes; followed by a final elongation step at 72°C for 7 minutes. All PCR products were visualised using gel electrophoresis, with a 1.5% agarose gel made from 1XTris-Borate-EDTA (TBE) buffer and stained with 1-5 µl of ethidium bromide (Sigma-Aldrich) per 100 ml of agarose gel. Three µl of a 1 Kb DNA ladder and 5 µl of each PCR sample was added to a well in the agarose gel and run at 120 volts for 30 minutes in a BioRad® PowerPac Basic™. The gel was then viewed in a gel documentation system (InGenius3 by Syngene, UK). Following this, PCR products were purified using the QIAquick PCR Purification Kit (Qiagen).

3.2.1.4 Cloning and Sequencing

The ligation step of the cloning was conducted using the Pgem Vector System (Promega). For each sample, 2.5 µl of 2x rapid ligation buffer, 0.5 µl of vector, 0.5 µl of T4 ligase and 1.5 µl of purified PCR product were added to an Eppendorf tube. This mixture was incubated overnight at 4°C. The following day, competent cells (*E. coli*) were thawed on ice and then 50 µl of competent cells

were added into each of the ligation mixtures. The combined mixture was mixed gently by pipetting and then incubated on ice for 15-20 minutes. The mixtures were then incubated at 42°C for 50 seconds and, following this, were placed immediately onto ice and left for 5 minutes. Seven hundred µl of LB liquid media was then added to each Eppendorf tube and these were incubated in a 37°C shaking incubator for 90 minutes.

LB agar plates were prepared by melting the agar in the microwave. Once melted and cooled, 50 µl of IPTG (0.1 M), 120 µl of X-gal solution (0.05 M) and 100 µl of ampicillin (100 mM) were added to the media per 100 ml of melted LB agar. The LB agar was then poured into Petri dishes (100 ml of LB agar made 4 plates). The plates were left to solidify and then 50 µl and 100 µl of each sample mixture was added onto a separate agar plate and spread with an L-shaped spreader. The plates were incubated at 37°C overnight.

The following day, PCR reactions were set up consisting of 12.5 µl 2xMangomix (Bioline), 1 µl of M13 forward primer (10 µM), 1 µl of M13 reverse primer (10 µM) and 10.5 µl of distilled water per reaction. A pipette tip was used to pick a white colony off of each plate and add it to the PCR mixture. PCR was conducted on 10 transformed colonies per sample. The PCR reaction was run at: 94°C for 3 minutes, followed by 30 cycles of; 94°C for 40 seconds, 58°C for 30 seconds and 72°C for 1 minute; followed by a final elongation step at 72°C for 10 minutes. PCR products were visualised using gel electrophoresis and a gel documentation system (see Chapter 3.2.1.3). PCR products were purified using the QIAquick PCR Purification Kit (Qiagen) and the purified products were sent to Eurofins for sequencing. NCBI Nucleotide BLAST was used to identify what PepMV genotype the nucleotide sequences belonged to (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE_TYPE=BlastSearch).

3.2.2 Symptom Assessment and Sampling of UK Tomato Crops

Symptom assessment was conducted on six different tomato crops within the UK with suspected or confirmed PepMV infection, as described in Chapter 2.1.1. Four of the crops were visited on two separate occasions approximately one month apart and the two remaining crops (with severe symptoms) were visited once. After the symptom assessment, approximately 30 leaf/fruit samples were collected (see Chapter 2.2.1) at each site and posted to Sutton Bonington for RNA extraction (Chapter 2.3) and RT-LAMP testing with the Ling *et al.* (2013)

CH2, EU and US1 primers (Chapter 2.4). This allowed for the confirmation of whether or not PepMV infection was present in each crop and, if infection was present, what PepMV genotype the crop was burdened with.

3.3 Results

3.3.1 Validation of RT-LAMP Primers

The Ling *et al.* (2013) primers were successful at detecting PepMV infection. RT-LAMP tests revealed that tomato plants on Sutton Bonington were infected with the CH2 genotype of PepMV. The amplification curves obtained from the RT-LAMP tests can be seen in Figure 3.1. The sample in well 4 amplified the fastest, after approximately 10 minutes. No amplification was achieved when testing the Sutton Bonington samples with the EU and US1 primers. In order to validate the Ling *et al.* (2013) and additional EU and US1 primers, EU and US1 RNA samples were obtained from Fera Science Ltd and tested with all primer sets. The RT-LAMP results from these tests can be seen in Table 3.2.

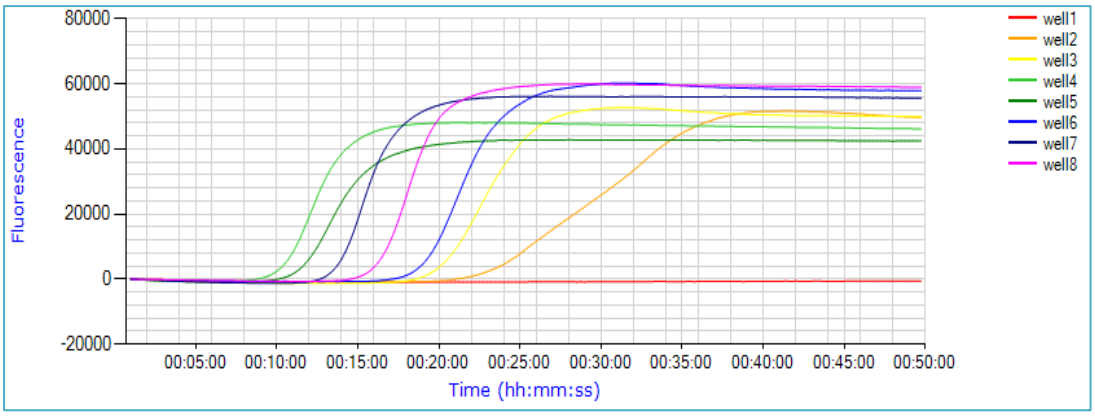

Figure 3.1- RT-LAMP amplification curves for Sutton Bonington glasshouse samples tested using the CH2 Ling *et al.* (2013) primer. Well 1= negative control, wells 2-8= RNA samples.

Table 3.2- RT-LAMP results for EU and US1 PepMV samples using Ling *et al.* (2013) and alternative PepMV primers.

	Amplification time with each primer (mm:ss)						
Sample	**Ling CH2**	**Alt CH2 1**	**Alt CH2 2**	**Ling EU**	**Alt EU**	**Ling US1**	**Alt US1**
EU 1	20:00	13:15	X	04:30	06:00	15:30	08:45
EU 2	26:45	17:45	X	04:15	05:45	15:30	08:30
EU 3	X	X	X	10:45	10:30	17:45	08:30
EU 4	X	X	X	03:30	05:00	17:30	10:00
EU 5	X	14:45	X	05:30	07:15	15:30	11:00
EU 6	25:15	15:00	X	11:30	11:15	16:15	14:15
US1 1	24:25	14:30	16:45	10:15	11:45	06:45	03:15
US1 2	27:30	14:15	X	10:30	14:15	05:15	02:30
US1 3	X	X	X	11:00	12:15	05:15	02:00
US1 4	X	X	X	11:30	12:15	06:15	03:00
US1 5	X	15:30	X	11:45	12:00	06:00	02:30
US1 6	X	X	X	14:30	21:15	06:15	03:00

X= no amplification

From Table 3.2 it can be seen that the Ling EU primer amplified all six EU samples rapidly, with the majority of the EU samples amplifying within 5 minutes using this primer. Two of the EU samples amplified more slowly (after 10 minutes) when using the Ling EU primer. This pattern is also reflected when using the alternative EU primer, with sample EU 3 and EU 6 amplifying after at least 10 minutes. RT-LAMP can be used quantitatively if a sample with a known viral RNA titre is used to create a standard curve by testing serial dilutions of the sample with RT-LAMP. This would enable the visualisation of how RNA concentration influences amplification time. A slower amplification time indicates that a lower RNA titre is present within the sample. Although a standard was not created in this experimental design and so specific quantification of viral concentrations was not possible, this concept was used to hypothesise that lower viral titres were responsible for the slower amplification times seen for the EU 3 and EU 6 samples.

The Ling and alternative US1 primers were successful in amplifying the US1 samples; the alternative US1 primer gave particularly rapid amplification, with samples showing as a positive result after 2 or 3 minutes. However, it can also

be seen from Table 3.2 that the EU and US1 samples were amplified by both the EU and the US1 primers. The EU primers amplified the US1 samples approximately two times slower than they amplified the EU samples. The US1 primers amplified the EU samples approximately three to four times slower than the US1 samples. There were two possible explanations for this pattern seen with the EU and US1 primers. Firstly, it was possible that cross-reactivity existed between the two primer sets, meaning that the EU and US1 primers detected both EU and US1 infection and so the primers were not genotype specific. Alternatively, the EU and US1 samples could have been contaminated with low titres of US1 and EU RNA, respectively. This would result in the samples giving positive results for both primers.

From Table 3.2 it can be seen that the CH2 primers detected some of the EU and US1 samples. The Ling CH2 primer detected three EU samples and two US1 samples, but at very slow amplification times of at least 20 minutes. The alternate CH2 1 primer detected the same samples as the Ling CH2 primer, plus an additional EU and US1 sample. These amplification results suggest that the EU and US1 samples are contaminated with some RNA of the CH2 genotype. The alternative CH2 2 primer only amplified one US1 sample, suggesting it was a less sensitive primer than the other two CH2 primers.

The anneal temperature can be used as a way of validating what genotype is present within a sample. After amplification, the RT-LAMP reaction involves an anneal step and the temperature at which the double stranded DNA re-anneals is displayed via florescence. Table 3.3 shows the anneal temperatures given by samples which tested positive for all three genotypes of PepMV. From this data it can be seen that the primers all gave different anneal temperatures; with the CH2 primer giving an anneal temperature of between 84-85°C, the US1 of approximately 86°C and the highest temperature (approximately 87°C) being given by the EU primer.

Table 3.3- RT-LAMP anneal temperatures given by the different Ling *et al.* (2013) PepMV primers when using Genie II.

	Anneal temperatures (°C) given from each primer		
Sample	**CH2**	**EU**	**US1**
1	85.00	87.34	86.10
2	84.84	87.43	86.09
3	84.94	87.52	86.24
4	84.85	87.48	86.10
5	84.79	87.47	86.13
6	84.77	87.30	85.96

However, when using different Genie machines (Genie II versus Genie III) it was noticed that the anneal temperature given by each primer varied between the machines. This is due to temperature differences between the heating blocks of the two machines. The Genie machines that were used in this study exhibited an approximate 2°C temperature difference, with the Genie III platform being approximately 2°C lower than the Genie II machine, as can be seen for the CH2 and EU primers in Figure 3.2.

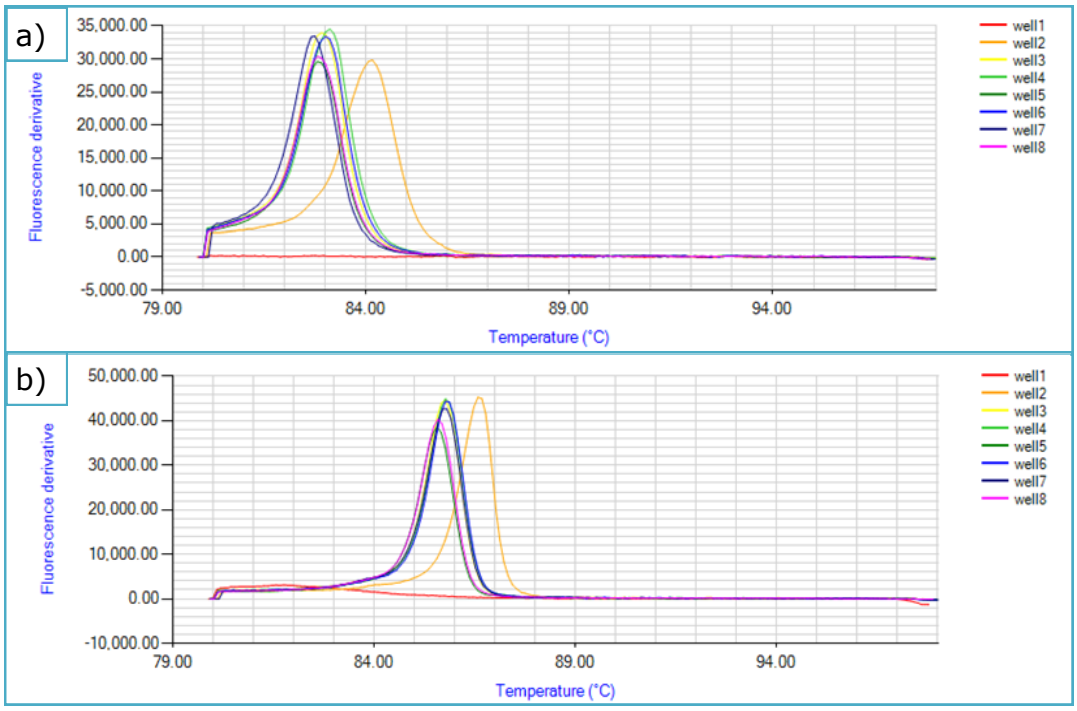

Figure 3.2- RT-LAMP anneal temperatures given for the same samples on Genie III for the CH2 (a) and EU (b) Ling *et al.* (2013) primers, approximately 2°C

lower than the temperatures given by the Genie II machine. Well 1= negative control, well 2= positive control, wells 3-8= PepMV samples 1-6.

As the EU and US1 Fera Science Ltd samples amplified with the EU and US1 Ling *et al.* (2013) primers (Table 3.2), cloning and sequencing was conducted to determine if the samples contained contaminating RNA. The results from cloning two EU samples and two US1 samples and sequencing ten transformed colonies for each sample can be seen in Table 3.4. The sequence results obtained from 20 colonies from cloning of the EU samples represented the EU genotype. This was also reflected in the US1 samples, with all colonies sequenced from the US1 samples belonging to the US1 genotype. These results suggest that there was no contaminating RNA present in the RNA samples. However, it may be that the contaminating RNA was present at a low level within the Fera samples, and so it may have been necessary to sequence many more transformed colonies per sample in order to find the contaminating RNA.

Table 3.4- Cloning and sequencing results of PepMV EU and US1 samples.

PepMV Sample	Number of transformed colonies sequenced	Genotype result of all colonies according to NCBI Nucleotide BLAST
EU 5	10	EU
EU 6	10	EU
US1 2	10	US1
US1 5	10	US1

As more tomato leaf and fruit samples were tested for PepMV infection, it became clear that there was no cross-reaction between the EU and US1 primers. A set of samples sent from Site 4 involved in the project (see Chapter 3.3.2) were tested with the CH2, EU and US1 Ling *et al.* (2013) primers. The RT-LAMP results can be seen in Figure 3.3 below. As can be seen from the figure, the samples from Site 4 showed amplification with the CH2 and EU primers but no amplification with the US1 primer (Appendix 1). If cross-reactivity existed between the EU and US1 primer, amplification would have also been achieved when using the US1 primer. It was therefore concluded that the Ling *et al.*

(2013) primers were genotype specific and low levels of contaminating RNA were present in the EU and US1 samples.

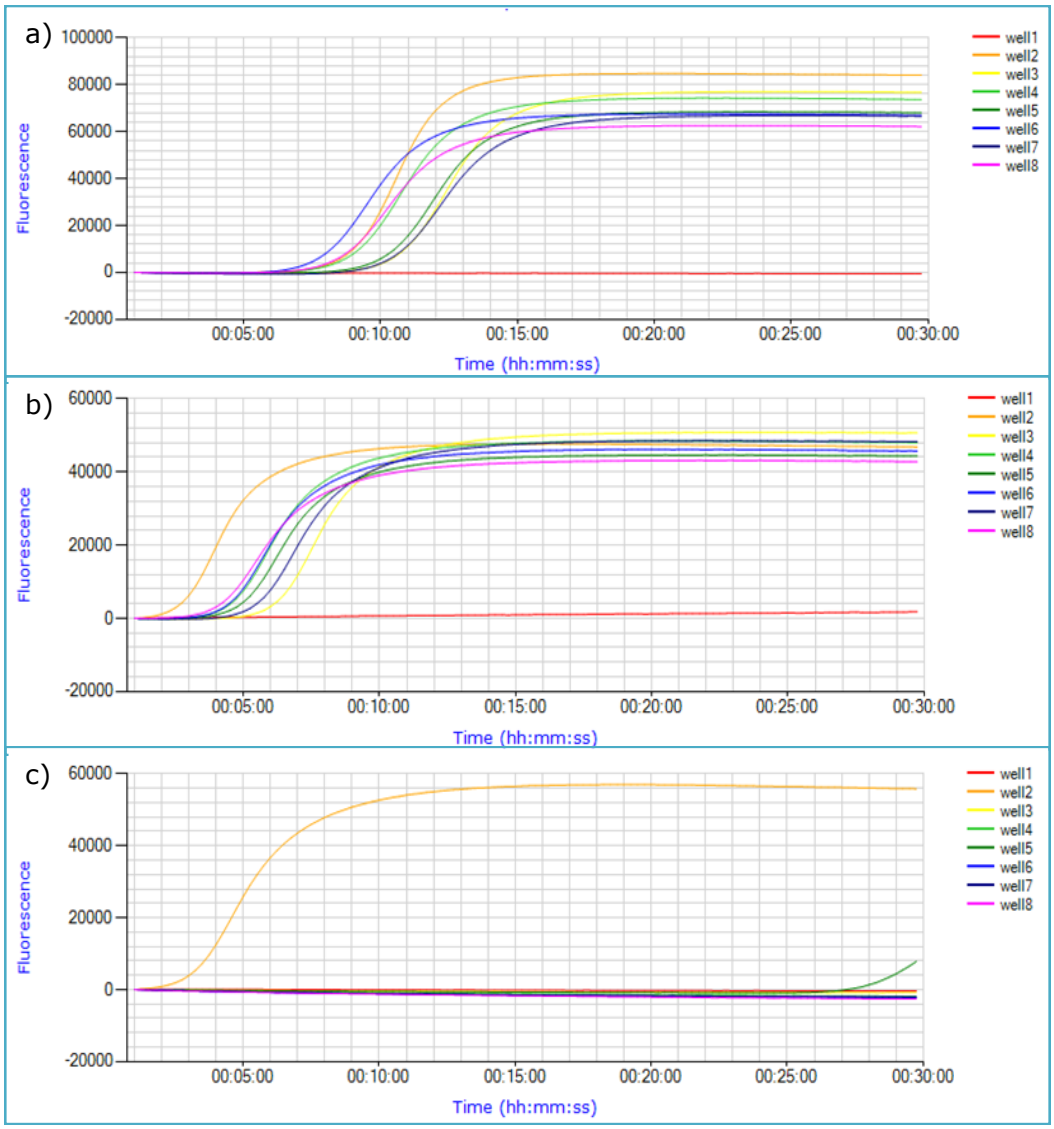

Figure 3.3- RT-LAMP amplification curves for six samples from Site 4 tested with the CH2 (a), EU (b) and US1 (c) Ling *et al.* (2013) primers. Well 1= negative control, well 2= positive control, wells 3-8= samples 1-6.

3.3.2 Assessment of PepMV Symptoms

The location and date of each symptom assessment/sample collection visit to each crop is displayed in Table 3.5, along with information on the appearance of the first PepMV symptoms and what tomato variety was affected. For anonymity, the site names have been replaced with numbers. Two visits were made to four of the crops and one visit was made to each of the two remaining crops where severe PepMV symptoms had been reported. Two different crops were visited at two of the sites (Sites 1 and 2). At each visit to each crop, a symptom assessment was conducted using a PepMV symptom rating scale adapted from Hanssen *et al.* (2009) and images from O'Neill (2014), as can be seen in Table 2.1 and Figure 1.4, respectively. At each visit to each crop, 100 tomato plants were selected at random and each plant was assessed on one stem for each symptom criteria. A rating of zero was given if a specific symptom was not present on the plant, and a rating of three was given if that symptom was particularly severe. The symptom assessments for each crop are summarised in Table 3.6.

Table 3.5- Symptom assessment and sampling visit information from tomato sites visited in the UK.

	Crop Number					
	1	**2**	**3**	**4**	**5**	**6**
Site name	1	2	3	1	2	4
Assessment and sampling dates	Visit 1: 09/09/15	Visit 1: 18/09/15	Visit 1: 19/10/15	Visit 1: 08/03/16	Visit 1: 15/03/16	Visit 1: 08/06/16
	Visit 2: 07/10/15	Visit 2: 12/10/15		Visit 2: 12/04/16	Visit 2: 20/04/16	
Appearance of first symptoms	Spring	Spring	Spring	Week 6 after planting	Not seen on first visit to crop, appeared on site at beginning of April 2016	November, crop 6 was an over-winter crop
Tomato variety/ Scion	Piccolo/ Maxifort	Roterno/ Maxifort	Piccolo/ Maxifort	Piccolo/ Maxifort	Sunstream/ Maxifort	Piccolo/ Emperador
Growing medium	Organic (soil)	Rockwool	Rockwool	Organic (soil)	Rockwool	NFT

Table 3.6- Symptom assessment summaries for 100 plants from each visit to each UK tomato crop.

	Crop 1				Crop 2				Crop 3		Crop 4				Crop 5				Crop 6	
	Visit 1		Visit 2		Visit 1		Visit 2		Visit 1		Visit 1		Visit 2		Visit 1		Visit 2		Visit 1	
Criteria Assessed	**I**	**S**	**I**	**S**	**I**	**S**	**I**	**S**	**I**	**S**	**I**	**S**	**I**	**S**	**I**	**S**	**I**	**S**	**I**	**S**
Chlorotic spots	26	1.08	17	1.12	54	1.07	46	1.13	40	1.65	67	1.43	25	1.1	7	1	10	1	17	1.2
Necrotic spots to leaf death	55	1.09	47	1.11	0	0	1	1	24	1.08	1	1	65	1.4	0	0	8	1	60	1.4
Fruit marbling	3	1	1	1	28	1.11	25	1.32	54	1.76	0	0	11	1	0	0	3	1	17	1.2
Fruit flaming	0	0	0	0	19	1.16	22	1.27	3	1	0	0	11	1	0	0	5	1	9	1
Open fruit	0	0	0	0	3	2	4	1.75	3	1.67	0	0	1	1	3	1	5	1	3	1
Petiole symptoms	14	1	25	1.08	7	1	10	1	17	1.06	32	1.69	13	1.4	5	1.4	30	1.3	39	1.5
Stem symptoms	9	1	0	0	3	1	0	0	3	1	9	1	0	0	0	0	4	1.5	6	1.5
Head symptoms	30	1	0	0	6	1	10	1.8	27	1.48	64	1.9	49	1.4	4	1	33	1.2	34	1.5
	Visit 1		**Visit 2**		**Visit 1**		**Visit 2**		**Visit 1**		**Visit 1**		**Visit 2**		**Visit 1**		**Visit 2**		**Visit 1**	
Number of trusses affected	3		1		49		50		92		0		23		3		11		33	
Total trusses with red fruit	222		210		216		109		234		0		199		175		192		281	
Proportion of trusses with symptomatic fruit (%)	1.35		0.48		22.69		45.87		39.32		0		11.56		1.71		5.73		11.7	

I= Incidence (%) of plants possessing the particular symptom **S=** Severity, average 0-3 rating of those plants affected with the symptom

Table 3.6 summarises the incidence (percentage of plants seen with each symptom) and severity (average 0-3 symptom rating of those plants affected with the symptom) of the PepMV symptoms observed at each visit to each crop. The symptom assessment from crop 1 indicates that necrotic spots (Figure 3.4) were the most commonly observed symptom on both visits, affecting approximately 50% of the plants assessed on both occasions. Fruit symptoms were not particularly pronounced in crop 1, with fruit marbling affecting only 3% and 1% of plants assessed on visit 1 and 2, respectively. The lack of fruit symptoms is also highlighted by the fact that only 1.35% and 0.48% of the trusses possessing red fruit from visits 1 and 2, respectively, exhibited fruit symptoms.

Figure 3.4- Tomato leaf from crop 1 exhibiting necrotic spots

All three fruit symptoms were observed on crop 2, resulting in 22.69% and 45.87% of the red-fruited trusses possessing symptomatic fruit, respectively. The second visit to crop 2 saw the greatest proportion of symptomatic fruit out of all crops assessed throughout the investigation. The incidences of each symptom seen in crop 2 remained relatively consistent in the time period between the two visits. Chlorotic spots were present on approximately 50% of the plants on both occasions. In contrast to crop 1, necrotic spots were not

common in crop 2, being observed on only one plant on the second visit to crop 2.

The most commonly observed symptom in crop 3 was fruit marbling, which affected over 50% of the plants assessed. This was the highest incidence of fruit marbling seen out of all of the assessments. Fruit exhibiting the marbling symptom in crop 3 had the highest average severity (1.76) out of all visits to all of the crops. The other fruit symptoms (flaming and open fruit) were not commonly observed in crop 3, with each being present on only three plants.

The most widespread symptoms seen on visit 1 to crop 4 were chlorotic spots (Figure 3.5) and head symptoms (such as nettle head), affecting 67% and 64% of the plants assessed, respectively. The incidence of chlorotic spots was reduced to affecting only a quarter of the plants on the second visit to crop 4. However, necrotic spots became more frequent on the second visit, affecting 64 more plants on visit 2 than it affected on visit 1, making necrotic spots the most profound symptom on the second visit to the crop. Interestingly, no fruit symptoms were seen on the first visit to crop 2; however, by the time of the second visit, all three fruit symptoms (marbling, flaming and open fruit) could be observed at a low incidence, possibly because there had been more time for fruit symptoms to develop. These fruit symptoms were present on 11.56% of the trusses possessing red fruit on visit 2.

Figure 3.5- Tomato leaf from crop 2 exhibiting chlorotic spots

From Table 3.6, it can be seen that, on the first visit to crop 5, only a small percentage of the plants assessed exhibited PepMV symptoms, with the most common symptom (chlorotic spots) affecting only 7% of the plants. It was noted on the first visit to crop 5 that there were not many observable symptoms on the crop; however, PepMV had been seen on site in other crops. On the second visit to the crop, the incidence of suspected PepMV symptoms had increased slightly, with all symptom types being able to be recognised on the crop. The symptoms that increased in incidence in particular were petiole and stem symptoms (such as twisted/distorted tissue), with the incidence of petiole symptoms being six times higher on visit 2 compared to what was seen on visit 1. Despite the fact that symptom incidence had increased in crop 5 in the time between the two visits, the majority of symptom incidences remained relatively low, with fruit symptoms affecting only 5.73% of the trusses possessing red fruit. Crop 5 was located on the same site as crop 2; however, fewer symptoms were observed in crop 5, particularly the chlorotic spot symptom which affected approximately 50% of plants in crop 2 on both visits, compared to only 7% and 10% in crop 5 on visits 1 and 2, respectively.

The symptom of highest incidence in crop 6 was necrotic spots, which were observed on 60 of the plants assessed. Petiole symptoms were present on 39% of plants in crop 6, meaning that this crop possessed the greatest incidence of petiole symptoms out of all crops involved in the study. All three fruit symptoms could be observed in crop 6, with fruit marbling being the most common fruit symptom, affecting 15% of the plants assessed in this crop.

No symptoms were present in any of the crops assessed at an average severity rating reaching 2 or above. In order to determine which crops had the most severe symptoms, Table 3.7 was produced which displays the proportion of plants exhibiting fruit symptoms with severity ratings of 2 and above instead of the average severity rating of all plants possessing each fruit symptom. From Table 3.7 it can be seen that only three crops, crops 2 (Site 2), 3 (Site 3) and 6 (Site 4), had symptom severity ratings of 2 and over. On average, fruit marbling was the most common fruit symptom present with a severity rating of 2 or greater. The occurrence of foliar symptoms with severity ratings of 2 or above was more common, with all crops containing plants exhibiting at least of one the symptoms with severity 2 or greater, as can be seen in Table 3.8. Stem symptoms appeared to be the least severe across all crops, with stem symptoms with a severity of 2 or greater found in low numbers in crops 5 and 6. The most

severe head symptoms were seen on the first visit to crop 4, with 37% of the plants exhibiting head symptoms with a severity rating of 2 or above. Crop 6 possessed plants with severity ratings of 2 or higher for all foliar symptoms assessed, this was not seen in the other five crops. When comparing Table 3.7 and 3.8, it can be concluded, overall, that foliar symptoms were more severe than the fruit symptoms in all crops.

Table 3.7- Summary of fruit symptoms in six crops with severity ratings of two or above.

		Proportion (%) of plants with a severity rating ≥ 2			
Crop	**Visit**	**Marbling**	**Flaming**	**Open Fruit**	**Proportion of trusses with an index ≥ 2 (%)**
1	1	0	0	0	0
	2	0	0	0	0
2	1	3	3	2	6
	2	7	5	3	9
3	1	19	0	1	19
4	0	0	0	0	0
	0	0	0	0	0
5	1	0	0	0	0
	2	0	0	0	0
6	1	3	0	0	9

Table 3.8- Summary of foliar symptoms in six crops with severity ratings of two or above.

		Proportion (%) of plants with a severity rating ≥ 2				
Crop	**Visit**	**Chlorotic spots**	**Necrotic spots**	**Petiole symptoms - distortion**	**Stem symptoms -distortion**	**Head symptoms -nettle head**
1	1	2	5	0	0	0
	2	2	5	2	0	0
2	1	4	0	0	0	0
	2	0	0	0	0	7
3	1	18	2	1	0	8
4	0	20	0	14	0	37
	0	3	21	4	0	18
5	1	0	0	2	0	0
	2	0	0	8	2	4
6	1	2	22	15	2	13

3.3.3 Occurrence of Mixed-genotype PepMV Infections in UK Tomato Crops

After conducting RNA extractions, the 30 samples (approximately) collected at each symptom assessment visit to each crop were tested for the presence of the CH2, EU and US1 genotypes of PepMV using the Ling *et al.* (2013) primers in RT-LAMP assays. The results from the RT-LAMP assays are summarised in Table 3.9, showing the proportion of each PepMV genotype that was present in each of the crops. As can be seen in Table 3.9, all sites possessed tomato plants with PepMV infection.

When tested in RT-LAMP assays, all samples from crops 1, 2, 3, 4 and 6 tested positive for CH2 infection according to the Ling *et al.* (2013) primers. The crop with the lowest incidence of PepMV infection was crop 5, with CH2 infection being detected in approximately 54% of samples from visit 1 and 30% of samples from visit 2. Crops 1 and 5 were infected with the CH2 genotype only. Mixed genotype PepMV infections were detected in crops 2, 3, 4 and 6. Crop 3 exhibited the greatest incidence of mixed infection, with almost 100% of samples testing positive for all three PepMV genotypes. One hundred % of

samples from crop 6 showed mixed infection with the CH2 and EU genotypes, the US1 genotype was only present in one sample from this crop. Similarly, the US1 genotype was detected, along with the CH2 genotype, in two samples from crop 2 and one sample from crop 4. From Table 3.9 it can be seen that there is no difference in the samples in which PepMV infection can be found, with PepMV being detected in symptomatic, symptomless, leaf and fruit samples.

Figure 3.6 was created in order to visualise the type of PepMV infection found (single or mixed infection) in all samples collected from all of the crops combined. From this Figure it can be seen that the CH2 genotype is the most common genotype found in tomato plants from the four different sites in the UK, with the single genotype CH2 infecting over 60% of the total number of tomato plants assessed. Mixed infection was found in approximately 20% of plants in total, with the most frequent type of mixed infection being caused by all three genotypes, closely followed by mixed infection by the CH2 and EU genotypes. Mixed infection by the CH2 and US1 genotypes only was less regular, being found in less than 2% of the samples. The EU and US1 genotypes did not exist as single infections. Approximately 13% of the plants assessed were free from PepMV infection.

Table 3.9- Proportion (%) of PepMV-positive samples of each genotype from each crop according to the Ling *et al.* (2013) RT-LAMP primers.

		Proportion (%) of PepMV-positive samples for each genotype									Total proportion (%) of Pep-MV infected samples
		Symptomless plants			Symptomatic plants			Symptomatic tissue			
		CH2	EU	US1	CH2	EU	US1	CH2	EU	US1	
Crop 1 (Site 1)	Visit 1	100	0	0	100	0	0	100	0	0	100
	Visit 2	100	0	0	100	0	0	100	0	0	100
Crop 2 (Site 2)	Visit 1	100	0	0	100	0	10	100	0	0	100
	Visit 2	100	0	3.33	100	0	0	100	0	0	100
Crop 3 (Site 3)	Visit 1	100	100	100	100	100	100	100	83.33	100	100
Crop 4 (Site 1)	Visit 1	100	0	0	100	0	0	100	0	0	100
	Visit 2	100	0	10	100	0	0	100	0	0	100
Crop 5 (Site 2)	Visit 1	58.33	0	0	55.56	0	0	50	0	0	54.84
	Visit 2	20	0	0	0	0	0	10	0	0	30
Crop 6 (Site 4)	Visit 1	100	100	0	100	100	0	100	100	10	100

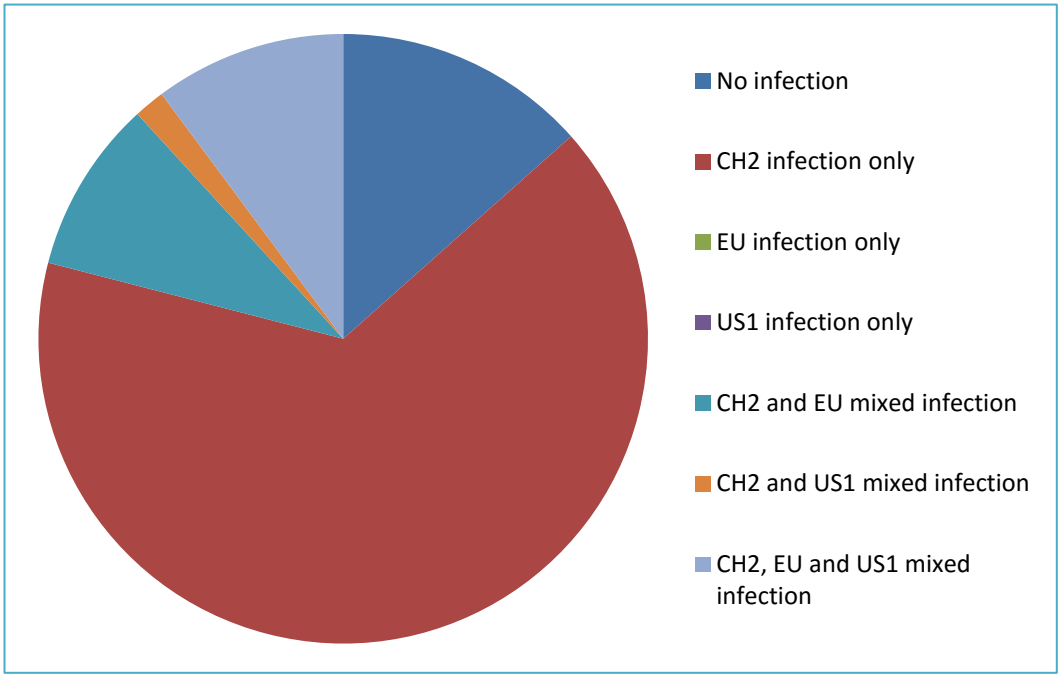

Figure 3.6- Proportion of each type of PepMV infection (single or mixed infection) found in all tomato crops assessed from four sites in the UK.

During the sampling visits to the four different sites, examples of plants with severe symptoms were sought after. These were found on Site 1, although at different locations on the site to where crop 1 and crop 4 were. The plants which were sampled for severe symptoms exhibited severely necrotic leaves and marbled fruit (Figure 3.7). These severe symptom samples tested positive for the CH2 genotype only, as can be seen in Table 3.10.

Figure 3.7- Severely marbled fruit from Site 1

Table 3.10- PepMV genotypes detected by RT-LAMP in additional samples with severe symptoms from Site 1 using the Ling *et al.* (2013) primers.

					Proportion (%) of samples positive for PepMV infection		
Sample	**Site**	**Date**	**Tomato variety**	**Symptom**	**CH2**	**EU**	**US1**
1	1	04/04/16	Lyterno	Necrotic leaves	100	0	0
2	1	31/05/16	Brioso	Marbled fruit	100	0	0

3.4 Discussion

Ling *et al.* (2013) developed RT-LAMP genotype-specific primers to allow for the discrimination between the CH2, EU and US1 genotypes of PepMV. One of the main objectives of this section of the project was to validate the Ling *et al.* (2013) primers with the aim to use them as a new on-site diagnostic tool for the detection of PepMV in tomato crops. Ideally, the primers would give rapid and efficient amplification of positive samples, while also confirming what genotype(s) was present in the crop.

Using the Ling *et al.* (2013) primers in RT-LAMP assays confirmed that the primers were successful at amplifying PepMV-infected tomato samples. All samples collected and tested from the Sutton Bonington glasshouses and crop 1 (Appendices 2 and 3) showed amplification with the CH2 primer, but not with the EU and US1 primers. In order to validate the EU and US1 primers, it was essential to test samples known to be infected with the EU and US1 genotypes. For this reason, EU and US1 RNA extracts were obtained from Fera Science Ltd. On testing these RNA extracts it was revealed that the EU primer was effective at detecting EU infection and the US1 primer was effective at detecting US1 infection. However, both primers also amplified all samples of the alternate genotype, but at a slower speed. This amplification pattern was also seen with the alternative EU and US1 primers. There were two potential explanations for this. Initially, it was hypothesised that the samples were contaminated with some viral RNA of the alternate genotype, for example, the EU samples also contained some US1 RNA. Alternatively, the primers could have been exhibiting some cross-reaction, meaning that they were in fact not genotype-specific.

In order to determine if the EU and US1 Fera Science Ltd samples were contaminated with US1 and EU RNA, respectively, cloning and sequencing was carried out. After conducting PCR and sequencing on ten transformed colonies for each PepMV sample, results indicated that the EU and US1 samples only contained RNA of their respective genotype and no contamination was present. Therefore, from these results it appeared that the EU and US1 Ling *et al.* (2013) primers were cross-reacting and detecting both EU and US1 genotype infection. However, RT-LAMP results from 30 tomato leaf and fruit samples collected from crop 6 (Site 4), which can be seen in Appendix 1, contradict the conclusion made from the cloning and sequencing results on primer cross-reactivity. All of the samples from crop 6 amplified with the Ling CH2 and EU primers; however, only one sample gave a positive result with the US1 primer. If there was cross-reactivity between the EU and US1 primers, all of the samples should have also amplified with the US1 primer.

A potential explanation for why the cloning and sequencing results indicated that no contaminating RNA was present in the EU and US1 samples could be that any contaminating RNA was present at very low levels within the samples. If this was the case, the contaminating RNA may not have been present in any of the ten transformed colonies used for PCR and sequencing. LAMP experiments that have used a standard quantification curve to determine the viral concentration within a sample suggest that there is an approximate 3-5 minute delay in amplification time with every 10-fold dilution of a sample. The EU Fera samples, for example, amplified approximately 10 minutes slower when tested with the US1 primer compared to when tested with the EU primer. This may suggest that the contaminating US1 RNA was present at levels approximately 100-fold lower than the EU RNA within the samples, and so it may have been necessary to sequence at least 100 clones to find the contaminating RNA. This was not possible within the time frame of this experiment; however, the RT-LAMP results from crop 6 strongly indicate that there was no cross-reactivity between the EU and US1 primers.

The anneal temperature given during an RT-LAMP reaction differed depending on what primer set was used (Table 3.3). The lowest anneal temperature was given by the CH2 primer, followed by the US1 and then the EU primer. The fact that the anneal temperatures differed meant that these temperatures could be used as a confirmation of what PepMV genotype was present within a sample. If a universal primer set was used that was able to detect PepMV, irrespective of

genotype, the anneal temperature could be used to determine which genotype was infecting a sample. However, a universal primer would not enable the identification of a mixed-genotype infection as only one anneal temperature would be displayed.

The Ling *et al.* (2013) primers were then used to test tomato leaf and fruit samples collected from six different crops in the UK. The symptoms observed in the crops assessed varied between sites and with sampling time (spring or autumn). The occurrence of fruit symptoms particularly differed between crops, with these symptoms being uncommon or absent in some crops (crops 1, 4 and 5) and frequent in others (crop 2). Differences were observed between symptom expression and severity of different crops located in different areas on the same site.

All samples collected from Site 1 (from crops 1 and 4) tested positive for the CH2 genotype of PepMV. One sample from crop 4 tested positive for the US1 PepMV genotype. Crops from Site 1 exhibited some severe foliar symptoms, particularly the nettle head symptom in crop 4. However, no severe fruit symptoms (with a severity rating of 2 or greater) were observed on the main crops assessed at this site. A potential explanation for why no severe fruit symptoms were observed in crops 1 and 4 could be that the crops became infected with PepMV early in the growing season, meaning that the plants had a greater time to grow through the PepMV symptoms before the trusses developed. This supports the claim that symptoms are less severe when crops become infected earlier in the growing season (Spence *et al.*, 2006). Two additional samples that did exhibit severe symptoms (necrotic leaves and fruit marbling) were collected from different locations on Site 1 and CH2 infection was confirmed using RT-LAMP. It is possible that the crops in this area of Site 1 became infected with PepMV later in the growing season, resulting in the exhibition of more detrimental symptoms. An additional reason for why more severe symptoms were observed in some locations on the site and not in others could be due to slight differences in growing conditions which can influence symptom expression.

As with the crops from Site 1, all samples from crop 2 (Site 2) tested positive for CH2 PepMV infection. Only one sample from the second visit to crop 2 showed amplification with the US1 primer. Crop 2 exhibited minimal foliar symptoms, however the plants also possessed some of the most severe fruit symptoms

assessed. Contrastingly, the second crop assessed on Site 2 (crop 5) did not exhibit disease symptoms at the time of the first visit (15/3/16), although approximately 50% of samples tested positive for infection with the CH2 genotype, highlighting that PepMV can still be present despite the absence of symptoms. The other 50% of the samples were negative for PepMV infection.

On the second visit to crop 5, the number of plants exhibiting possible disease symptoms had increased slightly but still remained low. However, only two samples were positive for PepMV infection (CH2 genotype only). The amplification times for these samples were slow (see Appendix 4) indicating that a low viral titre may have been present in these samples. It has been speculated that virus titre is correlated with symptom induction and severity; Soler-Aleixandre *et al.* (2005) found that higher PepMV titres within the plant were associated with severe symptoms, such as vascular necrosis. Additionally, Fakhro *et al.* (2011) found that increased concentrations of the LP PepMV isolate were linked to growth reduction in plants. Different tomato varieties were assessed at Site 2, which could explain why crop 2 (Roterno) exhibited more severe symptoms than crop 5 (Sunstream) if the different varieties respond differently to infection. Additionally, crop 5 was assessed in March/April, where light intensity would have been greater than in October when crop 2 was assessed. Previous research has shown that PepMV symptoms attenuate with increased light intensity (Jones and Lammers, 2005), which could explain why such contrasting symptom severities were seen on the same site.

Site 3 possessed the most severely symptomatic plants assessed in this study, with chlorotic spots and fruit marbling each being present at a severity rating of 2 or greater in approximately one fifth of plants assessed. Almost all samples tested positive for mixed PepMV infection with all three genotypes. The presence of mixed infection could be responsible for the broad variety and severity of symptoms observed at this site (Hanssen *et al.*, 2009). Similarly, mixed infection was found on Site 4 in crop 6; however, predominantly only involving the CH2 and EU genotypes (only one sample tested positive for all three genotypes). Although fruit symptoms were not particularly severe in crop 6, necrotic spots and petiole symptoms were of the highest severity recorded throughout the study. Overall, disease symptoms were more widespread at Site 3 compared to Site 4, which could be explained by the higher incidence of mixed infection with the US1 genotype at Site 3.

The tomato variety/cultivar may provide a potential explanation as to why symptoms varied so dramatically between crops, despite the fact that the majority of them were infected with the CH2 PepMV genotype. As mentioned in Chapter 1.9, different tomato cultivars exhibit different levels of yield loss as a result of PepMV infection (Schwarz *et al.*, 2010). From this study, it was observed that the Piccolo cultivar was more susceptible to foliar symptoms and less likely to develop fruit marbling symptoms. Fruit symptoms were only significant on Piccolo on Site 3 which was burdened with mixed infection from all three genotypes. The additional samples from Site 1 taken from Roterno and Brioso cultivars possessed the most severe fruit symptoms.

Additionally, even though the majority of crops were infected with the same PepMV genotype (CH2), the infection on each crop may have been caused by different subgroups of the genotype, resulting in a spectrum of different symptoms observed between crops. Recent research conducted by Davino *et al.* (2016) looked at genetic variation of PepMV in Sicily. Phylogenetic analyses revealed that all Italian PepMV isolates examined belonged to the CH2 genotype, however, two subgroups existed within the CH2 group. Subgroup A contained Sicilian isolates only and induced severe symptoms in tomato fruit. Subgroup B contained Sicilian and some Spanish isolates; however, members of this subgroup did not produce fruit symptoms. Therefore, it is possible that different subgroups of the CH2 genotype were infecting the crops assessed in this study, resulting in differences in symptom expression. This would also support suggestions by Hanssen *et al.* (2009).

Another potential explanation for why symptoms were so varied between crops could be that some of the crops may have been infected with multiple plant viruses. The more severe symptoms seen on the crops may have been the combined effects of two viruses. For example, Davino *et al*. (2008) found that tomato plants infected with PepMV and *Tomato chlorosis virus* (ToCV) exhibited fruit marbling and interveinal yellowing on the leaves, with these symptoms being characteristic of PepMV and ToCV infection, respectively. Research has shown more severe symptom expression in other crops, such as potato, infected with multiple viruses instead of just a single virus. Hameed *et al*. (2014) discovered that symptom severity was enhanced when potatoes were co-infected with *Potato virus X* and *Potato virus Y*. In this study, PepMV may have existed in co-infections with other viruses in some crops and not in others, which may have influenced the symptoms observed in the crops assessed in this investigation.

The EU genotype used to be dominant in Europe until a genotype shift occurred, with the CH2 genotype overtaking the EU (Hanssen *et al.*, 2008). This shift in dominant genotype is reflected in the results found in this study, with single genotype CH2 infection being found in over 60% of the tomato plants assessed. The EU genotype was never present on its own and was only found in mixed infections with CH2 or with CH2 and US1, supporting the findings of Gómez *et al.* (2009, Chapter 1.5).

Chapter 4: Sources of PepMV Inocula in the Glasshouse Environment

4.1 Introduction

Since the first discovery of PepMV on tomato plants, the virus has rapidly spread throughout tomato crops worldwide. The fast dissemination of the virus between regions and countries was highlighted recently by Davino *et al.* (2016). When they began their research in Sicily in 2011, PepMV was present in only one province of Sicily in 13% of tomato plants studied. By 2013, the incidence of PepMV had increased to 63% and was found in all Sicilian provinces involved in the study. The highly contagious nature of PepMV has facilitated its rapid spread, particularly during the hands-on practices required for tomato crop production. Contaminated clothing, tools, transport crates and seeds can then transmit the virus to other crops and regions.

It is essential for strict hygiene protocols to be followed within the glasshouse environment during crop production (see Chapter 1.10.1). It is vital to know where PepMV may occur within tomato nurseries so that disinfection procedures can be conducted efficiently to eliminate the virus. Research has shown that PepMV is capable of contaminating many glasshouse surfaces, such as concrete pathways, picking trolleys and waste containers (O'Neill *et al.*, 2003). Clean-up procedures to disinfect glasshouses are not always successful, particularly in areas that are difficult to clean, and so any PepMV remnants may function as sources of inocula for subsequent growing seasons.

Tomato crop waste created within glasshouses may be subjected to composting to be used for tomato crop production. Composting usually consists of three stages: a mixing period with mesophilic growth; a high-temperature period with thermophilic growth; and a lower temperature stage with mesophilic growth (Day and Shaw, 2001). The production of compounds such as ammonia, lytic enzymes and antibiotics by microorganisms during the composting process, as well as the high temperatures produced, are thought to antagonise plant pathogens (Bollen, 1985). Although there is no published data on the eradication of PepMV during composting, previous research has found composting to be successful in reducing levels of viruses, such as TMV and *Tomato mosaic virus*, below a detectable limit (Noble and Roberts, 2004). However, if composting is unsuccessful at removing PepMV, viral residues could contaminate new crops for which the compost is utilised.

Another potential source of inoculum in the glasshouse environment is water. As discussed in Chapter 1.8.3, research has shown that PepMV can survive in water and infect tomato crops via the plant roots. PepMV infested water creates a particular problem if water or nutrient solutions are recycled, as with hydroponic systems such as the NFT. Water may, therefore, provide a medium for rapid PepMV dissemination throughout a whole crop. Large volumes of water are used for the irrigation of crops, and so a concentration technique is required to allow for the detection of viral particles within the water. However, some concentration methods, such as ultracentrifugation and polyethylene glycol (PEG) precipitation (Lewis and Metcalf, 1988), are laborious and time consuming. Despite the fact that viral concentrations within a water system may be low, even the infection of one plant from contaminated water via the root system could result in the spread of the virus to multiple plants via mechanical means.

Knowledge of potential sources of PepMV inocula within the glasshouse, such as those discussed above, is essential in order to efficiently limit PepMV transmission. In this section of the study, glasshouse surfaces were tested for the presence of PepMV before and after crop removal and end-of-season clean-up practices. Additionally, PepMV survival was measured in tomato crop waste subjected to composting. Finally, water samples from sites with PepMV-infected crops were tested to determine if PepMV could be detected within the water. Results from this work will inform tomato growers on how effective glasshouse clean-ups are and what potential sources of PepMV inocula exist within the glasshouse.

4.2 Methods

4.2.1 Swab and Compost Sampling and PepMV Detection

Swab sample collection and compost sampling was conducted as described in Chapters 2.2.2 and 2.2.3, respectively. From the swab samples, 1.5 µl of phosphate buffer was used directly in the RT-LAMP reaction (Chapter 2.4). RNA extractions (Chapter 2.3) were carried out on the compost samples before RT-LAMP was conducted.

4.2.2 Water Sampling and Concentration

Water samples were collected from three tomato sites. One litre samples were collected from three locations on each site where possible: the water source (reservoirs); the drain water after the water had passed through the root zone of the crop; and the water after it had been treated with the site's disinfestation system. After collection, the samples were posted to Sutton Bonington where they were filtered twice through 0.8 µm filters. After filtration, the water samples were concentrated into 10 0.5 ml volumes using a method developed at the National Institute for Biology (NIB) Slovenia. The method cannot be described here as it is covered by a confidentiality agreement until the method is published. One in 10 dilutions of the concentrated water samples were made and 1.5 µl of the water dilutions were used directly in the RT-LAMP reactions (Chapter 2.4).

4.3 Results

4.3.1 The Occurrence of PepMV on Glasshouse Structures and Equipment after Crop Removal

Fifty swab samples were taken from glasshouse surfaces and equipment before and after end-of-season glasshouse cleanups from four sites with confirmed PepMV infection. The phosphate buffer from these swabs was used in RT-LAMP tests to determine the presence of PepMV, and so allowing for the determination of the efficiency of glasshouse disinfection techniques. All swab samples were tested for the CH2 genotype of PepMV using the Ling *et al.* (2013) primer as previous work (Chapter 3.3.3.) found that this was the most common genotype of PepMV found at the sites involved in this study. The proportion of samples

testing positive for PepMV before and after glasshouse clean-ups can be seen in Figure 4.1.

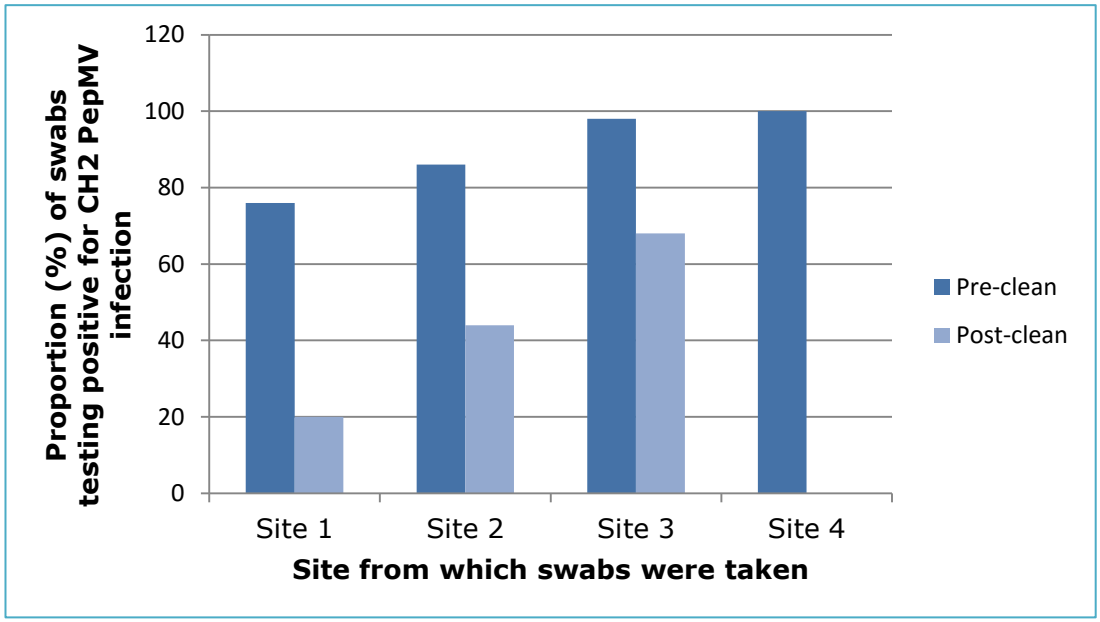

Figure 4.1- Proportion of swab samples from each site testing positive for CH2 PepMV infection before and after end-of-season cleanups.

As can be seen from Figure 4.1, a large proportion of pre-cleanup swabs from all sites tested positive for the CH2 genotype of PepMV, particularly Sites 3 and 4 where 98% and 100% of pre-cleanup swabs tested positive, respectively. There was a reduction in the proportion of PepMV-positive swabs after end-of-season cleanups were conducted; however, none of the sites were able to completely eliminate PepMV from their glasshouses and equipment as a number of swab samples still remained positive. It was not possible to collect post-cleanup swabs from Site 4 due to commercial reasons. From looking at Figure 4.1, it can be seen that Site 1 appeared to have the most efficient cleanup, with the proportion of positive swab samples being reduced by 56% after the cleanup was carried out. The cleanup at Site 3 did not appear to be overly effective, with 68% of surfaces/equipment swabbed still testing positive for the occurrence of PepMV. The specific surfaces/equipment swabbed, and those on which PepMV remained after cleanup, can be seen in Appendix 5.

The surfaces on which PepMV remained after glasshouse cleaning are summarised in Table 4.1. From this it can be seen that the surfaces on which PepMV remnants could still be detected were surfaces that had been in close contact with plant material, such as picking crates and trolleys, and also electrical equipment. Even though surfaces that came into close contact with plant material were disinfected (with Chlorine + Sanprox P/Virkon S/Horticide), PepMV was still able to be detected on them. Electrical equipment/surfaces are difficult to disinfect thoroughly and safely, which is the most likely explanation for why PepMV could still be detected on these surfaces. Additional surfaces on which PepMV could still be found were hand soap dispensers (Site 3) and first aid boxes (Site 1). It may not be immediately obvious to disinfect surfaces like these; however, the fact that they tested positive for PepMV highlights how contagious and mechanically transmissible the virus is.

Table 4.1- Surfaces that remained PepMV-positive after end-of-season glasshouse cleanups at each site.

Site	Surface Type	Disinfection used on surface
1	3 x trolley	Chlorine + Sanprox P
	2 x door handle	Chlorine + Sanprox P
	Main circuit board box	X
	Trolley of electrical equipment	X
	Pipe on wall	Chlorine + Sanprox P
	Metal bracket supporting pipe	Chlorine + Sanprox P
	First aid box	X
2	3 x concrete pathway	Virkon S
	Aluminium post	Virkon S
	3 x green waste cage	Virkon S
	Stanchion	Virkon S
	Mypex	Virkon S
	4 x trolley	Virkon S
	2 x heating pipe	Virkon S
	2 x bracket supporting pipe	Virkon S

	3 x electrical panel	X
	Electrical plug/switch	X
	Spray boom	Virkon S
3	2 x metal floor plate	X
	Heating pipe	Horticide
	Grow pipes	Horticide
	2 x packing crate	Jet wash/wipe
	4 x drip peg/line	Horticide
	Green waste bin	Jet wash/wipe
	Moth light	X
	2 x trolley	Jet wash/wipe
	Gutter support	Horticide
	Mypex	X
	Door handle	X
	Ceiling chain	Horticide
	2 x stanchion	Horticide
	2 x irrigation pipe	Horticide
	Row label	X
	4 x electrical charge point/switch	X
	2 x door switch	X
	2 x hand soap dispenser	X
	Whiteboard	Jet wash/wipe
	2 x forklift	Jet wash/wipe

X= hard to disinfect/not disinfected

The pre-cleanup swab samples from Sites 1 and 2, and a selection of post-cleanup swabs from Sites 1, 2 and 3 that tested positive for PepMV using RT-LAMP were sent to Fera Science Ltd where pre-cleanup swabs were tested using ELISA and sap inoculation tests onto tomato seedlings were conducted with the post-cleanup swabs. The results of the ELISA and sap inoculation tests are summarised in Table 4.2. It can be seen that ELISA tests were also able to detect PepMV in the pre-cleanup swab samples. However, ELISA detected fewer positive samples (46% and 40% less from Sites 1 and 2, respectively) compared

to the RT-LAMP tests. This suggests that the RT-LAMP assay used may be more sensitive than the ELISA tests used. Additionally, it can be seen in Table 4.2 that no positive samples were obtained from sap inoculation tests onto tomato seedlings using the post-cleanup swabs, suggesting that the virus detected by the RT-LAMP and ELISA tests may not have been viable or able to cause infection.

Table 4.2- Proportion (%) of pre and post-cleanup swabs testing positive for PepMV infection with each test.

	Proportion (%) of pre-clean PepMV-positive samples with each test		Proportion (%) of post-clean PepMV-positive samples with each test	
	RT-LAMP	**ELISA**	**RT-LAMP**	**Sap Inoculation**
Site 1	76	30	20	0
Site 2	86	26	44	0
Site 3	98	X	68	0

X= not tested

4.3.2 The Survival of PepMV in Composted Tomato Waste

In order to monitor the survival of PepMV in composted tomato waste, a commercial site (where composting was conducted on site) was visited at the time of crop pull-out. The crop (cultivar Piccolo) was chipped and ten samples of the chippings were taken. The time at which the crop was chipped was referred to as T0. The T0 samples were sent to Fera Science Ltd for ELISA tests, where it was confirmed that all ten replicates were infected with PepMV. Subsequent sap inoculation tests at Fera Science Ltd onto tomato seedlings and *N. benthamiana* plants indicated that all T0 chipping samples were infected with viable virus, as 100% of test plants became infected with PepMV, as can be seen in Table 4.3. As the composting process proceeded, the site was visited on nine further occasions (T1-9) and ten samples of the compost were collected each time. The RT-LAMP, ELISA and sap inoculation results for the samples from each visit and controls (chipped crop kept in a 24°C incubator) can be seen in Table 4.3.

From Table 4.3, it can be seen that 100% of compost samples and controls tested positive for PepMV-CH2 using RT-LAMP at T1 and T2. At T3, a drop in the

proportion of PepMV-positive compost samples to 70% was observed. At T4, the proportion of PepMV-positive samples, according to RT-LAMP, increased back up to 100%. At this time point (T4) the compost chippings were incorporated into windrows with other plant material. The incorporation of new plant material at this point could have introduced new virus into the compost, explaining the increase in the proportion of positive samples. No controls were tested at T4 as, at this time, the original controls were replaced with samples taken from the windrows. The proportion of compost samples testing positive for PepMV (according to RT-LAMP) decreased at T5 and T6, with only 30% of samples testing positive at T6. This low percentage of positive samples at T6 could be due to an uneven distribution of PepMV in the compost heap, as the proportion of positive samples increased to 70% again at T7 and T8. By the end of the composting process (dry phase), only one of the compost samples tested positive for PepMV using RT-LAMP.

Throughout the investigation, the proportion of control samples testing positive for PepMV with RT-LAMP remained consistently higher than the compost samples. The proportion of positive control samples remained at 100%, apart from at T6 where the proportion of positive controls dropped to 90%. It was not possible to test any control samples at T9 as the samples were lost in the post. From looking at the RT-LAMP results of the compost samples and controls over the 16 week sampling period, it can be observed that the composting process led to a reduction in the proportion of samples testing positive for PepMV, as almost all control samples not subjected to composting remained PepMV-positive over the 16 week period according to RT-LAMP. The lower levels of PepMV found in the compost samples could be due to the high temperatures created during composting, as can be seen in Table 4.3.

Although it was possible to detect PepMV in the compost and control samples at all time points using RT-LAMP, no compost samples or controls tested positive for the virus when using ELISA after T0. This pattern is also reflected in the sap inoculation results (Table 4.3). ELISA is a less sensitive assay than RT-LAMP which could explain why no samples tested positive with ELISA after T0. However, the fact that no positive results were obtained after sap inoculation tests indicates that the virus detected by RT-LAMP may not have been viable and suggests that viable virus had been eliminated by the T1 sampling time in both compost and controls.

Table 4.3- Proportion (%) of compost and control samples testing positive for PepMV when tested with each of the detection methods.

				Proportion (%) of positive samples using each test		
Stage of composting process	**Sample Time**	**Temperature of sample location (°C)**	**Sample Type**	**RT-LAMP**	**ELISA**	**Sap Inoculation**
Chipped	T0 (07/11/15)	-	Compost	X	100	100
		-	Control	X	100	100
Stack	T1 (18/12/15)	68	Compost	100	0	0
		24	Control	100	0	0
	T2 (26/01/16)	65.5	Compost	100	0	0
		24	Control	100	0	0
	T3 (23/02/16)	64.3	Compost	70	0	0
		24	Control	100	0	0
Compost rows	T4* (16/03/16)	60.3	Compost	100	0	0
		-	Control**	X	0	0
	T5 (23/03/16)	83.6	Compost	80	0	0
		24	Control	100	0	0
	T6 (30/03/16)	72.5	Compost	30	0	0
		24	Control	90	0	0
Phase 2	T7 (05/04/16)	54	Compost	70	0	0
		24	Control	100	0	0
	T8 (12/04/16)	51	Compost	70	0	0
		24	Control	100	0	0
Dry phase	T9 (26/04/16)	53.1	Compost	10	X	X
		-	Control	***	X	X

X=not tested *= samples incorporated into windrows with other plant material

=original control samples replaced with new compost mix from rows *=samples lost in postage

As mentioned in Chapter 3.3.1, the RT-LAMP amplification time can be used to quantify the amount of virus within a sample. This can be done by testing a sample with a known viral RNA concentration and making serial dilutions of the sample and running the undiluted sample with all its dilutions on RT-LAMP. This will allow for the creation of a standard curve, highlighting that the amplification time increases as the concentration of virus within a sample decreases. Due to the experimental design used in this study, quantification of the virus in the compost samples could not be achieved; however, the amplification time could still be used as an approximate indication of how the level of virus within the samples varied with each sampling time point. Therefore, with this in mind, the average amplification times of the ten samples and ten controls collected at each time point are summarised in Figure 4.2.

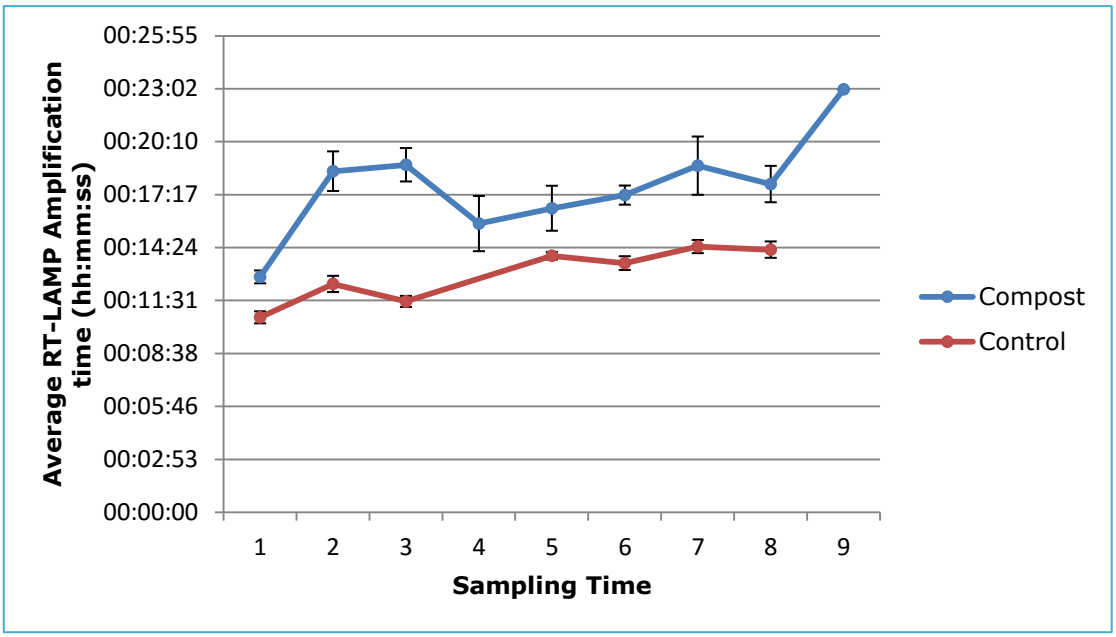

Figure 4.2- Average (± 1 standard error of the mean) RT-LAMP amplification times for 10 compost samples and 10 controls collected at each time point during the composting process.

From Figure 4.2 it can be seen how the average amplification times for the compost treatment and control samples changed over the 16 week sampling period. When looking at the average amplification times for the compost samples, it can be seen that from T1 to T3 the amplification time increased. At T4 the average amplification time decreased to 15:42 minutes, most likely due to the introduction of new virus into the compost from new plant material that was incorporated into the compost windrows. From T5-T9, the average amplification time for the compost samples increased again, reaching the highest amplification time of 23 minutes at T9, almost double the average amplification time exhibited by the compost samples at T1. These results indicate that as the composting process progressed, the level of PepMV that could be detected within the compost decreased.

In contrast, it can be observed that the average amplification times given by the control samples did not increase as drastically as the compost samples. From T1 to T9 the average amplification time for the control samples only increased by 3 minutes and 41 seconds, suggesting slower degradation of the PepMV within the control samples compared to the compost samples. Figure 4.2 highlights how the average amplification time for the control samples remained consistently lower than the amplification times for the compost samples at all sampling time points.

Due to the experimental design of this investigation, it was not possible to perform a statistical test to determine whether or not there was a significant difference between the average amplification times of the compost and control samples. In order to do this, samples and controls would have had to have been collected from multiple compost heaps. Instead, linear regression analysis was conducted to compare the change in amplification time of the three subgroups of samples (15 cm compost, 30 cm compost and controls) over the sampling period using Genstat®. The amplification times for each sample and control collected were converted into seconds, transformed into logs and plotted onto a scatter graph, as can be seen in Figure 4.3. The regression analysis showed that there was a significant increase in the amplification times observed over the sampling period ($p<0.001$). The regression analysis also indicated that, on average, the amplification times of the compost samples were higher than the controls ($p<0.001$). However, when looking at the change in amplification time of the three subgroups over time, the regression analysis indicated that no significant difference existed between the three slopes ($p=0.71$, Table 4.4). This implies that the level of PepMV does decrease over time; however, this rate of change

does not differ between the three groups. Therefore, it is not possible to conclude that the rate of PepMV decay is due to the composting process.

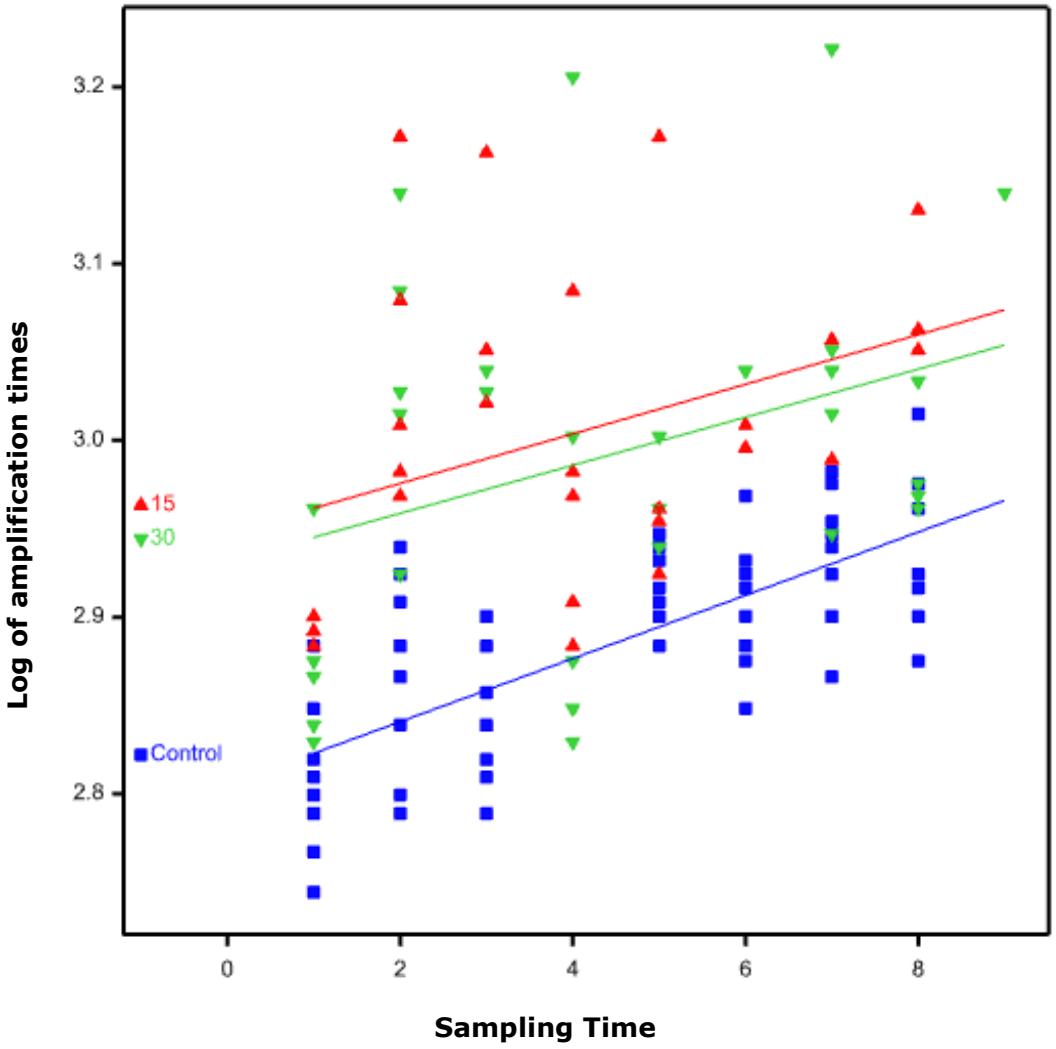

Figure 4.3- The change in the level of PepMV over the sampling period in the three subgroups of samples (15 cm compost, 30 cm compost and controls) according to the RT-LAMP amplification times.

Table 4.4- Accumulated Analysis of Variance (ANOVA) results given by the linear regression analysis in Genstat®, analysing the change in the dependent variable (amplification time) over time, over depth and over time and depth combined. A significance level of P<0.05 was used.

Change	Degrees of freedom (d.f)	Sum of squares (s.s)	Mean squares (m.s)	Variance ratio (v.r)	F probability
Time	1	0.150692	0.150692	32.00	<.001
Depth (15cm, 30cm, control)	2	0.448132	0.224066	47.59	<.001
Time + Depth	2	0.003224	0.001612	0.34	0.711
Residual	126	0.593286	0.004709		
Total	131	1.195334	0.009125		

4.3.3 The Detection of PepMV in Water Samples Collected from UK Tomato Nurseries

In spring 2016, three one litre water samples were collected from two tomato nurseries where recirculating irrigation systems were used. Two samples were collected from a third site which used the NFT for tomato crop production. After arrival at Sutton Bonington, the samples were filtered and concentrated using the concentration method developed in Slovenia. The water samples were concentrated into ten 0.5 ml elutions and these elutions were diluted and tested using RT-LAMP with the CH2 Ling *et al*. (2013) primer. The RT-LAMP results for each sample collected at each site and the sample type are displayed in Table 4.5.

Table 4.5- Detection by RT-LAMP of PepMV in water samples collected from three tomato nurseries in the UK.

Site	Irrigation	Disinfestation treatment	Sample	PepMV detected in pre-concentrated sample	Number of concentrated elutions testing positive for PepMV
1	Rockwool	Pasteurisation	Reservoir	×	1
			Pre-treatment	×	6
			Post-treatment	×	0
2	Rockwool	Pasteurisation	Reservoir	×	0
			Pre-treatment	×	0
			Post-treatment	X	X
3	NFT	None	Pre-plants (source and feed water)	×	1
			Post-plants (drain water)	×	7

X= not tested ×=no PepMV detected

The concentration method allowed for the successful detection of PepMV in four out of eight water samples collected. PepMV could not be detected in any of the water samples before the concentration method was conducted, indicating that the method was effective at concentrating PepMV in the samples. The reservoir water sample and the pre-treatment water sample (after the water had passed through the root zone of the plants) from site 1 both tested positive for CH2 PepMV infection. PepMV could not be detected in the post-treatment water at site 1, suggesting that pasteurisation is effective at removing PepMV from water. Only one concentrated elution fraction from the reservoir water tested positive for PepMV compared to six positive fractions from the pre-treatment water, suggesting that the level of PepMV particles within the water increased after the water had passed through the root zone of the plants. Contrastingly, the reservoir and pre-treatment water samples from site 2 did not test positive for PepMV infection. As PepMV could not be detected in the first two samples from

site 2, the third sample (post-treatment) was not tested. At site 3, both water samples collected tested positive for PepMV. PepMV was detected in more elution fractions from the sample collected after the water had passed through the crop compared to the source and feed water sample, with seven and one elution fractions testing positive, respectively. This site had no water disinfestation system, meaning that the PepMV infested water would be recirculated around the whole crop and possibly even more than one crop if the same irrigation loop was used to feed multiple glasshouses.

4.4 Discussion

Previous research, such as that conducted by O'Neill *et al.* (2003), has shown that PepMV is capable of surviving in dried sap and contaminating glasshouse structures. For these reasons it is essential that strict hygiene procedures are adopted within the glasshouse to avoid PepMV transmission. In order to evaluate the efficiency of end-of-season glasshouse cleanups, three commercial tomato sites were visited and swab samples taken from glasshouse surfaces before and after glasshouse cleanups were performed. These swabs were tested in RT-LAMP assays with the CH2 primer for the presence of PepMV.

PepMV was detected in a high proportion of the pre-clean swab samples collected from sites 1, 2 and 3, with 76%, 86% and 98% of swabs testing positive at the sites, respectively. A fourth site was also visited, where 100% of pre-clean swabs were PepMV-positive. However, a second visit to site 4 was not possible. The fact the PepMV was detected on such a high number and variety of glasshouse surfaces, including ones that did not come into direct contact with plants (for example electrical equipment/switches), highlights the highly contagious nature of the virus. The proportion of swabs testing positive for PepMV after the end-of-season cleanup did decrease; however, at least 20% of swabs remained positive post-cleanup. PepMV could still be detected on almost 70% of post-clean swabs at site 3. The surfaces on which PepMV could still be detected were those which had come into close contact with PepMV, such as green waste bins and picking trolleys, and surfaces which are hard to disinfect, such as electrical equipment. From the RT-LAMP tests of these swabs, it can therefore be concluded that, although the end-of-season cleanups did reduce the number of glasshouse surfaces testing positive for PepMV, they did not totally

eliminate PepMV from the glasshouse. This suggests that the surfaces on which PepMV remained could act as sources of inocula for PepMV transmission to other locations or to subsequent tomato crops grown within these glasshouses.

Although PepMV was detected in a high proportion of pre-clean swab samples when using RT-LAMP, a much smaller proportion of the swabs tested positive for PepMV when using ELISA. This result is likely due to the fact that ELISA is less sensitive than molecular methods such as RT-LAMP. Alternatively, the RT-LAMP assay may be more likely to detect fragments of viral RNA rather than a complete viral particle and ELISA works through the detection of antigens displayed on viral surfaces. It is thought that viral proteins degrade faster than viral RNA (Mehle *et al.*, 2014), and so if RT-LAMP was detecting viral RNA fragments rather than complete viral particles, this would explain why more positive results were obtained from the RT-LAMP tests compared to the ELISA tests.

For the post-clean swabs collected from the sites, a sub sample of swabs testing PepMV-positive according to RT-LAMP were sent to Fera Science Ltd for viability studies using sap inoculation tests. No test plants became infected with PepMV after sap inoculation using the swab samples. This suggests that the virus detected by RT-LAMP may not have been viable or able to cause infection. These results indicate that the RT-LAMP assay was most likely detecting PepMV RNA fragments instead of complete viral particles that were capable of inducing infection. From these results it can be concluded that although the end-of-season cleanups did not successfully remove all PepMV remnants, they were able to eliminate the virus' infectivity. The disinfectants used at the sites, such as Virkon S and Horticide, have been shown to be effective against PepMV contaminated surfaces in other studies (O'Neill *et al.*, 2003). Despite the fact that the PepMV detected was not viable, not all samples that tested positive with RT-LAMP were used for sap inoculation tests. Therefore, viable PepMV could have potentially remained on other glasshouse surfaces and on surfaces that were not swabbed as part of this investigation. Only a small amount of viable virus would have to remain within the glasshouse in order to transmit infection to a new crop.

The second part of this study investigated the survival of PepMV in composted tomato waste. Currently, there is no published data on the effects of composting on PepMV; however, there is some evidence that the composting process is

successful at eliminating other plant pathogens, such as *tomato mosaic virus*, to below levels of detection (Noble and Roberts, 2004). From conducting RT-LAMP tests on samples collected at nine time points during the composting process at a commercial tomato site and comparing them to un-composted controls, it was possible to monitor if the level of PepMV within the samples changed as the composting process progressed. Although the level of virus within the samples could not be quantified as a standard curve was not used, it was possible to observe an overall decrease in the level of PepMV within the compost samples over the 16 week sampling period due to the increasing RT-LAMP amplification times obtained. However, as only one compost heap was monitored, it was not possible to conduct a statistical test to determine whether the differences in amplification times between the compost and control samples were significant.

High temperatures are created during composting, which generate a favourable environment for antagonistic microorganisms. Peak compost temperatures of 68°C or more for 20 days are required to eliminate TMV from compost; however, the virus does degrade over time in compost kept at 31°C for a 26 week composting period (Noble and Roberts, 2004). In this study, the compost reached an average of 66°C for nine weeks whilst the compost was in the stack. This average temperature increased to 72°C for a three week period when the material was incorporated into windrows. Previous research would therefore suggest that these temperatures are sufficient to reduce the levels of viable virus. Although PepMV could be detected by RT-LAMP in the compost samples at all sampling time points, with one out of the 10 samples still being positive for PepMV at T9, the sap inoculation tests performed with T1-T9 RT-LAMP positive samples provided no positive results. This indicates that the virus detected by the RT-LAMP assay was not viable. It is therefore a possibility that the RT-LAMP assays were detecting PepMV RNA fragments rather than complete viral particles capable of inducing infection.

However, it is not possible to conclude that this loss of virus viability is due to the composting process as no viable virus was detected in the control samples which had not experienced such high temperatures. The virus still lost viability over time in the five week period between T0 and T1 when kept at a constant temperature of 24°C. PepMV may require lower temperatures than this to remain viable for extended periods of time; O'Neill *et al.* (2003) found that PepMV was able to remain viable on surfaces for up to four weeks when kept at 5°C.

Additionally, the virus may lose viability naturally over time, and this could occur at a faster rate at greater temperatures. The regression analysis conducted (Figure 4.3) suggested that the virus degrades/loses viability over time irrespective of whether or not the plant material is subjected to composting. Although, on average, higher amplification times were observed for the compost samples compared to the controls, from this investigation it cannot be concluded that the composting process is successful at eliminating infective PepMV. There may be another aspect that influenced the amplification times obtained. For example, inhibitors within the samples collected from the compost heap may have hindered the RT-LAMP reaction leading to increased amplification times. This may explain why multiple outliers from the regression lines with particularly high amplification times can be seen for the 15 cm and 30 cm compost samples in Figure 4.3. In order to more significantly determine the effects of composting on PepMV, samples and controls should be collected from more than one compost heap in order to increase the number of replicates for the investigation to allow for comparisons to be made and tests for statistical significance to be conducted.

The final part of this study involved the collection and concentration of water samples from commercial sites that utilised re-circulating irrigation systems. Where possible, three samples were collected from each site; from the source/reservoir water, after the water had passed through the root zone of the plants, and after the water had been treated. The water concentration method developed in Slovenia was successful in concentrating the samples to allow for the detection of PepMV in 50% of the samples collected. At site 1, PepMV was detected in the source and pre-treatment water but not in the post-treatment water. This would appear to suggest that the disinfestation method used at site 1 (pasteurisation) is effective at eliminating PepMV from the water. However, the fact that PepMV could be detected in the reservoir water could indicate that pasteurisation is not eliminating 100% of PepMV present. A disadvantage of physical treatments, such as pasteurisation, is that they have been found to lack a reservoir effect and are only effective in the immediate surroundings of their operating systems (Kraft, 2008). Alternatively, PepMV may have been contaminating the reservoir water via a different, unknown route. After completion of the water investigation, it was discovered that the reservoir water from site 1 was composed of rain water and condensation taken from the inside

of the glasshouse. This glasshouse condensation could potentially be the source of PepMV in the reservoir water.

No PepMV could be detected in the reservoir or pre-treatment water samples collected from site 2 and, because of this, the post-treatment sample was not tested. Sites 1, 2 and 3 were also tested as part of previous investigations into the distribution of PepMV in UK tomato crops (Chapter 3.3.3). The crops in the glasshouse from which the water samples were collected from site 2 exhibited relatively low levels of PepMV infection, whereas PepMV was found extensively in the crops at sites 1 and 3. This could explain why PepMV was not found in the water samples at site 2 but was present in those from sites 1 and 3.

At site 3, which utilised the NFT for irrigation, PepMV could be detected in the source/feed water and the drain water collected after the water had passed through the plants. This site did not have a disinfestation system and so it is likely that the PepMV accumulated by the water as it passed through the infected plants would be re-circulated around the plants again, or possibly even to another crop if the same irrigation loop fed multiple glasshouses.

The findings from this investigation support those of Mehle *et al.* (2014) who found that PepMV can survive and be transmitted in water. In their study, Mehle *et al.* (2014) conducted mechanical inoculations onto test plants using PepMV-infected water. It would have perhaps been beneficial to conduct mechanical inoculations using the concentrated water samples from this study in order to determine if the virus detected was viable and able to induce infection in healthy plants.

The fact that PepMV can be spread via re-circulating irrigation systems highlights the importance of the requirement of a disinfestation treatment to prevent spread of the virus from an infected plant to a whole crop. If pasteurisation is not 100% effective, it may be necessary to investigate other methods of removing PepMV from glasshouse irrigation systems. Recently, Bandte *et al.* (2016) tested a 'sensor-based' disinfection technique to inactivate PepMV and reduce its dispersal in hydroponic systems. The technique involved passing an electrical current through a solution made up of fresh water and low concentrations of potassium chlorite. This resulted in the formation of chlorine and the solution was then injected into nutrient solution tanks that were used for tomato plant irrigation. Similar techniques have been used for the disinfection of

waste water and drinking water. Bandte *et al.* (2016) found that weekly treatments of 0.2 mg of free chlorine per litre for 60 minutes were effective at inhibiting the dispersal of PepMV to tomato plants irrigated with PepMV-contaminated nutrient solutions. The treatment also decreased the number of unmarketable fruits, with only 5% of unmarketable tomatoes produced by the plants irrigated with treated water and 48% produced by the untreated control plants. This disinfection technique may therefore provide a promising method for preventing PepMV spread in recirculating nutrient solutions without compromising fruit quality and yield.

Chapter 5: Summary, Final Conclusions and Future Research

The overall aims of this study were to increase the understanding of PepMV infection in tomatoes, and to gain insight into symptom severity and persistence on tomato nurseries in the UK. PepMV is currently the most threatening viral pathogen to glasshouse tomato production, and so diagnosis of the virus in crops is essential to allow for preventative procedures against the spread of PepMV to be implemented. Chapter 3 of this thesis focussed on identifying mixed-genotype PepMV infection in UK tomato crops through the use of genotype-specific RT-LAMP assays. The Ling *et al*. (2013) primers were successfully validated and used to test a variety of tomato leaf and fruit samples from six crops collected from four UK tomato sites. The primers allowed for rapid confirmation of whether PepMV infection was present within a sample, and the three primer sets (CH2, EU and US1) were able to discriminate between infections by the different PepMV genotypes. The RT-LAMP tests revealed that PepMV was widespread in the crops tested, with the CH2 genotype of PepMV alone infecting over 60% of the crops. Mixed-genotype infections were discovered in approximately 20% of the samples tested, with infection by all three genotypes being the most common type of mixed-infection observed. Only 13% of the plants assessed were negative for PepMV infection.

Despite the fact that all infected samples assessed were infected with the same genotype (CH2) of PepMV, the symptoms observed in the different crops were variable. Fruit symptoms were common in some crops (crop 3), while almost absent in others (crops 1, 4 and 5). It may be that the more severe fruit symptoms are induced when a crop is burdened with mixed-genotype infection; almost 100% of samples from crop 3 were infected with the CH2, EU and US1 genotypes. Additionally, different tomato cultivars may respond differently to PepMV infection, with large fruit cultivars (such as Brioso and Roterno) being more susceptible to the development of fruit symptoms. Furthermore, mixed-infection with PepMV and other viruses may have influenced the differences in the symptoms observed between the crops assessed, as discussed in Chapter 3.4. The results obtained from Chapter 3 of this study show that PepMV is widespread in UK glasshouse tomato crops. The use of the Ling *et al*. (2013) primers in RT-LAMP assays would provide a valuable tool for tomato growers for the use in rapid on-site diagnosis of PepMV infection within a crop. Early PepMV identification will allow for the correct measures to be taken to limit the damage caused by the virus and to prevent its dissemination.

Chapter 4 of this project was directed at identifying sources of PepMV inocula within the glasshouse environment. The efficacy of end-of-season glasshouse cleanups were assessed by swabbing glasshouse surfaces/equipment before and after the cleanups were conducted. The high percentage of pre-clean swabs which tested positive for PepMV highlighted the highly contagious nature of the virus. PepMV was found on surfaces that did not come into contact with plants directly, such as door handles, first aid boxes and electrical equipment. This shows how easily PepMV can be mechanically transmitted, especially when carrying out necessary hands-on practices required for glasshouse tomato production. In order to prevent the spread of PepMV to other glasshouses and crops it is essential that the end-of-season cleanups are effective at eliminating PepMV from the glasshouse. Although PepMV was still detected on a number of post-cleanup swabs, sap inoculation tests indicated that the PepMV detected by the RT-LAMP assays was not viable. This suggested that the end-of-season cleanups conducted by the growers involved in this study were successful at eliminating infective PepMV. However, not all surfaces within the glasshouses were swabbed. If viable PepMV remnants existed on these un-swabbed surfaces, this may be sufficient to contaminate subsequent crops grown in these glasshouses.

The second source of potential PepMV inoculum that was investigated was compost. The survival of PepMV in composted tomato waste was monitored over an approximate 16 week period. RT-LAMP was used to test compost samples and controls (kept in a 24°C incubator), with the RT-LAMP amplification times being used as an indication of the level of PepMV that was present within the samples. The RT-LAMP amplification times, overall, showed that the average amplification times for the compost samples were higher than the controls, indicating that lower concentrations of PepMV were present in the compost samples. However, statistical analysis using linear regression revealed that the rate of change of PepMV concentration in the compost samples and controls did not differ significantly over time. Therefore, it could not be concluded that the composting process was successful at eliminating PepMV from this particular compost heap, but instead PepMV degraded and lost viability over time, irrespective of the conditions it was exposed to. As there is currently no published data on the effects of composting on PepMV and in order to improve this experiment, it would be valuable to conduct a replicated study using multiple compost heaps

and controls, instead of just one heap, so that the effect of composting on PepMV could be compared more effectively to un-composted controls.

The final part of this investigation looked at the detection of PepMV in water used to irrigate tomato crops. As large volumes of water/nutrient solution are used in crop irrigation, often a concentration method is required before detection of viral particles is possible. The National Institute of Biology (NIB) Slovenia have developed a successful technique which allows for the concentration of PepMV particles from large volumes of water. This method was used to concentrate water samples collected from three commercial UK tomato sites. The concentrated water samples were then tested using RT-LAMP which revealed 50% of the samples to be infected with the CH2 genotype of PepMV. PepMV was detected in the reservoir water of site 1; however, the virus was not detected in the post-treatment water (which had been pasteurised), suggesting that their water disinfestation technique was successful at eliminating PepMV from water. PepMV was detected in the two samples collected from the NFT site and this site utilised no disinfestation treatment for their water. The nutrient film technique is a particularly popular irrigation method used for commercial tomato production in the UK and involves recirculation of the nutrient solution around the crop and possibly to other crops. The findings from this study show that PepMV can be found in water/nutrient solutions, supporting findings by Mehle *et al*. (2014) and Schwarz *et al*. (2010). The irrigation systems used in commercial tomato production therefore represent a medium for PepMV transmission and are likely to aid the dissemination of the virus from one or few infected plants to a whole crop, especially if no treatment is used to disinfect the water.

Currently, the most common way of reducing PepMV infection and transmission is the implementation of hygiene procedures within the glasshouse. However, the rapid global spread of the virus since its first emergence in Europe in 1999 would suggest that these cultural hygiene practices are not always completely effective. Therefore, more research should be conducted into ways of more efficiently preventing PepMV dissemination. The use of cross-protection may provide a promising way of reducing yield loss caused by PepMV. In recent months the use of PMV-01 (Chapter 1.10.3) against PepMV in the UK has been approved, and so it will be interesting to see how successful this cross-protection treatment is at preventing crop damage by necrotic PepMV strains.

Instead of relying on the use of mild field PepMV isolates for use in cross-protection, it has recently been shown that cross-protective PepMV variants can be created using mutagenesis. Chewachong *et al*. (2015) engineered a mild PepMV isolate from a necrotic PepMV isolate through the use of site-directed mutagenesis of the CP gene. The sites at which mutagenesis was targeted were selected by comparing the PepMV CP sequence with the CP sequences of five other closely related potexviruses. Variable amino acids/clusters that existed between the CP sequences were thought to contribute to the virus' adaptation to its specific host, and so it was speculated that altering these amino acids could lead to a reduction in fitness of that virus within its host. The mutated PepMV isolate created possessed two CP differences to the wild type PepMV; the conversion of threonine to lysine at amino acid 66 and the conversion of alanine to aspartate at amino acid 67. When used to inoculate *N. benthamiana* and tomato plants, the mutated isolate exhibited low accumulation of viral RNA and CP within the test plants and also induced very mild disease symptoms. Furthermore, both test plants were protected against secondary infection by the wild type necrotic PepMV. Chewachong *et al*. (2015) concluded that their mutagenesis method was simple and successful at developing a PepMV isolate capable of cross-protection. Their method did not require any prior knowledge of mild strain isolates and, therefore, this method could possibly be used for cross-protection in other plant viruses. Furthermore, the relative speed of this procedure may allow for a rapid response to other emerging viral pathogens.

Although the use of PepMV isolates, such as the mutated isolate discussed above and that used in PMV-01, have been shown to be successful in trials, research so far indicates that the cross-protection isolate only confers protection against secondary infection by the same genotype (De Nayer *et al*., 2012). Although the CH2 genotype appears to be the most common cause of PepMV infection in Europe, approximately one fifth of tomato fruit/leaf samples assessed in the current investigation were burdened with mixed-genotype infection. Therefore, it is unlikely that cross-protection would be successful when trying to prevent mixed-genotype infections and so strict glasshouse hygiene procedures would still have to be maintained in order to minimise the possibility of a different PepMV genotype infecting the cross-protected crop.

A potential risk of the use of cross-protection is that synergism could exist between the mild PepMV isolate and other plant viruses. Synergism often leads to the increase of viral titre of at least one of the viruses infecting the plant. This

usually results in more severe symptom expression than that which would be observed due to single infection. If this was the case, symptom severity could be enhanced in crops that were co-infected with PepMV and other viral pathogens. Alfaro-Fernández *et al*. (2010) observed synergism at the ultrastructural level of tomato leaf tissue co-infected with PepMV, *Tomato torrado virus* (ToTV) and *Tomato chlorosis virus* (ToCV). Tissues with the triple virus infection possessed greater numbers of necrotic cells and cells with cytoplasmic disruption than tissues with single infection. Synergism has also been observed in viral infections of potato, as mentioned in Chapter 3.4.

Another consideration is that the population of PepMV is highly genetically heterogeneous. Gómez *et al*. (2012) calculated that the mutation rate of PepMV is 5.570×10^{-3} substitutions per site per year, approximately an order of magnitude higher than that of other RNA plant viruses reported recently. The high mutation rate means that PepMV has an increased propensity to evolve into new isolates and strains. This is highlighted by the fact that the first isolates found in Europe shared 99% genetic similarity; however, as the virus spread across the globe new isolates emerged (US1 and US2) which shared only 80% sequence homology with the original EU isolates. It is possible that the dominant CH2 strain to which cross-protection has been developed against could evolve into a new strain, rendering the cross-protection isolates ineffective.

There is a positive relationship between the speed of molecular evolution and the mutation rate (Sanjuán, 2012). Furthermore, the ability to evolve faster increases the likelihood of horizontal transmission and viral adaptation to other host plants. Blystad *et al*. (2015) recently showed, through inoculation studies, that eggplant (*S. melongena*) could be a systemic host of the CH2, EU and US1 genotypes of PepMV, although symptom expression was variable. This supported earlier work by Gómez *et al.* (2009) who noted that eggplant could serve as a PepMV reservoir in fields surrounding tomato crops. Blystad *et al.* (2015) also found that potato and sweet pepper can be experimentally infected with PepMV; however, systemic symptoms were often not observed. As well as taking action to prevent the dissemination of PepMV between tomato crops, it may also be beneficial to take precautions against the spread of the virus to other crop types as research has shown other potential PepMV hosts to exist.

Perhaps the most desirable method for reducing crop damage and yield loss caused by PepMV infection is the use of resistant tomato varieties. Soler-

Aleixandre *et al.* (2007) screened a collection of accessions from different *Solanum* species in an effort to identify sources of PepMV resistance. Inoculation experiments with the EU genotype of PepMV revealed that one accession of *S. pseudocapsicum* exhibited complete resistance to PepMV, with no symptom development observed. However, the use of this *Solanum* species is limited as it cannot be bred with cultivated tomato species. Soler-Aleixandre *et al.* (2007) found that some accessions of *S. chilense* and *S. peruvianum* were resistant to infection by the EU strain. *Solanum chilense* has been used for breeding resistance against other viral pathogens such as *Tomato yellow leaf curl virus* (TYLCV) and *Cucumber mosaic virus* (CMV) and so the authors concluded that *S. chilense* represents the most promising species for potential sources of PepMV resistance.

In a similar study, Ling and Scott (2007) assessed 109 tomato accessions for their resistance to PepMV infection. Mechanical inoculation with isolates representing the EU (a mixture of CH1 and CH2) and US1 (a Texan isolate) PepMV strains revealed that two accessions each of *S. peruvianum* and *S. chilense* had moderate levels of resistance. The highest levels of resistance were observed in three accessions of *S. habrochaites*, and these accessions were used for further seed production. Secondary screening of the progenies of the *S. habrochaites* accessions revealed that accession LA1731 exhibited the highest levels of resistance, with all progeny plants being symptomless when inoculated with EU and US1 strains. However, the level of viral accumulation varied greatly between the progeny plants, with low titres of PepMV being detected in only 9 out of 35 progeny plants. This suggests that resistance to PepMV may be polygenic, with a certain gene or genes conferring resistance to viral replication and other genes being responsible for symptom suppression. Polygenic resistance traits may be difficult to introgress into commercial tomato cultivars; nevertheless, the findings of Ling and Scott (2007) are promising and have identified a *Solanum* accession with resistance traits against multiple PepMV isolates.

While efforts are made to find more effective ways of reducing damage caused by PepMV infection, it is vital that current preventative measures are strictly followed. The results obtained from this investigation highlight the wide distribution of PepMV in UK tomato crops and the ease of viral transmission via mechanical means. Glasshouse hygiene protocols should be abided by in order to prevent the spread of PepMV via contaminated clothing, equipment or tools to

other uninfected glasshouses. Rigorous disinfection of all glasshouse surfaces and equipment should be conducted at the end of each growing season, even on surfaces that are unlikely to have come into contact with plant material. Tomato sites which utilise recirculating irrigation systems should ensure that a suitable water disinfestation treatment is employed, such as pasteurisation or sensor-based disinfection (Chapter 4.4). This will facilitate the prevention of dissemination of PepMV via water/nutrient solutions from an infected plant/crop to separate crops in other glasshouses where mechanical transmission can be avoided. The use of genotype specific primers, such as those designed by Ling *et al*. (2013), in RT-LAMP assays should be utilised by tomato growers for early identification of PepMV-infected crops. Early detection will allow for the implementation of control measures to limit PepMV transmission, minimise crop damage and yield loss and, ultimately, aid in maximising productivity.

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Appendices

Appendix 1- RT-LAMP results for samples from crop 6 using the Ling *et al.* (2013) primers

		RT-LAMP Amplification time (mm:ss)		
Sample	**Description**	**CH2 Primer**	**EU Primer**	**US1 Primer**
1	Symptomless plant	12:00	07:15	X
2	Symptomless plant	10:15	05:45	X
3	Symptomless plant	11:30	06:00	X
4	Symptomless plant	09:15	05:30	X
5	Symptomless plant	11:45	06:45	X
6	Symptomless plant	09:45	05:15	X
7	Symptomless plant	08:45	06:00	X
8	Symptomless plant	09:15	07:30	X
9	Symptomless plant	09:30	07:00	X
10	Symptomless plant	09:00	06:00	X
11	Symptomatic plant	11:00	07:15	X
12	Symptomatic plant	09:30	05:15	X
13	Symptomatic plant	09:00	05:30	X
14	Symptomatic plant	10:15	06:45	X
15	Symptomatic plant	09:30	05:45	X
16	Symptomatic plant	10:30	05:45	X
17	Symptomatic plant	10:45	05:45	X
18	Symptomatic plant	11:00	07:45	X
19	Symptomatic plant	08:45	06:00	X
20	Symptomatic plant	08:15	06:30	X
21	Symptomatic tissue- leaf with chlorotic spots	07:45	05:45	X
22	Symptomatic tissue- leaf with necrosis	06:45	04:30	X
23	Symptomatic tissue- leaf with necrosis	07:45	05:45	X
24	Symptomatic tissue- leaf with necrosis	08:00	05:45	X
25	Symptomatic tissue- leaf with necrosis	07:45	05:30	19:15
26	Symptomatic tissue- leaf with chlorotic spots	06:45	04:45	X
27	Symptomatic tissue- leaf with necrosis	08:15	05:30	X

28	Symptomatic tissue- leaf with necrosis	06:45	04:45	X
29	Symptomatic tissue- marbled fruit	09:30	06:30	X
30	Symptomatic tissue- marbled fruit	09:30	07:30	X

Appendix 2- RT-LAMP results for samples from crop 1, visit 1 using the Ling *et al.* (2013) primers

		RT-LAMP Amplification time (mm:ss)		
Sample	**Description**	**CH2 primer**	**US1 Primer**	**EU Primer**
1	Symptomless Plant	09:15	X	X
2	Symptomless Plant	10:30	X	X
3	Symptomless Plant	09:45	X	X
4	Symptomless Plant	10:00	X	X
5	Symptomless Plant	09:30	X	X
6	Symptomless Plant	08:15	X	X
7	Symptomless Plant	09:45	X	X
8	Symptomless Plant	10:15	X	X
9	Symptomless Plant	09:45	X	X
10	Symptomless Plant	09:30	X	X
11	Symptomatic Plant	08:00	X	X
12	Symptomatic Plant	08:15	X	X
13	Symptomatic Plant	08:45	X	X
14	Symptomatic Plant	09:15	X	X
15	Symptomatic Plant	08:30	X	X
16	Symptomatic Plant	07:45	X	X
17	Symptomatic Plant	08:45	X	X
18	Symptomatic Plant	08:45	X	X
19	Symptomatic Plant	08:00	X	X
20	Symptomatic Plant	07:30	X	X
21	Symptomatic Tissue- leaf with chlorotic spots	08:00	X	X
22	Symptomatic Tissue- leaf with chlorotic spots	08:30	X	X
23	Symptomatic Tissue- leaf with chlorotic spots	07:30	X	X
24	Symptomatic Tissue- leaf with chlorotic spots	08:15	X	X
25	Symptomatic Tissue- leaf with chlorotic spots	08:15	X	X
26	Symptomatic Tissue- leaf with chlorotic spots	07:15	X	X
27	Symptomatic Tissue-unripe fruit on	11:15	X	X

	fully ripe truss			
28	Symptomatic Tissue -unripe fruit on fully ripe truss	10:00	X	X
29	Symptomatic Tissue-unripe fruit on fully ripe truss	10:45	X	X
30	Symptomatic Tissue- marbled fruit	09:30	X	X

Appendix 3- RT-LAMP results for samples from crop 1, visit 2 using the Ling *et al.* (2013) primers

		RT-LAMP amplification time (mm:ss)		
Sample	**Description**	**CH2 primer**	**US1 Primer**	**EU Primer**
1	Symptomless Plant	08:00	X	X
2	Symptomless Plant	08:00	X	X
3	Symptomless Plant	08:00	X	X
4	Symptomless Plant	08:00	X	X
5	Symptomless Plant	07:00	X	X
6	Symptomless Plant	08:45	X	X
7	Symptomless Plant	07:15	X	X
8	Symptomless Plant	08:30	X	X
9	Symptomless Plant	08:15	X	X
10	Symptomless Plant	07:00	X	X
11	Symptomatic Plant	08:15	X	X
12	Symptomatic Plant	07:30	X	X
13	Symptomatic Plant	08:45	X	X
14	Symptomatic Plant	08:30	X	X
15	Symptomatic Plant	08:30	X	X
16	Symptomatic Plant	07:00	X	X
17	Symptomatic Plant	08:45	X	X
18	Symptomatic Plant	08:15	X	X
19	Symptomatic Plant	10:00	X	X
20	Symptomatic Plant	10:00	X	X
21	Symptomatic Tissue- leaf with chlorotic spots	06:45	X	X
22	Symptomatic Tissue- leaf with chlorotic spots	06:45	X	X
23	Symptomatic Tissue- leaf with necrotic spots	07:00	X	X
24	Symptomatic Tissue- leaf with chlorotic spots	08:30	X	X
25	Symptomatic Tissue- leaf with chlorotic spots	06:15	X	X

26	Symptomatic Tissue- leaf with chlorotic spots	07:30	X	X
27	Symptomatic Tissue- leaf with chlorotic spots	06:45	X	X
28	Symptomatic Tissue- leaf with necrotic spots	06:30	X	X
29	Symptomatic Tissue- leaf with necrotic spots	06:30	X	X
30	Symptomatic Tissue- marbled fruit	06:15	X	X

Appendix 4- RT-LAMP results for samples from crop 5, visit 2 using the Ling *et al.* (2013) primers

		RT-LAMP Amplification time (mm:ss)		
Sample	**Description**	**CH2 primer**	**EU primer**	**US1 primer**
1	Symptomless Plant	15:00	X	X
2	Symptomless Plant	X	X	X
3	Symptomless Plant	X	X	X
4	Symptomless Plant	17:30	X	X
5	Symptomless Plant	X	X	X
6	Symptomless Plant	X	X	X
7	Symptomless Plant	X	X	X
8	Symptomless Plant	X	X	X
9	Symptomless Plant	X	X	X
10	Symptomless Plant	X	X	X
11	Symptomatic Plant	X	X	X
12	Symptomatic Plant	X	X	X
13	Symptomatic Plant	X	X	X
14	Symptomatic Plant	X	X	X
15	Symptomatic Plant	X	X	X
16	Symptomatic Plant	X	X	X
17	Symptomatic Plant	X	X	X
18	Symptomatic Plant	X	X	X
19	Symptomatic Plant	X	X	X
20	Symptomatic Plant	X	X	X
21	Symptomatic tissue- leaf	X	X	X

22	Symptomatic tissue- leaf	X	X	X
23	Symptomatic tissue- leaf	X	X	X
24	Symptomatic tissue- leaf	X	X	X
25	Symptomatic tissue- leaf	X	X	X
26	Symptomatic tissue- leaf	X	X	X
27	Symptomatic Tissue- unripe fruit	X	X	X
28	Symptomatic Tissue- unripe fruit	25:30	X	X
29	Symptomatic Tissue- unripe fruit	X	X	X
30	Symptomatic Tissue- unripe fruit	X	X	X

X= PepMV-negative sample

Appendix 5- All on site locations swabbed before and after end-of-season cleanups and the RT-LAMP results for the presence of the CH2 genotype

Site 1

	Result of RT-LAMP assay	
Location swabbed	**Pre-clean**	**Post-clean**
Concrete at row 39	✓	×
Water cooler	×	×
Concrete at row 34	✓	×
Trolley number 364	✓	×
Ladder rungs trolley 362	✓	✓
Inside door handle	✓	✓
Inside main circuit board box at row 41	×	✓
Small switch row 39	✓	×
Glass at end row 43	×	×
Glass at end row 46	×	×
Heating pipe row 33	✓	×
Heating pipe row 46	✓	×
Metal between pipes row 45 (4 down)	✓	×
Drip line row 45	×	×
Drip line roe 34	×	×
Drip peg row 34	×	×
Drip peg row 39	×	×
Stanchion row 10, between metal plate and post	✓	×
Large pipes on back wall by door	×	✓
Grey irrigation pipes/rig row 39	✓	×
Stanchion row 39/41, pathway	✓	×
Purple picking crate	✓	×

Purple picking crate	✓	×
Under heating pipe row 42	✓	×
White in crop pipe row 37, inside end	✓	×
White in crop pipe row 38, inside end	✓	×
Metal support for white pipe	✓	✓
Under heating pipe row 47	✓	×
Pipes on glass at end of row 47	✓	×
Centre of concrete path row 43	✓	×
*Taps on water cooler / grower phone	✓	×
*Plant support / plastic jug to catch drips	✓	×
Spray trolley inc. fabric straps	✓	×
Floor of trolley 362	✓	✓
Outside of electrical box row 41	✓	×
Metal plate to hold wires behind stanchion row 41, with holes	✓	×
Waste bin	✓	×
Rubber irrigation pipe	✓	×
Glass joining concrete, end row 41	×	×
Wheels of trolley 362	✓	✓
Small trolley carrying electricals	✓	✓
Glass end of row 28	×	×
Glass in door	✓	×
Outside door handle	✓	✓
Grey pipe floor row 39	✓	×
Wooden pallet	✓	×
Picking crate handle	✓	×
Stanchion row 40, middle or row	✓	×
First aid box	✓	✓
Hand sanitiser by door	×	×

***not available at second visit / swabbed instead**

Site 2

	Result of RT-LAMP assay	
Location swabbed	**Pre-clean**	**Post-clean**
Concrete pathway by row 106	✓	✓
Concrete pathway by row 173	✓	✓
Glass at end of 106	✓	×
Glass at end of 105	×	×
Aluminium post at 101	✓	✓
Drip line 173	×	×
Drip line 172	×	×
Drip peg 173	✓	×

Drip peg 172	✓	×
Inside door handle	✓	×
Alcohol gel dispenser	✓	×
Leafing cage 34	✓	✓
Ladder on crop trolley 37	✓	✓
Heating pipe 177 (bottom)	×	✓
Heating pipe 177 (top)	✓	×
Black picking crate	✓	×
Green picking crate	✓	✓
Outside door handle	✓	×
Small electrical panel 161	✓	✓
Electrical panel	✓	✓
Mypex row 173	×	✓
Mypex row 176	×	×
Water cooler	✓	×
Pipes on back wall	✓	×
Between/below slabs 177	✓	×
Spray trolley 2	✓	×
Spray trolley 4	✓	×
Pen from site	✓	×
Aluminium post 1st on right	✓	×
Black de-leafing bin	✓	✓
Glass at end of row 173	✓	×
Metal on floor 173	✓	✓
Hook at end of 173	✓	×
Wheels of leafing cage 17	✓	✓
Trolley 33	✓	✓
Leafing cage 17	✓	×
Plugs/switch at 169	✓	✓
Below gutter 173	✓	×
Fruit trolley (no number)	✓	✓
Fruit trolley (no number)	✓	✓
Concrete pathway	✓	✓
Control box	×	✓
Leafing cage (no number)	✓	×
Plant support bracket	✓	✓
Spray boom	✓	✓
Inside door handle	✓	×
Heating pipe surface	✓	✓
Black picking crate	✓	×
Outside door handle	✓	*
Concrete pathway by door	✓	×

***Not Tested, swab missing**

Site 3

	Result of RT-LAMP assay	
Location swabbed	**Pre-clean**	**Post-clean**
138 Concrete	✓	×
136 Contrete	✓	×
Glass at end of 137	✓	×
Glass at end of 139E	✓	×
Heating pipes Row 135	✓	×
Heating pipes Row 139	✓	✓
Metal floor plate 135	✓	✓
Metal floor plate 139	✓	✓
Grow pipes 141	✓	×
Grow Pipes 142	✓	✓
Green packing crate	✓	✓
Big Black packing crate	✓	×
Blue packing crate	✓	✓
Drip peg half row 130	✓	✓
Drip peg half row 130	✓	✓
Drip line half row 130	✓	✓
Drip line half row 130E	✓	✓
Water cooler small tap	×	×
Water cooler big tap	✓	×
Green waste bin - inside bottom	✓	✓
Moth light, row 131	✓	✓
Picking trolley	✓	×
Crop work trolley floor	✓	✓
Crop work trolley rails	✓	✓
Gutter support 137	✓	✓
Gutter 134	✓	×
Gutter 135	✓	×
Mypex 131	✓	✓
Door handle inside	✓	×
Door handle outside	✓	✓
Ceiling chain 139 2 in	✓	✓
Stanchion 140 end row	✓	✓
Stanchion row 135 6 in	✓	✓
Stanchion row 135 half way	✓	×
Irrigation pipe under gutter row 152	✓	✓
Irrigation pipe under gutter row 129	✓	✓
Electrical trolley charge point 125	✓	✓
Electrical trolley charge point 121	✓	✓

Tag row 138	✓	×
Tag row 142	✓	✓
Hand sanitiser	✓	✓
Whiteboard for jobs	✓	✓
Green button	✓	✓
Green button	✓	✓
Electricals outside	✓	✓
Electric pallet truck folks	✓	✓
Electric pallet truck handles	✓	✓
Electrical switch nearest door	✓	✓
Soap dispenser in wash area - middle	✓	✓
Door and handle and key pad to office	✓	×

✓ = PepMV detected

× = PepMV not detected