IDENTIFICATION, PREVALENCE AND IMPACTS OF VIRAL DISEASES OF UK WINTER WHEAT

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Abstract

The potential for viruses to be causing the plateau in the yield of UK wheat (*Triticum aestivum*) was investigated. Mechanical inoculation of Cynosurus mottle virus to wheat cv. Scout and cv. Gladiator caused 83% and 58% reduction in the number of grains produced, highlighting the potential of viruses to cause disease and yield loss. Viruses historically detected in cereals in the UK were not found to be prevalent following real time reverse transcriptase polymerase chain reaction testing of 1,356 UK wheat samples from 2009-2012 using eleven assasys developed in the project. This included an assay for Cynosurus mottle virus, which was based on its complete genome sequence which was obtained for the first time in this project. Viruses detected were *Barley yellow dwarf virus-MAV* (6 samples) (BYDV-MAV)*, Barley yellow dwarf virus-PAV* (6 samples) (BYDV-PAV) and *Soil-borne cereal mosaic virus* (12 samples) (SBCMV). There was a higher prevalence of viruses in the south, thought to be due to warmer temperatures which benefitted insect vectors and the molecular processes of infection. Viruses were most commonly detected in the variety JB Diego, perhaps because this variety has no known resistance to viruses.

The low prevalence of known viruses could also have been because they were outcompeted or replaced by previously unknown ones. Next generation sequencing was used to test 120 samples from an organic site, including wheat, weeds and insects, to search for novel viruses. Testing of twelve storage regimes for insect traps using BYDV-PAV infected *Sitobion avenae* for recovery of PCR amplifiable RNA using 18S rRNA and BYDV-PAV assays found that 0.5 M EDTA was the most successful regime which was therefore used in the collection of samples for sequencing. Known viruses such as BYDV-PAV were detected along with some additional potentially novel viruses (eight possibly novel viruses or strains of viruses with four in wheat). One such virus was apparently present in 25% of all wheat

samples tested, making it potentially very significant. This could be important for unlocking the yield potential of wheat because it could be a cryptic virus which is highly prevalent.

In order to control the spread of viruses their methods of transmission must be understood, therefore testing of seeds and resulting plants from Cynosurus mottle virus infected material was done. Tests did not detect the virus, therefore it was concluded that seed transmission does not occur. However, further tests are required.

In conclusion this study indicates that known viruses are not currently a major problem for UK winter wheat. However, novel viruses that are a problem may be detected in the future perhaps by next generation sequencing. Additonal viruses from abroad would add to the threat. The impact of all viruses in wheat may be greater in the future due to climate change.

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List of viruses

The names and acronyms of viruses referred to frequently are listed. Formatting is accordance with the current ICTV (International Committee on Taxonomy of Viruses) information.

Name of the virus	**Acronym of the virus**
Acute bee paralysis virus	ABPV
Agropyron mosaic virus	AMV
American wheat striate mosaic virus	AWSMV
Acyrthosiphum pisum virus	APV
Aubian wheat mosaic virus	AWMV
Barley mild mosaic virus	BaMMV
Barley stripe mosaic virus	BSMV
Barley yellow dwarf virus GAV, GPV, MAV, PAS, PAV, RMV, RPV and SGV	BYDV-GAV/GPV/MAV/PAS/PAV/RMV/RPV and SGV
Barley yellow mosaic virus	BaYMV
Barley yellow striate mosaic virus	BYSMV
Black queen cell virus	BQCV
Brazilian wheat spike virus	BWSV
Brome mosaic virus	BMV
Brome streak mosaic virus	BrSMV
Cannabis cryptic virus	CCV
Chinese wheat mosaic virus	CWMV
Citrus leprosis virus C	CLVC
Cocksfoot cryptic virus	CCV
Cocksfoot mild mosaic virus	CfMMV
Cocksfoot mottle virus	CfMV
Cocksfoot streak virus	CSV
Cynosurus mottle virus	CnMoV
European wheat striate mosaic virus	EWSMV
Festuca leaf streak virus	FLSV
Flame chlorosis virus	FCV
Foxtail mosaic virus	FoMV

Freesia mosaic virus	FMV
Grapevine virus B	GBV
High plains virus	HPV
Indian peanut clump virus	IPCV
Indian wheat dwarf virus	IWDV
Iranian wheat stripe virus	IWSV
Iranian maize mosaic virus	IMMV
Japanese wheat mosaic virus	JWMV
Johnsongrass mosaic virus	JGMV
Maize dwarf mosaic virus	MDMV
Maize mosaic virus	MMV
Maize rough dwarf virus	MRDV
Maize streak virus	MSV
Maize yellow stripe virus	MYSV
Mal de Rio Cuarto virus	MRCV
Northern cereal mosaic virus	NCMV
Oat chlorotic stunt virus	OCSV
Oat mosaic virus	OMV
Oat necrotic mottle virus	ONMV
Olive leaf yellowing-associated virus	OLYaV
Parsnip yellow fleck virus	PYFV
Peanut clump virus	PCV
Raphanus sativus cryptic virus 1/2/3	RSCV1/2/3
Rice black-gall dwarf virus	RBGDV
Rice black-streaked dwarf disease	RBSDD
Rice hoja blanca virus	RHBV
Rice stripe virus	RSV
Rosy apple aphid virus	RAAV
Rhopalosiphum padi virus	RPV
Ryegrass mosaic virus	RgMV
Soil-borne cereal mosaic virus	SBCMV
Soil-borne wheat mosaic virus	SBWMV
Soybean dwarf virus	SDV
Sugarcane mosaic virus	SCMV

Tobacco mosaic virus	TMV
Triticum mosaic virus	TrMV
Turnip mosaic virus	TuMV
Wheat dwarf virus	WDV
Wheat spindle streak mosaic virus	WSSMV
Wheat spot mosaic virus	WSpMV
Wheat streak mosaic virus	WSMV
Wheat yellow mosaic virus	WYMV
White clover mosaic virus	WCMV

List of abbreviations

Deoxyribonucleotide triphosphates	dNTPs
Double antibody sandwich enzyme linked immunosorbent assay	DAS-ELISA
Enzyme linked immunosorbent assay	ELISA
Polymerase chain reaction	PCR
Real time reverse transcriptase polymerase chain reaction	qRT-PCR
Transmission electron microscopy	TEM

Chapter 1 - Introduction

1.1 The importance of wheat

Wheat is an important crop in the UK and globally. It has many uses including animal feed, a source of bioenergy and human food. It is the most widely grown crop worldwide in terms of harvested area and was the third most produced crop after rice (*Oryza sativa*) and maize (*Zea mays*) in 2010 (Leff *et al*., 2004; Web reference - FAOstats). The International Grain Council record that an average of 676 million tonnes (MT) of wheat were produced per year from 2010 to 2012 (Web reference – IGC). Wheat currently provides an average of 20% of calories consumed by humans (Web reference - FAOstats); the stability and development of the UK economy therefore, depends in part on wheat.

1.2 Wheat production in the UK and globally

The UK produced approximately 2% of the total global yield of wheat in 2010 (14.9 MT) and was the 14th producer in terms of weight worldwide (Web reference – FAOstat). Approximately 40% of the wheat grown in the UK is used as feed, 31% for milling with the remainder used for seed, brewing and export (Web reference – Grain). According to the HGCA British Cereals Update, typically 15-20% of the wheat produced in the UK is exported, the majority of which is used overseas for milling. For example between 2010 and 2011 2.4 million tonnes (MT) were exported out of a total production of 15.3 MT (Web reference – HGCA exports). Top customers in 2011 were Holland (0.75 MT), Spain (0.37 MT), Germany (0.12 MT) and Portugal (110,000 tonnes). UK wheat is also sold out of the EU to the USA (150,000 tonnes), Algeria (25,000 tonnes) and Morocco (11,640 tonnes) (Web reference – HGCA BCU). According to Defra in 2009/10 global wheat stocks were used for food (70%), feed (16%), industrial (2%), biofuel (1%) and other (11%) (Web reference – Defra grain

markets). The global population is increasing dramatically and as a result it has been estimated that the amount of agricultural produce needs to double by 2050; this applies to wheat in the UK (Foresight, 2011).

There has been a trend towards increased area for wheat growth in the UK. The HGCA Winter Planting Survey for 2011, based on 3000 farm businesses from England and Wales, states that the total area of land planted with cereals and oilseeds rose by 5% from December 2010, to 2.98 million hectares. The area for wheat specifically increased by 3% to 1.86 million hectares. In comparison, barley (*Hordeum vulgare*) was planted on just 345,000 hectares of land. The HGCA state that the increase in the area of wheat planted was due to favourable autumn weather conditions which allowed planting to go ahead and high forecasts for wheat prices encouraging farmers to plant more (Web reference – Planting 2011). The area of winter wheat sown each year fluctuates for these reasons, for example the amount of wheat planted in 2012 was reduced by 19% compared to 2011 because of unfavourable weather conditions (Web reference – Planting 2012). While increasing the area of land allocated to wheat farming will increase production, it will not solve the underlying issue of the plateau in wheat yields (see Section 1.3). This needs to be investigated and solutions developed in order to meet the future demands.

1.3 The changing yield of wheat in the UK

The yield of wheat in the UK has been dynamic in previous years for various reasons. Figure 1.1 shows the yield of wheat in the UK between 1880 and 2013. The first increase in yield around the 1940s coincided with the increased use of chemical fertilisers such as nitrogen, phosphorus and potassium and herbicides being more widely available (Semenov *et al.*, 2012). After the second world war efforts turned to farming, which became more intensive and there was greater mechanisation (Robinson and Sutherland, 2002). The plateau following this increase was probably

due to the effects of these factors combined producing the maximum possible yield at that time. The next increase in yield is likely to have been due to the 'green revolution' which saw advances in wheat breeding. The development of new varieties was incentivised by the 1964 Plant Varieties and Seeds Act which developed plant breeders rights by royalties being paid for later use of the product (Hedden, 2010). The new varieties benefitted from better fertilisers and pesticides with more effective targeting, which allowed them to yield more grain (Knight *et al*., 2012). However, this made the plants heavy and more prone to lodging; therefore, semi-dwarf varieties were developed by researchers such as Borlaug (Evans, 1998; Hedden, 2010). Improvemnts in fungicides alsonadded to the achievement of higher yields of wheat (Semenov *et al*., 2012). The second plateau, which appears to have begun around the year 2000 could be due to the same reasons as the first plateau, but additionally there were fungicide reduction requirements from 1990-2003 (Knight *et al*., 2012; Moray Taylor, Fera, personal communication). This plateau has continued to date, showing stagnating yields with minor fluctuations; for example, in October 2012 Defra statistics stated that the yield of wheat in the UK was the lowest for 23 years, at 6.7 tonnes per hectare, despite an increased area of land planted (Web reference – FarmingUK). According to the HGCA this was due to poor weather in the spring and summer months, including wet periods which contributed to added disease pressures (Web reference – Poor harvest). It is possible to achieve higher yields of wheat than this. For example, in Scotland in 1989 13.99 tonnes per hectare were produced, and on a worldwide scale a yield of 15 tonnes per hectare was reported in New Zealand (Armour *et al*., 2004; Scottish Crop Research Institute Annual Report 1998-1999). Therefore, a plateau has been reached and wheat is not simply being produced to the maximum possible yield.

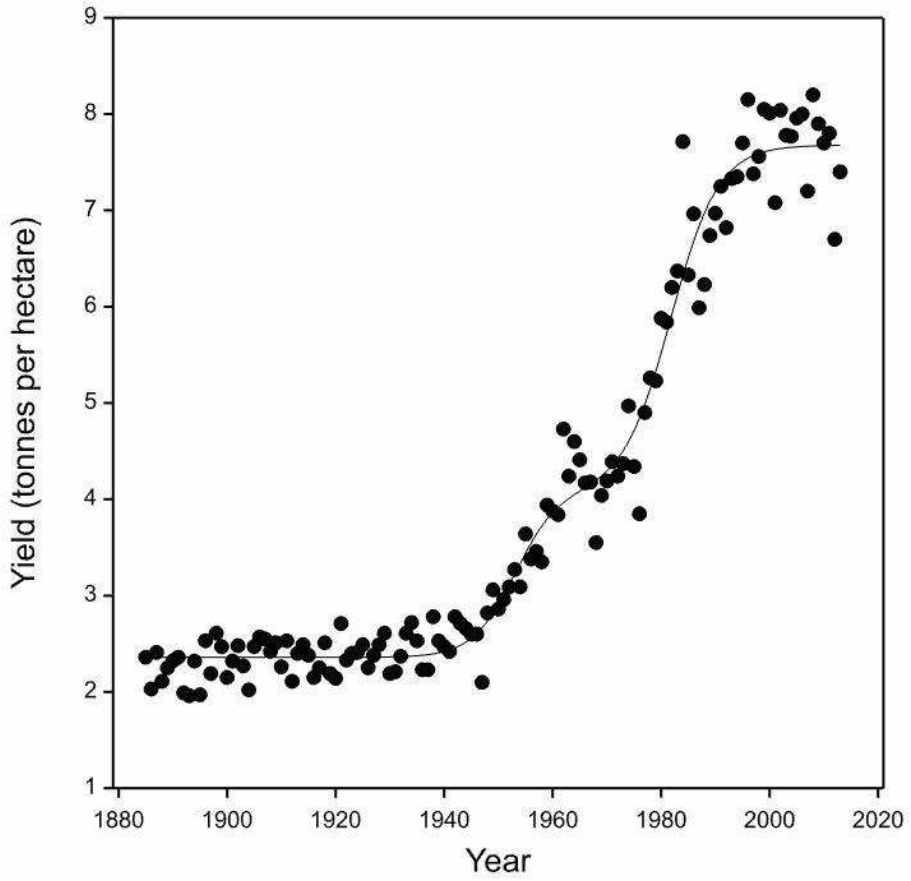


Figure 1.1. The yield of wheat achieved in the UK between 1880 and 2013. Modified from Defra statistics (Stephane Pietravalle, Fera, personal communication, data for 1880-2005). Data added for 2006-2013 from Defra farming statistics (Web reference – Defra farming statistics).

1.4 Could viruses help explain the current plateau in the yield of wheat?

The agronomic and political influences on yield that have been discussed do not seem to be great enough to fully explain the plateau; another contributing factor could be the impact of viruses. Viruses can cause a range of symptoms in wheat and ultimately severe reductions in yield. For example, in the USA yield losses of up to 100% have been attributed to a virus (*Wheat streak mosaic virus*) and *Barley yellow dwarf virus* (BYDV) has caused yield loss of 86% (Budge *et al.,* 2008; McNeil *et al*.,

1996; Miller and Rasochova, 1997). Viruses have had significant financial impacts and total losses worldwide of all plants are estimated at 6 x 10^{10} billion dollars per year (Cann, 2005). The initiative by Rothamsted Research which aims to achieve a yield of twenty tonnes per hectare of wheat in the UK by the year 2022, includes strategies to maximise yield potential, determine soil resources and interactions using systems approaches to crop improvements, and finally protect yield potential. The final category which includes protection from pests and disease, and therefore viruses, is predicted to result in a 5-10% increase in yield if resolved (Web reference – Rothamsted). This means that approximately 1.49 million extra tonnes of wheat could have been produced in the UK in 2010 if pests and disease were not an issue. This would have met the import demands of Canada, India and China in 2010/2011.

Aphids such as *Rhopalosiphum padi* and *Sitobion avenae* are known vectors of viruses of wheat, but also cause direct damage as they exude honeydew on to plant surfaces which encourages sooty moulds and blocks stomata, thereby decreasing photosynthesis (Ajayi and Dewar, 1983; Rochow, 1969). The plant also suffers damage as aphids overwhelm its resources, by diverting energy from plant growth and development to the themselves and to plant defence mechanisms against them (Nault, 1997). There is evidence that virus free *R. padi* can cause root and shoot reductions in wheat and chlorosis, necrosis and deformations have been observed in cereals (van Emden and Harrington, 2007). These are similar symptoms to those caused by viruses (see Section 1.10). Therefore it can be difficult and likely not possible, to separate the impacts caused by an aphid, in to that caused by direct feeding and that as a result of virus inoculation in natural samples which require diagnosis. However, comparisons using viruliferous and non-viruliferous aphids in laboratories would be able to investigate this.

The other pests and diseases that are likely to be contributing to the plateau in the yield of wheat will not be dealt with in this project, but briefly include examples such as wheat bulb fly (*Hylemia coarctatain*) and orange blossom midge (*Sitodiplosis mosellana*). The latter causes damage when the larvae, which hatch inside plants, exude enzymes that break down cell walls and convert starch to sugars. These pests in connection with viruses are estimated to cause a yield loss of 10% or more (Web reference – HGCA 3 and HGCA 5). Fungal diseases such as *Septoria tritici* and *Fusarium* spp. also cause significant problems with yield losses of 10-40% attributed to the latter example (Willocquet *et al*., 2008).

Viruses have been reported in wheat in the UK in the past, but not at a high level of prevalence which in part is likely due to a lack of screening, which means that they have not been considered a major problem (Clover *et al*., 1999a). In comparison, surveys have been performed for non-viral diseases of UK winter wheat such as fungal diseases whose symptoms are more easily identifiable without the need for laboratory testing (Cook *et al*., 1991; Polley and Thomas, 1991). The viruses known to be in the UK could be contributing to the yield loss of wheat and it is also possible that there are many viruses present in wheat in the UK that have not yet been detected. These could be viruses that are currently known in other species, such as barley. Historically tools for diagnosis of viral infections have not been readily available for large scale screening of viruses. Now, such tools are available (see Section 1.10), the main tool being next generation sequencing which could detect thousands of previously unknown plant viruses (Roossinck, 2013). In addition, scientists have not routinely tested for viruses that are not typically associated with wheat which means interactions may have been missed. For example Horvath (1983) confirmed 1,312 novel interactions between angiosperm species and 24 viruses for which he tested. That equates to 12%, and because to date there are approximately

250,000 species of angiosperm and 900 known plant viruses, there are potentially 25 x 10^6 new host and virus interactions which are currently unknown. While the early date of this work partially explains this result as fewer viruses had been described, it does highlight the point that if tests are not carried out for wheat and specific viruses, we simply cannot know if the interaction occurs.

Viruses could have entered the UK from overseas, via trade and travel which are becoming more common place (West *et al*., 2012). In the past such viruses and their vectors may not have been able to survive in the climate of the UK, such that the virus would not have become established. However, climate change predictions suggest that this may not be the case in the future. It then becomes important to understand transmission of individual viruses in order to predict and manage their spread and attempt to control them. Therefore a study of viruses in wheat in the UK is important, with regard to managing and sustaining the yield of wheat in the UK. These issues will be discussed.

1.5 Introduction to viruses and their impacts on wheat

Viruses that infect wheat are made of one or more nucleic acid template molecules, which are normally encased in a protective coat or coats of protein or lipoprotein. They must exist and replicate within suitable host cells where they use the host's protein synthesizing machinery (Hull, 2004). Viruses cause a range of visual symptoms in wheat which can include yield loss due to chlorosis or reddening of leaves and stems in mosaics, mottles of stripes, or by local necrosis, stunting and deformations of leaves such as twisting, which can reduce the surface area available to capture light, and thus photosynthesis. In addition, viruses such as BYDV cause decreased root mass and transpiration in susceptible plants (Erion and Riedell, 2012). Viruses can cause complete plant death, but this is rare as this would effectively remove the virus from existence as it relies on its host for survival (Hull, 2004).

Examples of symptoms are shown in Figures 1.2, 1.3, 1.4 and 1.5. The visual symptoms occur due to changes at the cellular level. For example the virus diverts resources from the plant for its own replication. As the virus replicates the particles form aggregates (see Figure 1.6). The plant also diverts energy to defence against the virus rather than yield (see Section 1.7). There are examples of synergistic relationships of viruses of wheat which leads to a greater impact than single infections, such as *Wheat streak mosaic virus* and *Triticum mosaic virus.* Dual infection causes worse symptoms including bleaching, stunting and deformation and up to 7.4 fold more virus to accumulate than in single infections (Tatineni *et al*., 2010). It is known that the HC-Pro encoded by *Potyviridae* can benefit other viruses which do not have it by aiding their development and avoidance of defence mechanisms. Both *Wheat streak mosaic virus* and *Triticum mosaic virus* are *Potyviridae,* therefore it is possible that their HC-Pros complement each other or that other encoded proteins from both benefit the other virus (Stenger *et al*., 2005; Tatineni *et al*., 2010). The additonal viral load will massively tax the plant, further reducing its energy for growth and development including yield production. Therefore infection by more than one virus is a significant threat to wheat yield. This could become more likely if more viruses are introduced to the UK from abroad.

Figure 1.2. Cynosurus mottle virus causing shortening of internodes which results in stunting in wheat cv. Gladiator.

Figure 1.3. Cynosurus mottle virus causing a severe chlorotic mottle (central image) and necrosis (background) in wheat cv. Scout.

Figure 1.4. Barley yellow dwarf disease causing reddening of the tips of wheat leaves (Web reference - KMLE).

Figure 1.5. *Soil borne cereal/wheat mosaic virus* causing chlorosis and stunting in patches in a field of wheat (Web reference – APS).

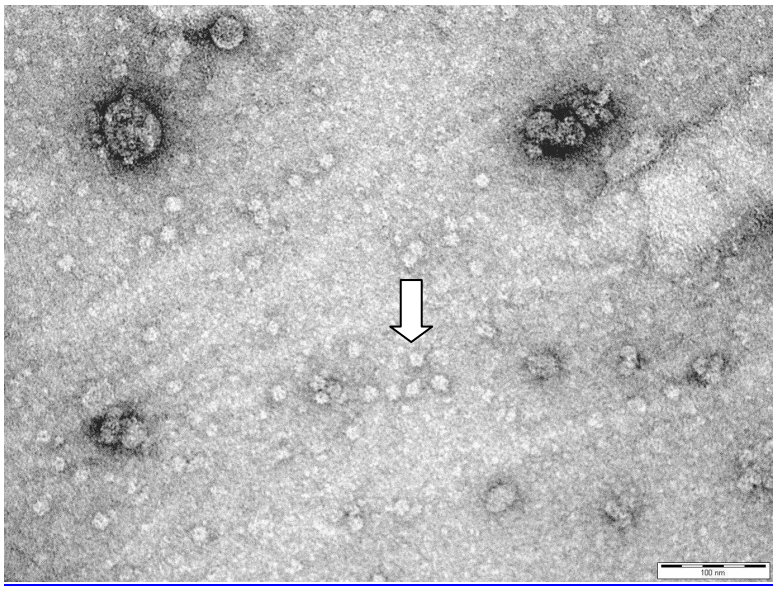


Figure 1.6. Virus particles of Cynosurus mottle virus in wheat cv. Einstein, note the arrow which highlights a cluster of viral particles (taken by author during this project).

1.6 Transmission methods of viruses

There are several natural methods by which viruses of wheat can spread and some are aided by human activities. Some viruses may only use one specific method, while others can use a range. An understanding of the methods a specific virus can use is important and is required to manage and limit its spread and therefore prevalence. In wheat, natural methods include spread by insects such as aphids, leafhoppers, planthoppers, beetles and mites as they feed on the plant (Cann, 2001; Fereres and Moreno, 2009). Insects themselves can be dispersed in the wind and travel to other locations, up to 1,000 kilometers away and across seas (Compton, 2002). The location of certain insects varies and as there are specific relationships between insects and the viruses they can transmit, so too does the location of reports of viruses in wheat (van Emden and Harrrington, 2007). There is evidence that viruses can manipulate their insect host to increase their spread. For example, it has been observed that *R. padi* that are carrying BYDV preferentially feed on wheat plants

which are not infected with the virus, but the opposite is true for insects which are not carrying the virus (Ingwell *et al*., 2012; Mauck *et al*., 2010). Ingwell *et al*. (2012) state that it is surprising that given the potential significance of this theory, that little research has been done to understand it. However, there are suggestions that the different profiles of volatile organic compounds from plants which are perceived by the insects (including blends of chemicals such as the following in BYDV infected wheat - nonanal, (Z)-3-hexenyl acetate, decanal, caryophyllene, and undecane) which virus infected plants produce compared to non-infected plants, cause increased settling of insects which are not currently carrying the virus (Ingwell *et al*., 2012; Jimenez-Martinez *et al*., 2004; Medina-Ortega *et al*., 2009). It has been shown in several different plant host-virus-insect vector relationships that virus infected plants are more suitable for insect growth and reproduction, and this is perhaps due to the increased levels of available nutrients such as amino acids from phloem. This has been suggested because less honeydew was exuded from aphids feeding on BYDV infected plants compared to healthy ones, perhaps because they needed to consume less sap to receive the required nutrients as it was of higher solute content (Hull, 2004). Additionally evidence suggests that BYDV infection can disrupt the development of the braconid parasitoid of aphids therefore increasing their health (Christiansen-Weniger *et al*., 1998). The increased fecundity also observed causes overcrowding, which leads to emigration, and therefore virus spread beginning sooner than on virus free plants (Hull, 2004). There is little evidence to suggest why virus infected insects prefer to transfer to healthy plants, but it is perhaps due to a change in preference for volatile organic compounds, to avoid the more heavily populated virus infected plants, or because there may be more healthy plants than infected thus increasing the chance of landing on one (McElhany *et al*., 1995).

Viruses of wheat can also be transmitted by soil dwelling organisms; such as *Polymyxa graminis*. This is a Plasmodiophorid that is a root-infecting obligate parasite (Kanyuka *et al*., 2003). The level of *P. graminis* may increase in soil over time (Ordon *et al*., 2009), where it can remain active for decades (which causes problems for control of such infections, see Section 1.7) (Adams *et al*., 1993; Kanyuka *et al*., 2003). Seed-borne transmission of viruses is possible amongst the Graminieae and as the infection begins in young plants the effects are often more severe than they would be in older plants to which viruses were transmitted because younger plants are more vulnerable and have not developed mature plant resistance (Gray and Banerjee, 1999; Lindblad and Sigvald, 2004). Reservoir plants (plants that can become infected with a virus, but may not suffer from it directly) are important in natural virus transmission cycles as they allow survival of viruses during periods when their major host, wheat, is not present (Hull, 2004).

Some viruses can be mechanically inoculated to certain cultivars of wheat to cause infections, for example Cynosurus mottle virus can be mechanically inoculated to wheat cv. Scout (see Chapter 6). The ease with which wheat can be mechanically inoculated with viruses has been exploited by humans who use the technique in laboratory studies. For example to investigate symptoms and for use as a diagnostic tool for test samples which form the homogenate which is used in the inoculation, and any resulting symptoms are compared to historical records of virus infections (Cann, 2001). Humans have also aided the spread of viruses by providing opportunities to break natural barriers though trade and travel. This has increased in recent years and continues to do so as the global community expands (Bateman *et al*., 2001). The virus may enter in virus infected plants, soil or via an insect vector. This could allow foreign viruses to enter the UK; and wheat currently grown may not be able to withstand them. For example it is suggested that a case of *Wheat streak*

mosaic virus in wheat in Australia occurred because infected seed was brought in from the USA. This was because there was evidence of spread by trade routes which all originated from a port (Dwyer *et al*., 2007).

1.7 Control measures for viral diseases of wheat

Wheat plants themselves employ several of the known methods that plants use to defend themselves from viruses, beginning with the physical barrier of the cell wall and including methods such as the hypersensitive response, RNA silencing and releasing volatile organic compounds that encourage predators of aphids to prey upon them thus removing them (Tatineni *et al*., 2012; Web reference - APS).

With regard to human intervention in wheat and virus cycles, the best way to control viruses of plants is to prevent them and their vectors from entering in the first place (Bacon *et al*., 2012). Therefore thorough inspection and quarantine measures at points of entry to the UK are important. An example has been set by New Zealand, which has strict measures in place, to ensure that diseased plant material or that which is carrying vectors of disease does not enter the country and threaten the native species (Web reference – Biosecurity). Currently viruses that are unknown could be entering the UK in imports, because testing using targeted tests for known viruses is missing them. In the future tools such as next generation sequencing could be used to avoid this (see Chapter 4). Good farming practice is important to prevent the spread of any viruses that are present; and equipment, clothing and machinery should either be dedicated for a certain area (eg a field with virus) or should be thoroughly cleansed with an anti-viral agent before and after use (Web reference – Clt). However, it is unlikely that these practices of good hygiene occur in reality, perhaps due to time or financial pressures or ignorance of risk of disease spread. This has been confirmed to an extent in discussions with local farmers who do not carry out such practices (Anonymous, personal

communications), therefore leaving entire farms and other users of the same equipment (such as shared combine harvesters) vulnerable to any viral infections present in cereals in one region of the farm.

Varieties of wheat differ in their susceptibility to certain viruses, which can be exploited for control. For example in the case of *Soil-borne cereal mosaic virus* (SBCMV) which is transmitted by *P. graminis*, resistant cultivars should be grown to prevent yield loss in wheat (Budge *et al*., 2008). If varieties which have resistance to viruses and the genes which are responsible can be identified, the eventual aim would be to use them in breeding or genetic modification to introduce the genes into varieties of wheat in which they were lacking, but which were still desirable for other traits. They can also be used to screen potential new varieties for the beneficial genes. Two major genes were identified in SBCMV resistant varieties of wheat, these were *Sbm1* and *Sbm2* (Bayles *et al*., 2007). These genes cause restriction of the movement of virus particles from below ground to above ground and impaired replication (Kanyuka *et al*., 2003). *Sbm1* is effective in producing resistance alone but there are additive effects when *Sbm2* is also present. However, *Sbm2* cannot cause resistance without *Sbm1*. Therefore while breeders may aim to target both genes, the focus has been on the latter. Current wheat breeders DSV and Limagrain use screening and selection for *Sbm1* and *Sbm2* genes (Edward Flatman, Limagrain; Matthew Kerton, DSV United Kingdom Ltd, personal communications). Bayles *et al*. (2007) also developed an accelerated test for screening new wheat lines for the genes. A reason for targeting both genes is the improved performance, but also the potential for increased durability in the field because polygenic resistance has been shown to be more durable than monogenic resistance in other plant-virus interactions (Bayles *et al*., 2007; Palloix *et al*., 2009). Bayles *et al*. (2007) investigated the possibility that the resistance *Sbm1* and *Sbm1* confer to some cultivars of wheat

may give them resistance to other soil borne viruses of wheat. It was concluded that there was likely to be resistance to SBWMV as well as SBCMV, due to them having resistance mechanisms which operated in a similar way by limiting movement and replication in roots. However, the same was not so for WSSMV resistant plants, for which an alternative resistance method is suggested to occur. Budge *et al*. (2008) reached the same conclusions and highlighted that the cultivar Aztec was resistant to WSSMV, but susceptible to SBCMV, which was thought to be due to them having different resistance genes. There is no evidence that can be found in published literature of how this may apply to viruses that are not soil borne, however it seems unlikely that *Sbm1* and *Sbm2* which have effects in the roots would be successful in controlling viruses introduced to aerial parts of a plants through insect feeding.

Pesticides are currently used in agriculture in the UK to reduce the number of insects in wheat fields and to limit their spread (Azzam and Chancellor, 2002; Web reference HGCA2). However, there are concerns over the levels of pesticides used so modelling is increasingly being used to enable targeted application which reduces the total amount of pesticide required whilst still being effective (Philips *et al*., 2011). This involves predicting when and where infestations of the insect viral vectors will occur and applying pesticides to coincide, therefore not applying chemicals unnecessarily thus reducing environmental impact, time and financial losses. The Rothamsted insect survey which comprises 16 insect trap collection sites across the UK and has been running since 1964 provides a valuable tool on which predictions can be made. It can also be used to suggest where insecticide resistance is occurring and to which products, therefore informing changes in chemical choices for farmers (Web reference – RIS). The HGCA and other farming publications also release information and alerts about levels of insects and provide suggestions of when to apply pesticides (Web references – FWI aphids and HGCA aphids).

Once a wheat plant is infected with a virus there are several courses of action that can be taken, and while these may not save the wheat plant, they can help to prevent future infections of emerging wheat. For example, it may be possible to isolate infected plants and remove and destroy them. The area can then be left fallow for a number of years with no possible reservoir plants nearby, with the field not used for wheat (Azzam and Chancellor, 2002). This is perhaps unrealistic because if there are infections of just a few plants in a field they are likely to go un-noticed. It would probably be necessary to destroy the whole crop because transmission methods of viruses mean it is likely that more than just a few plants are affected (even if not all symptomatic at the same time – perhaps due to different times of inoculation by insects) which farmers would not want to do, and in addition not all reservoirs for viruses of wheat are known therefore they cannot all be removed with certainty. Also, as soil-borne viruses can remain infective while associated with *P. graminis* within soils for many years, this may not always be practical as farmers may want to use the field for cereals again during that time (Kendall and Lomell, 1988). *Polymyxa graminis* cannot be removed from soil in a safe or economically viable way, therefore currently the only option is to plant resistant cultivars, such as for SBCMV as discussed (Budge *et al*., 2008; Kanyuka *et al*., 2003; Ordon *et al*., 2009).

In the future, methods may be developed that help control the spread or effect of viruses. For example Borodavka *et al*. (2012) suggest that antiviral drugs could be used to target the process of virus coat removal. Nanoparticles have been suggested for targeted application of additives or as part of fertilizer applications to plants (Gogos *et al*., 2012) and this method could also be used for antiviral drugs.

1.8 Climate change – to date and for the future

There is no doubt that the climate in the UK is changing. It is predicted that in the future there will be more severe and more frequent droughts (due to less rain in the

summer), floods (due to more rain in the winter), higher temperatures (an increase of up to 8°C in the summer) and more freak natural weather events such as snow or wind (Gornall *et al*., 2010; Web reference - CCRA; Web reference – Met Office). However, these predictions are general, and local conditions are likely to vary. These changes will have an impact on wheat itself, the ecosystems that support it and pests and diseases (Web reference - Thornton). Some projections suggest that 100-200 million additional people could be at risk of hunger due to climate change by 2050, due to its impact on areas such as food security (Web reference – Met Office).

Predictions of long-term climate trends threaten to reduce wheat yields, or retard yield growth by faster growth with reduced tillering, negative effects on photosynthetic pathways, closure of stomata to decrease water loss (at the same time decreasing photosynthesis), direct damage to plant cells such as freezing and the increasing survival of pests and diseases of agricultural crops; thus viruses are likely to cause worse symptoms during adverse weather conditions (Batts *et al*., 1997; Gourdji *et al*., 2012; Lobell and Gourdji, 2012; Web reference – HGCA). It has been estimated that 30% of the wheat grown in the UK is planted on drought prone land, such that wheat is already vulnerable to drought, and it has been shown that 10-55% achievable yield can be lost dependent on the growth stage (early stem extension, flowering and grain filling are sensitive development stages) and cultivar of wheat (Dodd *et al*., 2011; Foulkes *et al*., 2007; Whalley *et al*., 2006). Periods of drought have other affects that are not directly related to the wheat plant; for example, they harden the ground surface and increase soil strength making future watering and root growth more difficult, and there is also evidence that drought causes abscisic acid to be produced in roots, which is transported to shoots where growth and water loss are limited (Dodd, 2005; Whalley *et al*., 2008). As periods of drought are predicted to occur more frequently in the future, the yield of wheat will

be more vulnerable if the same drought intolerant wheat varieties are used. However, newer varieties which are being developed currently either through breeding, or perhaps genetic modification in the future, may be able to withstand periods of drought more effectively, thus having less impact on yield (Budak *et al*., 2013). Conversly, flooding of farmland like that widely known in 2012 could also become more commonplace, thereby destroying wheat and yields.

Lobell and Gourdji (2012) analysed the changing global temperatures and impacts on crops in previous years and found that there have been large differences in changes to minimum and maximum temperatures in the UK (see Figure 1.7). Increased temperatures can have a positive impact on wheat growth depending on growth stage; for example, temperatures up to 20°C are beneficial during the vegetative stages, but temperatures above 16°C have a detrimental effect during reproduction and any warming during the grain-filling stage has a negative impact on yield. It is predicted that an increase of 2°C globally will cause loss of approximately 11% current yields across the globe (Gourdji *et al*., 2012). Contrastingly, the UK climate change risk assessment conducted by Defra (Web reference – CCRA) predicts that due to increased temperatures, if water is not limiting, the yield of wheat could increase by 40-140%. Overall, the situation is uncertain but it is likely that projected warming will have a negative impact on wheat yields around the globe (Deryng *et al*., 2011), although localised benefits that outweigh the negative impacts could be seen due to elevated CO_2 levels (Easterling *et al*., 2007). It is clear that the increasing global demand for wheat is threatened by a multicomponent challenge which includes the direct impact of climate change and in connection with that, other pests and diseases which may be exacerbated by climate change. This project aims to investigate the impact of viruses, which currently add to the challenge. If in the future these can be controlled so that they do not reduce the yield of wheat it will

increase our chances of meeting global wheat demands by reducing the total challenge that wheat faces.

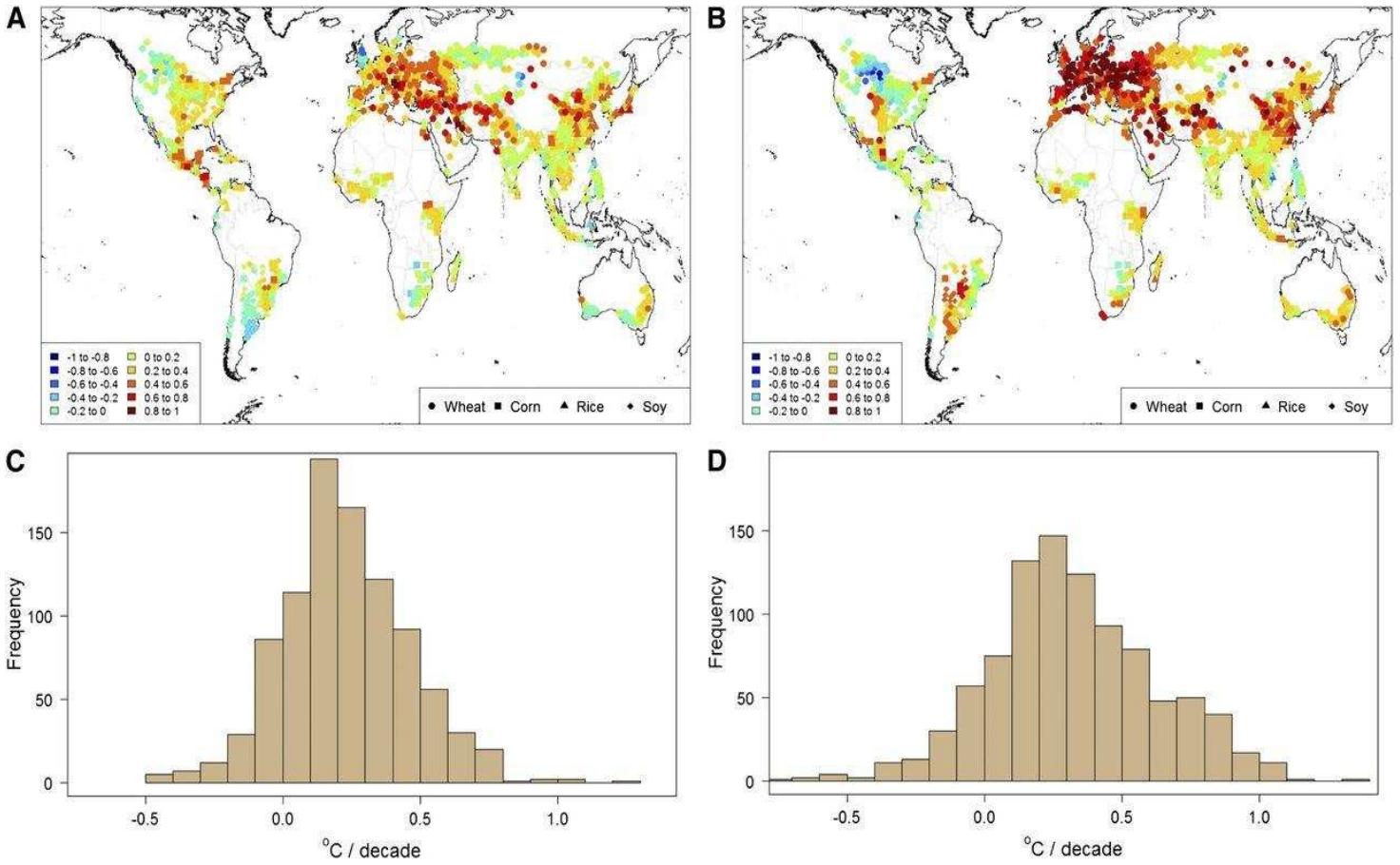


Figure 1.7. The changing temperature worldwide with relation to crops including wheat (Lobell and Gourdji, 2012). Decadal warming trends (°C per decade) since 1980 in growing season daily T_{min} (left) and T_{max} (right) in major global cereal cropping regions, displayed on maps (A and B) and as histograms (C and D).

With regard to the impact of climate change on viruses, in general an increase in temperature will increase replication and spread through a plant, and also increase the severity of symptoms (Dahal *et al*., 1998; Hull, 2004). The growth and symptoms of virus infected plants is temperature dependent, and barley seedlings infected with *Barley mild mosaic virus* will not grow when the temperature is between 5 and 10°C and symptoms are not observed when the temperature is above 20°C (Hill and Evans, 1980; Huth, 1988). Evidence suggests that increased production of short interfering RNAs by the plant at higher temperatures is responsible for this (Chellappan *et al*., 2005). Therefore, in contrast to predictions about temperature regarding wider spread and greater survival of insect vectors, this suggests that higher temperatures may actually reduce the problem of viruses in some cases. However, countries with higher temperatures than the UK, such as the USA, suffer problems due to viruses in wheat, suggesting this lowering of the impact of viruses may not become a reality (McNeil *et al*., 1996). It is possible that in the future alternative viruses which have higher optimum temperatures may evolve, therefore the problem of viruses may continue. In contrast to the most common view that viruses have harmful effects on wheat, interestingly there are suggestions that they can have mutualistic relationships with plants which help them adapt to adverse climates. For example, improving drought or freezing tolerance. This is due to increased levels of salicyclic acid, osmoprotectants and abscisic acid, with the former beneficial trait attributed to better water retention due to stomatal closure. Additionally there is evidence that virus infected plants are stimulated to respond better than non-infected when resource constraints such as CO_2 are removed (Malmstrom *et al*., 2011; Xu *et al*., 2008). These benefits are due to the defence response of the plant and apparent increases in the resilience of the plant which allows them to benefit when other constraints are removed. It could be considered to inoculate wheat with a virus

found to cause these effects, to exploit these benefits in the future. The virus would have to cause limited damage, while preparing the plants defence mechanisms in case of future infection by a more damaging virus, similar to a human vaccine. However selection of a suitable virus would be difficult. There would be a high level of risk because such a virus could form synergistic relationships or recombine with other viruses to produce a harmful form which could get out of control. There could also be transfer of the virus between wheat or other plants for example by insects which could cause widespread disease. It would not be possible to predict this because not every virus-plant combination could be tested and there are likely unknown viruses which could contribute to relationships. However, the addition of some form of manipulated attenuated virus, (perhaps with lower rates of viral replication to prevent large scale systemic spread thereby overwhelming the plant) may be both effective in causing the plant to respond providing the beneficial effects, and resistant to the disadvantages. There is currently no published work which has found such a virus and it seems unlikely that it will happen in the near future, mainly due to the risks to wheat and other crops.

Insects (some of which can transmit viruses to wheat), such as aphids, are ectothermic meaning that they are significantly affected by climatic conditions, which dictates where they can survive (Alford *et al*., 2012). Global warming could make environments, such as the UK, which were previously too cold to support some insect vectors of viruses more conducive to their survival (Ordon *et al*., 2009). The Met Office and data that have been collected as part of the Rothamsted insect survey, support the theory that increasing temperatures cause insects such as aphids to emerge earlier in the spring (see Figure 1.8) and to reach higher numbers sooner in the year (see Figure 1.9) (Richard Harrington, personal communication; Web reference – Met Office). For example in 2008, the first sighting of *Myzus persicae* was

in April, which was four weeks earlier than the long term average. For every 1°C increase in temperature for January and February combined, aphids arrive two weeks earlier than the long term average. The mean temperature of autumn and spring in the UK has increased in recent years, however the winter temperature has not and infact fluctuates year to year, therefore so does the time of first record of aphids in suction traps, due to differing aphid survival over winter (see Figure 1.10). Despite this as discussed the first sighting each year is getting earlier in general. If the winter is mild and there is a favourable spring there is likely to be earlier emergence along with a greater total number of aphids at the start of the year, which is important for the spread of viruses of wheat to an extent. However, the most important time for insect transmission of viruses such as *Barley yellow dwarf virus* (BYDV) to wheat is in the autumn. This is because there are likely to be higher populations of aphids which have built up during the year. Also the wheat is at its most vulnerable to viral infections when it is young compared to older wheat which may have mature plant resistance (Doodson and Saunders, 1970; Web reference BBSRC). For example Lowles *et al*. (1996) confirm that higher BYDV rates have been attributed to higher autumn temperatures in the UK. Additionally, experiments by Smyrnioudis *et al*. (2001) found increases in temperature increase aphid movement, and therefore spread of viruses amongst wheat plants. Lucio-Zavaleta *et al*. (2001) also found increases in acquisition and inoculation of aphids transmitting BYDV at higher temperatures. The colder temperatures of winter then reduce aphid populations. For example, the lethal temperature (temperature at which 50% of the population cannot survive) for a grain aphid is -8°C and 0.5°C for bird cherry aphids (Web reference – HGCA3).

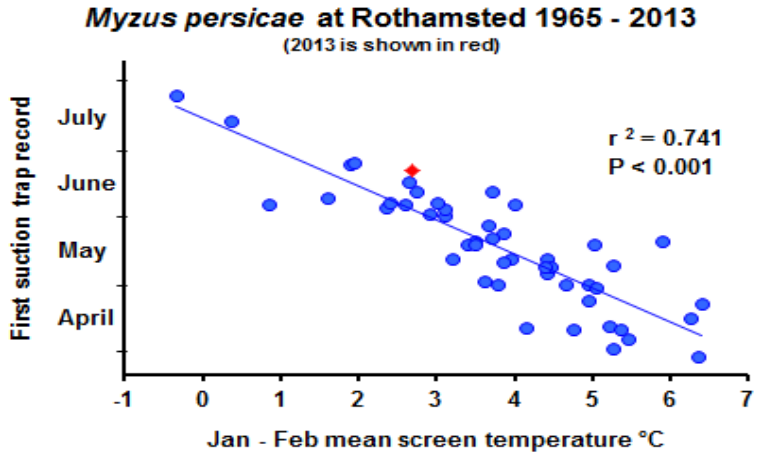


Figure 1.8. The time for first report of *Myzus persicae* in suction traps at Rothamsted, versus mean temperature in January and February (1965-2013) (Richard Harrington, Rothamsted insect survey, personal communication).

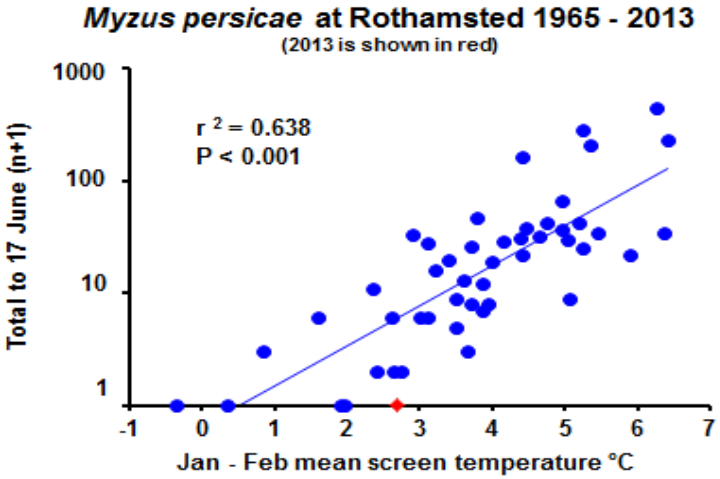


Figure 1.9. The total number of *Myzus persicae* caught in traps at Rothamsted until 17th June each year from 1965-2013, versus the mean temperature during January and February (Richard Harrington, Rothamsted insect survey, personal communication).

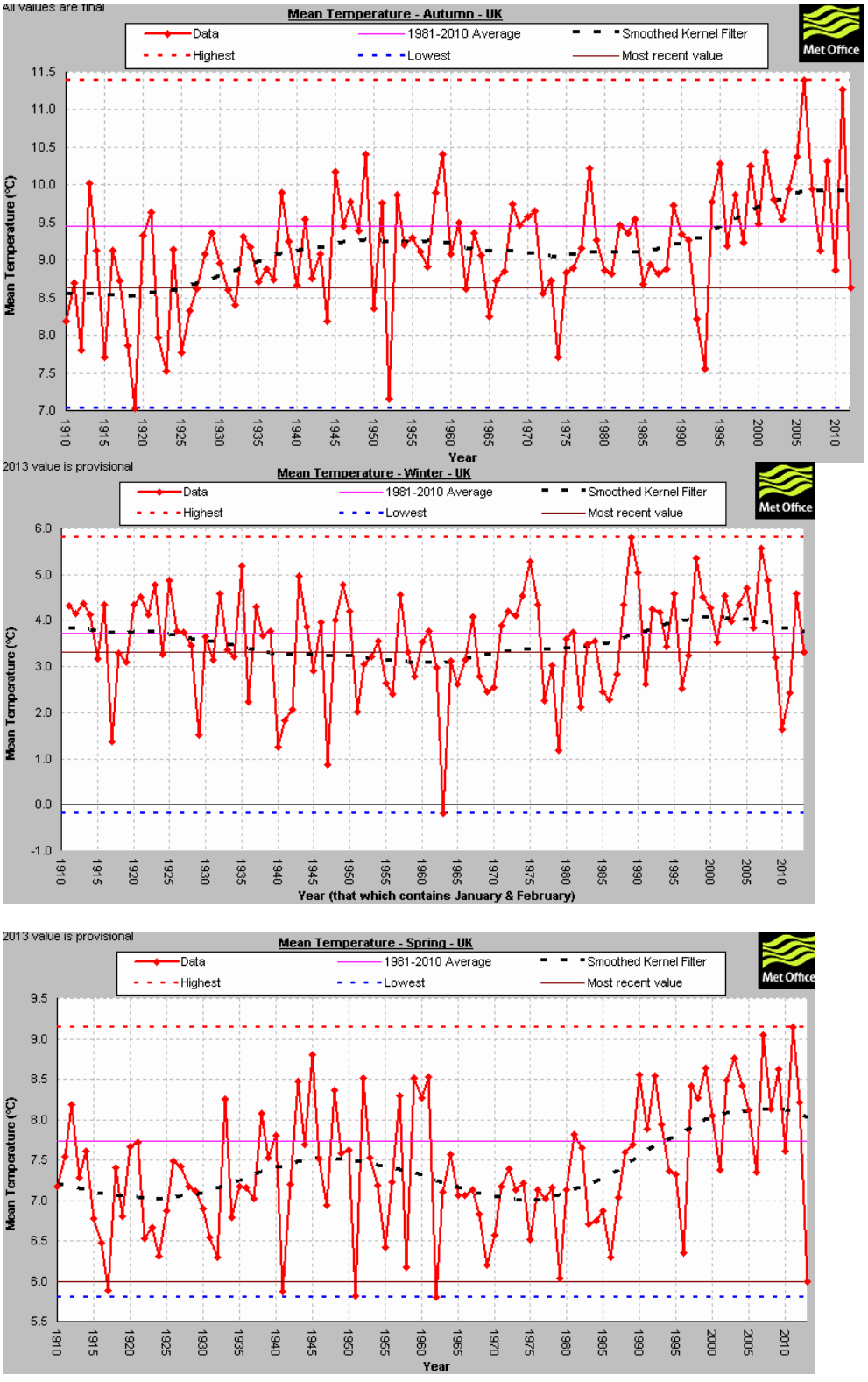


Figure 1.10. Mean temperature graphs for the UK (1910-2013) for autumn (top), winter (middle) and spring (bottom) (Web reference – Met Office temperatures).

Climate change will also have an impact on soil-borne viral vectors, such as *P. graminis* which benefits from warmer soils, and these could become more common place in the UK on a regional basis (Ledingham, 1939). According to Kanyuka *et al*. (2003) the zoospores of *P. graminis* penetrate root or epidermal cells during periods when soils are 'moist but not cold' and near saturated soil conditions cause active movement to the plant (Campbell, 1996). However, drying soil does not reduce infectivity when moisture is resumed (Web reference – DPV Polymyxa). Cycles of rain have been reported as favourable for its spread, and such conditions could occur in the future as severe rain is likely to occur sporadically (Gornall *et al*., 2010; Kanyuka *et al*., 2003; Web reference - CCRA; Web reference – Met Office).

1.9 Trade-offs when attempting to improve wheat yields

The state of global food production has been described as 'being on a knife-edge' by Thwaites (2011). He states the reason for this as the struggle between reducing plant disease and improving crop yield against the requirement to reduce water use, ensure continued soil quality and reduce chemical inputs. For example, a HGCA report by Knight *et al*. (2012) recommends planting wheat earlier than has been normal in previous years to lessen the impact of drought. However, by doing this, there is likely to be increased exposure to insects and any viruses they may be carrying. Additionally practical limitations such as having completed the harvest before needing to drill seed again make this difficult. Actions proposed to help solve other problems, such as nutrient depletion in soils by transferring soils from nutrient rich areas to poorer ones may achieve the intended effect; however, such actions risk soil borne virus introduction (Barrow *et al*., 2008; de Vries *et al*., 2012). Another aspect that should be considered is that increasingly the government encourages lower pesticide use (Web reference – Defra). While this has some benefits, including environmental and social, there is also a possibility that this will result in increased

vector numbers and spread of viruses in wheat crops, thereby increasing the prevalence and impact of viruses in wheat. Conversley, the decrease in pesticides may allow natural predators of insect viral vectors, such as Ladybirds (*Coccinella septempunctata* L.) to survive, and therefore vector numbers may not escalate to such high levels as expected (Kaplan and Eubanks, 2002).

1.10 Diagnosis of viruses of wheat

The principle aim of viral diagnostics is to investigate symptomatic or abnormal plant material for which the prescence of viruses are a possibility, and to suggest if they are the causal agent of the symptoms. A range of detection methods can be employed to clarify if a specific virus or a combination of viruses are present, or certain viruses are not present. The reason for seeking the diagnosis is to inform downstream control measures which are appropriate for the causal agent and limit spread. In addition to this, non symptomatic samples can be screened for target viruses, with the objective of ascertaining if they are free of the virus(es), therefore they can be used in future work and the spread of viruses canbe limited.

Diagnostics uses historical knowledge of which viruses have been found in certain plant hosts in the past. This is used to suggest which viruses could be the causal agents of symptoms and targeted tests for such viruses are carried out. However, just because a virus is detected, it does not necessarily mean that it the causal agent of disease. Other, undetected viruses may be the true cause of symptoms, or there may be other viruses present which are participating in synergistic relationships or acting as helper viruses with the detected virus, which are required for symptoms. Therefore, this type of testing can be useful but the results do have caveats.

The visible symptoms caused by viruses of wheat are often similar such that diagnosis based on symptoms alone is difficult. In addition, symptoms such as

chlorosis can also be caused by a range of other factors including nutrient deficiency. Prabha *et al*. (2013) list the requirement for suitable high quality indicator plants, correct environmental conditions and subjective result interpretation as disadvantages of the method. Despite this, visual observations can be used in diagnosis, but in connection with other methods to confirm conclusions. Inoculation of sap from an infected plant to indicator plants followed by observation, can give information about which virus may be present, based on historical knowledge of specific virus and plant host interactions. This method can take weeks to perform and is not amenable to high throughput testing. Transmission electron microscopy (TEM) can be used to look for virus particles within samples of plants, using knowledge of possible viral targets, which may lead to a diagnosis. The method is time consuming, requires skilled staff and again, is not amenable to high throughput work. Disadvantages also include the variability of results with different staining techniques, a requirement for relatively high titre virus particles and that it does not provide confirmation of cause of disease (Prabha *et al*., 2013). Enzyme linked immunosorbent assays (ELISA) are a reliable and accurate way of diagnosing viral infections of wheat (see Figure 1.11). This method takes a mamxium of two days and can be used for larger sets of samples than the methods discussed previously. However, more modern methods such as polymerase chain reaction (PCR) and its derivatives such as real time reverse transcription polymerase chain reaction (qRT-PCR), a form of which is called Taqman (see Figure 1.12 and Figure 1.13) can be more sensitive than ELISA (see Chapter 5; Haber *et al*., 1995; Huth *et al*., 1984; Lebas *et al*., 2009). Sequencing products of such reactions can be useful in diagnosis, with results being compared to databases of known viral sequences such as GenBank, to suggest which virus could be present.

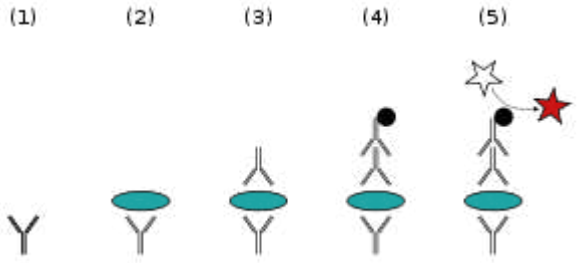


Figure 1.11. The principle of double antibody sandwich enzyme linked immunosorbent assay. (1) Plate is coated with a capture antibody; (2) sample is added, and any antigen present binds to capture antibody; (3) detecting antibody is added, and binds to antigen; (4) enzyme-linked secondary antibody is added, and binds to detecting antibody; (5) substrate is added, and is converted by enzyme to detectable form (Web reference – ELISA).

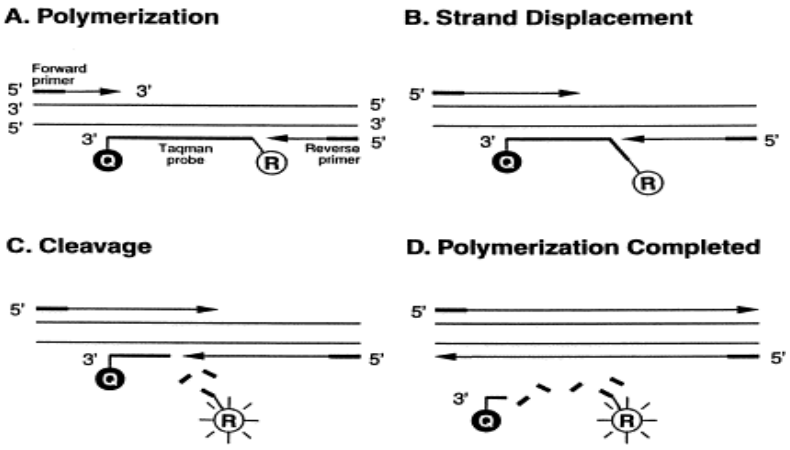


Figure 1.12. A schematic illustration of 5’ nuclease assay for TaqMan real-time PCR. (A) A TaqMan probe labeled with a reporter fluorescent dye (FAM) at the 5’ end and a quencher-fluorescent dye (TAMRA) at the 3’ end hybridizes to the target cDNA. When the probe is intact, the reporter dye emission is quenched, owing to the physical proximity of the reporter (R) and quencher (Q) dyes. (B–D) During the polymerization chain extension, the 5’ nuclease activity of the DNA polymerase cleaves the hybridized probe and releases the reporter dye from the probe. A sequence detector can now detect the emission of the released reporter dye, and the relative signal increases in real-time during PCR amplification (Li and Wang, 2000).

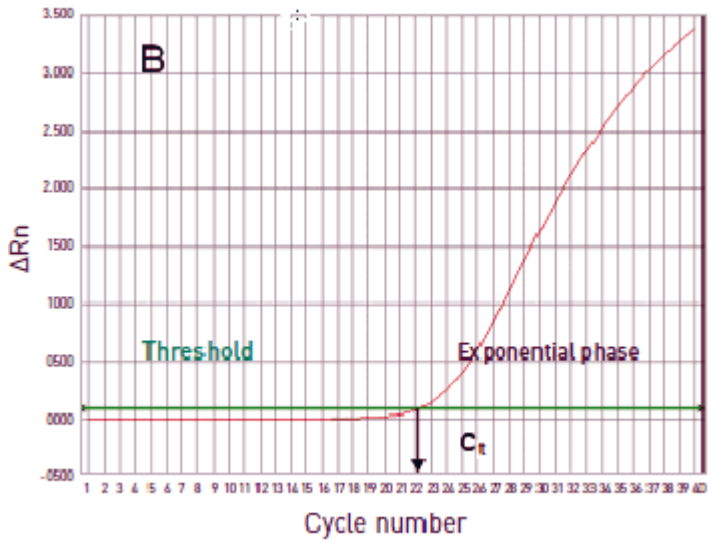


Figure 1.13. Graphical representation of real-time PCR data. Rn is the fluorescence of the reporter dye divided by the fluorescence of a passive reference dye; i.e.,Rn is the reporter signal normalized to the fluorescence signal of ROX™. ΔRn is Rn minus the baseline; ΔRn is plotted against PCR cycle number. The Ct value is the number of PCR cycles required before the level of fluorescence is greater than the threshold (the threshold is set by the software, at the beginning of the exponential phase) indicating a positive result (Web reference – Lifetechnologies).

In recent years, the opportunity for large scale testing of plant samples for viruses has been dramatically increased by the use of robotics in applications such as extraction of total nucleic acids (Kingfisher 96 – Thermoscientific) and preparation of reaction plates for diagnostic tools such as qRT-PCR (Hamilton). Machines such as the ViiA7 (Applied Biosystems) now offer the opportunity to test samples in a 384 well plate (190 samples tested in duplicate with controls) in two hours in qRT-PCR reactions which require no further wet laboratory work (Adams *et al*., 2013; Van Gent-Pelzer *et al*., 2007). Therefore qRT-PCR is favourable over PCR which requires agarose gel electrophoresis which is time consuming and requires the use of dangerous chemicals such as ethidium bromide and Gel Red (Biotium) (the latter

does not give such good results but is safer than the former (Ian Adams, Fera, personal communication). For nucleic acid sequencing, the Sanger method used to be the gold standard, and fragments of up to 800 base pairs could be generated offering good amounts of data. However, next generation sequencing such as pyrosequencing can offer deep sequencing in an un-biased manner (not requiring targeted primers to begin sequencing) for numerous samples at one time in a matter of days, with a cheaper cost per base and with better automation (Siqueira *et al*., 2012). It can also be used to sequence whole genomes, for example the human genome, with a requirement for one thousand fewer sequencing runs, costing seventy times less financially and providing three times as much sequence data (Metzker, 2010). However, Sanger sequencing still has a role to play, for example in resolving sequencing uncertainities (see Chapter 5). In contrast to the earlier discussed target biased methods this tool is not restricted to searching for specific targets. Therefore it allows virtually anything which is present in the sample to be sequenced. Therefore any potential causal agents of disease can be detected, not just those we have knowledge of from the past. One limitation of the method, which means that we cannot say everything in the sample can be detected, is that results are compared to a database such as GenBank which in the case of viruses, is required to search for homology to known viruses. Therefore there is some bias in that only viruses with some homology to known ones will be detected. However, the homology required can be low level (see Section 4.3). The method involves clonal amplification of DNA fragments with sequential addition of pyrophosphate bases which when added release phosphate that is used in the form of adenosine triphosphate in a reaction which emits light in luciferase conversion. The light is captured by a camera which relates it to number of bases added (see Figure 1.14).

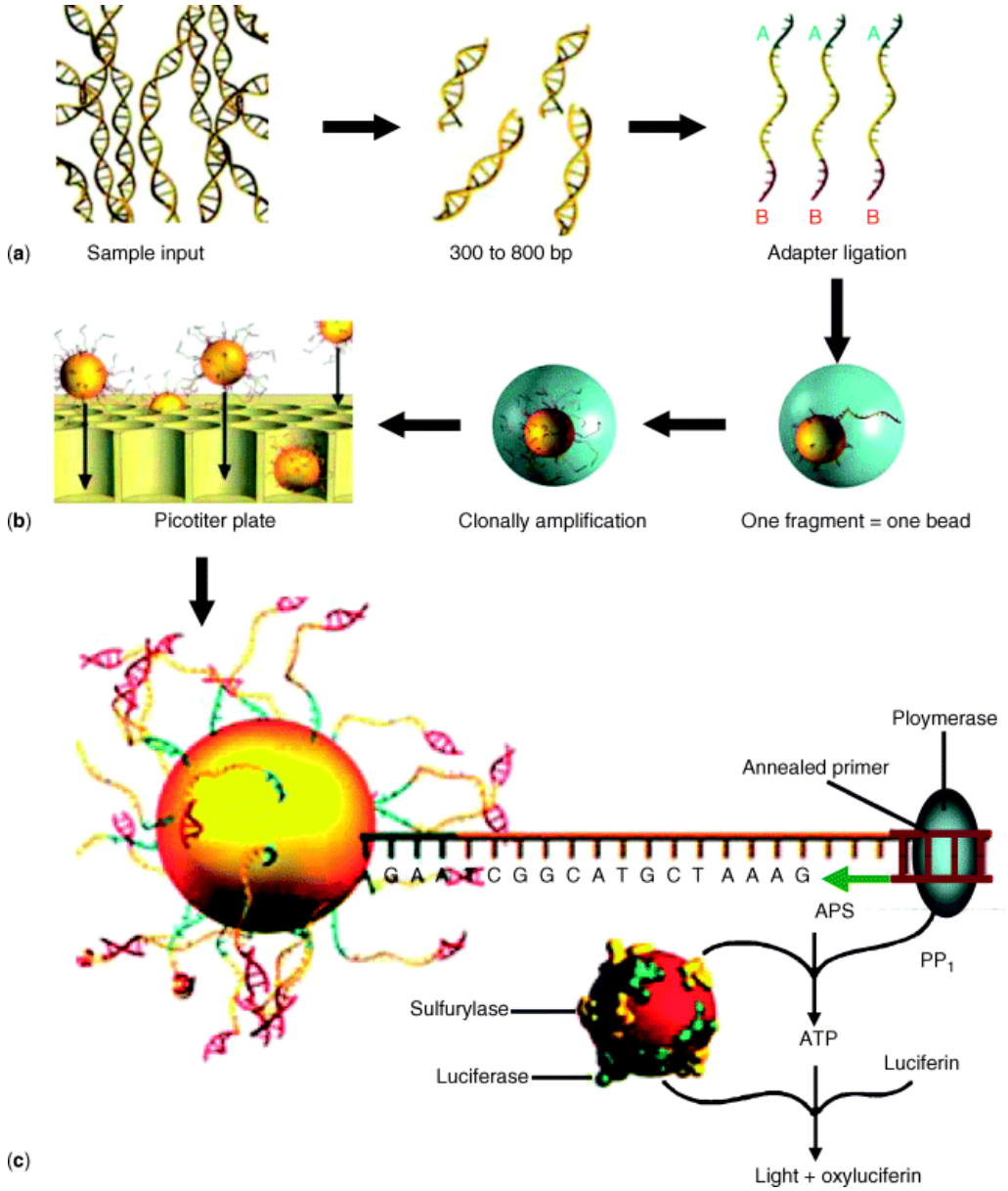


Figure 1.14. Overview of the Roche/454 GS FLX system workflow: (a) fragmentation of DNA and ligation to Roche specific adapters (denoted as blue A and orange B); (b) clonal amplification by emulsion PCR; and (c) real time sequencing-by-synthesis (Su *et al*., 2011).

The technology was introduced in 2004 (Margulies *et al*., 2005; Voelkerding *et al*., 2009) and since then five main platforms have been used, including the 454 pyrosequencer from Roche. The average length of reads and total amount of data has increased from 100 base pairs (bp) to 1000 bp (maximum) and 60 megabytes

(mb) to 500 mb, showing that improvements have been made and this trend is likely to continue (Siqueira *et al*., 2012). The cost of such technology was initially very high, therefore limiting its use. However, it has decreased and will eventually become affordable for relatively widespread use. Reductions in reagent costs coupled with being unable to charge such a premium due to competition have caused this to occur. This provides a significant opportunity to investigate what is in a sample of almost anything, in this case a virus in wheat. Databases such as GenBank and initiatives such as Q-bank mean that there is now an ever increasing wealth of sequence data available for many subjects including plant viruses (Web references – GenBank and Q-bank). This valuable information can be used with a range of bioinformatics tools to assemble and compare newly sequenced samples. Therefore, while older methods of diagnostics are still valuable and are often used in conjunction with more modern methods and indeed are required to confirm the results of next generation sequencing, the high throughput, relatively low cost, accurate and precise options that have been developed in more recent years are driving plant virology forwards.

1.11 A review of viruses of wheat and other members of the Gramineae

There have been numerous reports of viruses affecting wheat in the UK, Europe and worldwide to date. These are discussed below in terms of the symptoms and yield loss, to consider what threat they pose to wheat; locations where they have been found and their methods of transmission to consider likelihood and extent of spread. As discussed, there are likely to be many more virus and host interactions than mentioned here, as a lack of testing allows them to go undetected. Viruses of other Gramineae are included for this reason, as such viruses may pose a threat to wheat now and in the future.

1.11.1 Viruses of wheat reported in the UK, and their incidence in Europe and globally

In 1958, Slykhuis and Watson (1958) reported a striate mosaic virus in the UK. Five percent of the wheat fields in England contained plants that showed striate symptoms, followed by stunting, chlorosis and in some cases death. Other plants also hosted the virus, such as oats (*Avena sativa*), barley, rye (*Secale cereale*) perennial rye grass (*Lolium perenne*) and Italian ryegrass (*Lolium multiflorum*). The vector of the virus appeared to be *Delphacodes pellucida* (Fabricius), which was later renamed *Javesella pellucida* (Fabricius) by Le Quesne and Payne (1981). European wheat striate mosaic virus was apparently vectored by the same insect (see 1.11.1.8). It is possible then that these viruses were the same virus. However, the symptoms described are typical of many viral infections that are known today. It is possible that the striate mosaic virus still occurs in the UK, but is known by a different name, or that it was not one specific virus and so is now known by a range of names. It is also possible that the proposed vector was incorrect as it was only noted as appearing to be the vector. It could be the case that infact another virus such as *Soil-borne cereal mosaic virus* was being observed. This highlights the point that the improved diagnostic techniques available today can provide more specific diagnoses of viral infections of wheat than were possible in the past. Tools such as next generation sequencing help vastly improve comparison of viruses present within samples avoiding erroneous identification or duplication of names.

1.11.1.1 Agropyron mosaic virus

Agropyron mosaic virus (AMV) was found in couch grass (*Elymus repens*) in the UK with subsequent inoculation tests showing that wheat is susceptible to the virus (22/23 cultivars including 8 winter wheat, tested were susceptible), developing a mild pale green mosaic which became less conspicuous with age. One cultivar of wheat,

Cardinal, developed a more persistent and more severe yellow mosaic (Catherall and Chamberlain, 1975). Transmission of the virus in the UK was by the eriophyid mite, *Abacarus hystrix,* to wheat, and by sap-inoculation to 17 other festucoid species of Gramineae (Catherall and Chamerlain, 1975).

There have also been reports of the virus in Finland (Bremer, 1964) and Germany (Schumann, 1969). The UK isolate of AMV was serologically related to an isolate from Canada, suggesting transfer over a large geographical region. In Canada, the virus was found in wheat and agropyron. In the former it was not considered economically important in nature and in the majority of cases caused only mild symptoms, although there were some cases of wheat showing severe chlorosis and stunting. Inoculation of these severe isolates to winter wheat caused a yield loss of 42%, with symptoms most prominent at 15°C (Slykhuis, 1962a). The virus has also been found in wheat in Colorado (Seifers *et al*., 1992). In the latter case there was an estimated 80-85% loss of yield of marketable grain. It is possible for the virus to infect other plants such as silverbeard grass (*Andropogon saccharoides*) and yellow Indian grass (*Sorghastrum nutans*) (Montana *et al*., 1994).

1.11.1.2 Aubian wheat mosaic/Bedford virus

In Bedford, UK in 1995 a field of wheat cv. Riband had patches of yellow plants, which investigations concluded were caused by a virus. A specific diagnosis was not made but it was suggested that the virus was probably a strain of *Soil-borne wheat mosaic virus*, or a novel virus. Limited information is available with no knowledge of any yield loss or other susceptible plants; however, it was suspected that *P. graminis* was involved in the spread of the virus. In contrast to other soil-borne viruses the symptoms of disease were seen just once in the field (Clover *et al*., 1999b). Later studies by Hariri *et al*. (2001) found that the virus reacted positively in ELISA tests to antisera raised against Aubian wheat mosaic virus (AWMV), which was a virus that

had been detected in France. It was concluded that the French AWMV isolate and that from Bedford were biologically and serologically different to other soil-borne mosaic viruses, and were in fact a new un-described virus. Mechanical inoculation of AWMV was possible to broad bean (*Vicia faba*) and lettuce (*Latuca sativa*), and it is possible that the same results would be found in trials with the virus found in Bedford if they are in fact the same virus. Attempts in this PhD project to resolve the relationship of the virus from Bedford and AWMV proved unsuccessful, as bait tests in stored infected soil could not produce infected plants, due to poor storage of the soils (data not shown).

1.11.1.3 Barley yellow dwarf virus

Barley yellow dwarf virus (BYDV) is known to occur across the world, and it was noted just outside the top ten most scientifically/economically important plant viruses in 2010 (Scholthof *et al*., 2011). In 2011 the virus was said to pose a serious threat to wheat production with evidence that the threat is increasing (Siddiqui *et al*., 2012). There is evidence that BYDV can exacerbate the impacts of other diseases including *Fusarium culmorum* (Koch and Huth, 1997). It is one of the most well known viruses to farmers in the UK, and this statement is based on discussions with farmers and industry representatives at the agricultural event Cereals in 2010, 2011 and 2012. It is also one of only two viruses that are commonly tested for when symptomatic wheat is submitted to the virology department of Fera, and the only virus on which assessments are made in the annual winter wheat survey by the Plant Protection and Disease team at Fera. Thus, this is considered an important virus in the UK currently.

There are several related ssRNA viruses that cause the disease known as Barley yellow dwarf disease. These include the species GAV, GPV, MAV, PAS, PAV, RMV, RPV and SGV. The MAV, PAV and RPV species have been found in the UK (D'Arcy and Domier, 2005). This project found several cases of BYDV-MAV in 2010

and 2012 and BYDV-PAV in 2012, in wheat from the UK, with similar results from the virology department of Fera (see Section 3.3).

Several crops are affected by BYDV, but wheat suffers the worst symptoms (Smith, 1972). Different genotypes of wheat vary in their susceptibility to BYDV (Szunics *et al*., 1991). Suppressed heading, sterility and failure to fill grains occur in the most severe cases. In the field, symptoms usually appear as yellow or red patches of stunted plants (Hoffman and Kolb, 1997; Mastari *et al*., 1998). The yield loss is greatest when wheat is infected during its early growth stages (Doodson and Saunders, 1970). This is supported by Kennedy and Connery (2001), who reported higher numbers of aphids on plants and virus infections in barley that was sown in September compared to that sown in October. Due to the large range of isolates and fluctuating incidence, it was not possible to give definite yield loss figures for the UK or worldwide (Plumb *et al*., 1986). However, modern research has suggested that the estimated average worldwide yield loss is 11-33%, although some areas report an 86% yield loss (Miller and Rasochova, 1997).

Transmission of the virus is by aphids and there are specific relationships between virus strain and aphid species: BYDV-MAV is primarily transmitted by *Sitobion avenae*, but can occasionally be transmitted by *Rhopalosiphum padi, Rhopalosiphum maidis* and *Schizaphis graminum*. BYDV-PAV is primarily transmitted by *R. padi* and *S. avenae* but can occasionally be transmitted by *S. graminum* but rarely by *R. maidis* and BYDV-RPV is primarily transmitted by *R. padi*, but can erratically be transmitted by *S. graminum* and rarely by *R. maidis* and *S. avenae* (Rochow, 1969). In other areas of the world where BYDV is detected the vectors may be different depending on which insects exist there; for example, *Hysteroneura setariae* is a vector of BYDV-MAV in Australia (McKirdy and Jones, 1993). Increases in

temperature in places such as the UK improve aphid survival, therefore increasing the chance of virus spread.

The strain of the BYDV virus found differs according to the location in the UK, which depends on where the specific insects that transmit them are found. In experiments in the Vale of York, Leeds University Farm, North Yorkshire, in 1984 and 1985, high numbers of *S. avenae* were found. The findings were in contrast to those in the south of England, where *R. padi* was the vector that was most commonly found (McGrath and Bale, 1990). The is supported by Henry *et al*. (1993), who reported that in 1987 and 1988 BYDV-MAV, BYDV-PAV and BYDV-RPV were all found to be widely distributed, although BYDV-MAV was found more in the north, BYDV-PAV in the south and there was little difference with BYDV-RPV. In south west and central Scotland in 1988 and 1989 BYDV-MAV, BYDV-PAV and BYDV-RPV were found, with the most common type varying according to region; BYDV-RPV was the most common in Ayrshire, BYDV-PAV in Wigtownshire and BYDV-MAV in Dumfriesshire and Stirlingshire (Dempster and Holmes, 1995). There is a link between prevalence and severity of cases of BYDV and aphid numbers in a location. This was shown in Canterbury, New Zealand (Web reference - MOVE) in 2005 when there was an outbreak of BYDV which coincided with higher numbers of aphids than had been seen in previous years. It was suggested that this was due to warmer temperatures (Teulon *et al*., 2008).

Other plants can host BYDV, thereby acting as reservoirs (Mercer and Ruddock, 2004). These reservoirs of virus are important because they can retain the virus and the aphid vectors when wheat plants are not present and interact with the wheat as soon as it begins to grow. Aphids thatare maintained in this constant way have a higher chance of infecting new crops than those hatching newly from eggs (Plumb *et al*., 1986). Perennial ryegrass (fom Wales and Scotland), maize (from Exeter

and Devon), barley (from Northern Ireland) and perennial ryegrass (from central Scotland) were diagnosed with BYDV of various strains (Dempster and Holmes, 1995; Holmes 1991; Pearson and Robb, 1984). The reservoir hosts vary according to what is present in an area. For example, in south western and western areas of Australia, bermuda grass (*Cynodon dactylon*) and weeping lovegrass (*Eragrostis curvula*) are two hosts of BYDV-MAV (McKirdy and Jones, 1993).

Barley yellow dwarf virus of various strains has been reported in many countries, and here follows a selection; Hungary (first report 1972) (Szunics *et al*., 1991), Czech Republic (high levels in wheat, barley and oats) (Kundu *et al*., 2009), Latvia (in 2000 and 2002 in spring cereals and pasture grasses) (Bisnieks *et al*., 2006), France (in surveys of winter cereals, crop stubble and grasses, with the highest prevalence in stubble) (Henry *et al.,* 1993), Spain (in three of the main cereal growing regions, the importance of reservoir plants was noted) (Comas *et al*., 1996; Fereres *et al*., 1989). Examples from outside Europe include the USA - Alabama (a major problem facing winter wheat) (Bowen, 2009), Colorado (confirmed infections at later growth stage causes less severe yield loss) (Hammon *et al*., 1996), Washington (confirmed aphid numbers affect the number of infected plants) (Halbert and Pike, 1985); Australia - western Australia (yield gaps, percentage of shrivelled grain and 500-seed weight problems due to BYDV) (McKirdy *et al*., 2002), southwest Australia (reported greatest BYDV spread and yield loss was predicted to occur if there was a high proportion of immigrant aphids with BYDV, crops were sown early, and aphids arrived early compared to the sowing date) (Thackray *et al*., 2009) and for every 1% increase in virus incidence the crop yield decreased by 55-72 kg per hectare, when aphids migrated to crops early in their growth stage and stayed active on them over winter, thereby spreading the virus to wheat at young growth stages so yield loss was more significant (Thackray *et al*., 2005) and southern New South Wales (vectored by

R. padi) (Milne and Delves, 1999); in India, a serious outbreak of BYDV-MAV was reported in the Central Himalayas in 1994 (Khetarpal *et al*., 1994); in Tunisia the first report showed that a low percentage of plants were affected (Makkouk *et al*., 2001); in the Yemen (Kumari *et al*., 2006), and the virus has also been reported in New Zealand (Lebas *et al*., 2009) and Iran (Pakdel *et al*., 2009).

There are strains of BYDV that are less common, such as BYDV-PAS, which was reported for the first time in the Czech Republic in 2008. The affected wheat plants showed typical symptoms associated with BYDV, but sequencing confirmed this strain was present rather than the more established ones such as BYDV-MAV (Kundu *et al*., 2008). Another strain, BYDV-RMV, was reported in Germany (in over summering grasses with no symptoms), the south west of Western Australia (in 1992, where *R. padi*, *R. maidis* and *H. setariae* were proposed to be vectors), the USA, Uzbekistan and Tunisia (Helmke and Huth, 1996; Makkouk *et al*., 2001; McKirdy and Jones, 1993). Symptoms caused are the same as other BYDV strains, but BYDV-RMV specifically is weakly virulent in oats cv. Coast Black (Rochow, 1969). *Barley yellow dwarf virus–RPS* is one of the major problems facing winter wheat in Alabama (Bowen, 2002). The virus has also been found in China, Mexico, California and Australia (Lapierre and Signoret, 2004). *Barley yellow dwarf virus–RPV* has been reported in Alabama, USA (Bowen *et al*., 2003), Australia (Mckirdy and Jones, 1993), Uzbekistan (from a low number of fields) (Makkouk *et al*., 2001), Iran (one of the main causes of barley yellow dwarf disease) (Pakdel *et al*., 2010; Rastgou *et al*., 2005) and Tunisia (low percentage (0.7%) of plants sampled were affected) (Makkouk *et al*., 2001). The SGV strain of BYDV has only been found outside Europe to date. In 2001, BYDV-SGV was detected in wheat from twelve fields from two cereal-growing regions in Uzbekistan (Makkouk *et al*., 2001). In the same year surveys in Tunisia found the strain for the first time where it was present in 17.2% of cereal samples (Makkouk *et*

al., 2001). More recently the strain has been found in the central and southern areas of Iran (Pakdel *et al*., 2010).

As mentioned previously BYDV is found globally and there are reports from the vast majority of wheat or barley growing countries. There is evidence of long distance spread of the strains of the virus. For example, in Germany, which has seen sporadic epidemics due to changing environmental conditions, the worst infections are in milder areas that favour aphid survival. The BYDV-RMV strain reported in Germany has a serological relationship with that found in the USA, suggesting long distance movement (Helmke and Huth, 1996; Huth, 2000; Koch and Huth, 1997). In addition BYDV-PAV detected in Iran had some sequence similarity to isolates from the USA, France and Japan (Rastgou *et al*., 2005). It is possible that these isolates all originated from the same place and that extensive spread has occurred. In Illinois, 27 isolates of BYDV-PAV were identified in wheat and oat fields. The dynamics of the specific isolates of BYDV changes and novel types are found. For example in Illinois, two of the isolates differed from the majority and were found to be a new and rare isolate (Moon *et al*., 2000). However, studies in Canada suggest that host ranges may be more static (Cheour *et al*., 1993). This suggests that it is important to constantly survey for novel isolates, but it should not be unexpected that once discovered there may be little change in susceptibility for each specific species or cultivar of plant.

1.11.1.4 Cocksfoot mild mosaic virus

Cocksfoot mild mosaic virus (CfMMV) was detected in cocksfoot (*Dactylis glomerata*) in Scotland (Torrance and Harrison, 1981). Symptoms varied according to the host plant, and a systemic mottle and necrosis was observed in foxtail millet (*Setaria italic*a) and cocksfoot (Torrance and Harrison, 1981); however, other hosts, such as wheat inoculated with the Scottish isolate of CfMMV by Torrance and Harrison (1981) showed no symptoms. *Cocksfoot mild mosaic virus* is transmitted by aphids

such as *M. persicae* (Chamberlain and Catherall, 1976) and mechanical transmission is also possible to wheat, oat, barley and timothy grass (*Phleum pratense*) (Torrance and Harrison, 1981). There have been no reports of natural infections of CfMMV of wheat in the UK to date (Lesley Torrance, The James Hutton Institute, personal communication).

Cocksfoot mild mosaic virus has also been reported in Germany (Huth, 1968), where the symptoms on hosts were different to those caused by the Scottish isolate; for example, wheat did not become infected with the German isolate. The virus has also been found further from the UK; the first report of CfMMV in the southern hemisphere was in spring 2003, and then 2004. Brome grass (*Bromus diandrus*) with conspicuous mosaic symptoms from New Zealand, was infected with the virus. Mechanical transmission was possible to some species such as italian ryegrass but not to others, such as wheat (Guy, 2006).

1.11.1.5 Cocksfoot mottle virus

Cocksfoot mottle virus (CfMV) has been found in the central and southern areas of England, in cocksfoot and areas containing cocksfoot mixed with legumes (Serjeant, 1964). Hosts that have been cut are affected to a greater degree than those that have been grazed (Upstone, 1969). This is perhaps due to the greater wounding and therefore increased opportunities for entry of the virus. Symptoms include chlorotic streaks on leaves, chlorotic or green mottling of leaves, whitening or necrosis of older leaves, stunting and reduced tillering. Symptoms specific to wheat are conspicuous chlorotic mottling and necrosis, with seedlings dying within 6-8 weeks (Serjeant, 1967). The beetle *Oulema melanopa* is a vector of the virus (adults to a greater extent than larvae) (Serjeant, 1967). Other hosts are oats, barley, and brome grass (Paul *et al*., 1980; Serjeant, 1967; Smith, 1952). The virus has also been found in Norway and Germany (Huth and Paul, 1977; Olspert *et al*., 2010).

1.11.1.6 Cocksfoot streak virus

Cocksfoot streak virus (CSV) was detected in cocksfoot in Scotland (Torrance *et al*., 1994). Symptoms include chlorotic, light green or dark green streaks on leaves, fewer tillers and less fertile seeds (Catherall and Griffith, 1966). The virus can be transmitted artificially by mechanical inoculation and naturally by the vector *M. persicae, Macrosiphum euphorbiae* and *Hyalopteroides humilis* (Smith, 1952; Torrance *et al*., 1994; Watson and Mulligan, 1960). According to Smith (1972) wheat is not susceptible to CSV. However, Schumann (1969) found that an isolate of the virus from Germany could infect wheat. Other hosts include chase (*Paspalum ceresia*) and plains bristle grass (*Setaria leucopila*) (Ohmann-Kreutzberg, 1963). *Cocksfoot streak virus* has been reported widely across Europe (Gotz and Maiss, 2002; Mühle and Schumann, 1959).

1.11.1.7 Cynosurus mottle virus

This virus has been found in the UK (A'Brook, 1972; Serjeant, 1967). It was originally named Lolium mottle virus by A'Brook, but was later changed to Cynosurus mottle virus (CnMoV) by Catherall *et al*. (1977), due to ryegrass (*Lolium spp.*) being resistant to infection with the virus. While this specific relationship was not investigated further, Catherall (1985) did research the varietal resistance of other grasses to Sobemoviruses, such as *Cynosurus cristatus* to CnMoV. The resistance was thought to be due to the release of antiviral agents in response to virus, which resulted in restricted multiplication and movement of the virus. A different mechanism of resistance occurs in *Lolium perenne* varieties which are resistant to *Ryegrass mosaic virus*, and in that case inhibitors are produced which are present in plant sap (Salehuzzaman and Wilkins, 1983). It was suggested that these may prevent attachment of the virion to a suitable site initially, or the release of viral RNA. The resistence is additive, therefore a range of resistances are possible (Salehuzzaman

and Wilkins, 1984). Therefore it is possible that a mechanism similar to these occurs to render *Lolium spp.* resistant to CnMoV. More recent research by Xu *et al*. (2001) into the resistance to RgMV by *Lolium perenne* concludes that RNA degradation followed by post transcriptional gene silencing occurs along with inhibition of replication of viral RNA. An understanding of these methods of resistance would be important should the viruses become major problems, at which point the genes responsible could be screened for in wheat in breeding or genetically engineered into currently susceptible varieties.

Typical symptoms of a CnMoV infection can include chlorotic mottling 1-3 weeks post inoculation, extensive necrotic streaks and plant death (Catherall, 1985). Conspicuous yellow streaks are seen on infected wheat plants and yield loss occurs (see Section 6.3.1). In the UK, transmission occurs in a semi-persistent manner via *O. melanopa* (Catherall *et al*., 1977; Serjeant, 1967) and mechanical transmission is also possible (Brunt *et al*., 1996), however seed transmission does not seem to occur (see Section 6.3.2). Crested dogs-tail (*Cynosurus cristatus*), common bent (*Agrostis capillaris*) and creeping bent (*Agrostis stolonifera*) are natural hosts of CnMoV (Brunt *et al*., 1996; Catherall, 1985). Infection of wheat, barley and oats is possible (Brunt *et al*., 1996; Mohamed and Mossop, 1981). Cynosurus mottle virus has also been reported in Germany (Huth and Paul, 1977), and more distantly in New Zealand, where transmission is by aphids where *O. melanopa* does not occur (Mohamed, 1978).

1.11.1.8 European wheat striate mosaic virus

Slykhuis and Watson (1958) discovered and described European wheat striate mosaic virus (EWSMV). It was found in many locations across Europe, including England. Diagnosis was based on host range, symptoms and the ability to continually infect plants with the insect vectors only. Therefore it is possible that this virus is still in

existence today but due to modern diagnostic methods, it is known as one or more viruses. A fine chlorotic striate can be seen on the leaves of infected wheat plants. This develops to general chlorosis, stunting and plant death (Lapierre and Signoret, 2004). Transmission is by insect vectors, which include *Javesella pellucida* and *Javesella dubia* (Lapierre and Signoret, 2004). Natural infections by insect vectors are possible to wheat and oats (Ajayi and Plumb, 1981) and barley, rye, perennial ryegrass and Italian ryegrass can also host the virus (Slykhuis and Watson, 1958). European wheat striate mosaic virus has been found in Denmark, Germany, Spain, the Czech Republic, Finland, Turkey and Sweden (Lapierre and Signoret, 2004).

1.11.1.9 Oat chlorotic stunt virus

The first report of *Oat chlorotic stunt virus* (OCSV) in oats was in 1986, at the Welsh Plant Breeding Station in Aberystwyth, and it was reported there again the following year (Catherall, 1986; Thomas, 1987). In oats, there may be striking chlorotic streaks that become necrotic with age. Newly emerging leaves are twisted, a darker green and broader than healthy plants and show severe stunting (Boonham *et al*., 1997). Transmission in soil by *P. graminis* was suggested by Catherall (1986), and Boonham *et al*. (1997) were able to infect oat plants by growing them in infected soil. Importantly for this study, wheat, barley and annual meadow grass (*Poa annua*) are also susceptible to OCSV, but viral load does not reach such high titre as in oats (Boonham *et al*., 1997). The virus has only been reported in the UK to date.

1.11.1.10 Oat mosaic virus

Oat mosaic virus (OMV) was first detected in the UK in winter oats by Macfarlane *et al*. (1968). Since then, it has been reported in Ireland (Kavanagh and Lahert, 1990), Wales (Catherall and Hays, 1970) and England (Monger *et al*., 2001). In the UK, eyespot symptoms are most commonly observed (green spots with yellow/grey borders which are more prominent in older leaves); however, apical symptoms (light

green or yellow patches at the tips of the top few leaves) also occur but less frequently (Catherall and Hayes, 1970; McKinney, 1946). Yield losses can range from 25-100% depending on the variety of oat and its susceptibility to OMV (Herbert and Panzio, 1975). Soil and mechanical transmission are methods by which the virus can spread (Toler and Hebert, 1963). Following mechanical inoculation wheat was not successfully infected with OMV (Toler and Hebert, 1963); however, McKinney (1946) reported mosaic symptoms on wheat cv. Michigan Amber after inoculation. *Oat mosaic virus* has also been found in oats in France (Hariri *et al.,* 1996; Monger *et al.,* 2001), Italy (Rubies-Autonell *et al*., 1992) is widespread across the USA (Atkinson, 1945; Hebert and Panizo, 1975) and it has tentatively been reported in New Zealand (Slykhuis, 1962b).

1.11.1.11 Ryegrass mosaic virus

Ryegrass mosaic virus (RgMV) was detected in ryegrass in England, where light green or chlorotic mosaics were observed (Slykhuis, 1958). In England the eriophyid mite *A. hystrix* is the vector of RgMV (Mulligan, 1960). Mechanical transmission is possible (Web reference –Pvo). The British strain can infect numerous members of the Gramineae but has not been reported in wheat (Mulligan, 1960); however, the US strain could be inoculated to wheat cv. Michigan Amber wheat (Bruehl *et al*., 1957). An isolate of RgMV has also been found in Denmark (Schubert *et al*., 1995), the Czech Republic and Bulgaria, where it was transmitted by eriophyid mites, causing 'tremendous yield loss' (Xu *et al*., 2001). Global reporting countries include New Zealand (Guy, 2006), South Africa, Canada and the USA. There have been cross reactions between the Canadian, South African and Welsh isolates (Salm *et al*., 1994), suggesting that they may originate from the same location.

1.11.1.12 Soil-borne wheat mosaic virus/Soil borne cereal mosaic virus

Soil-borne viruses are transmitted via *Polymyxa graminis* to wheat. Infections differ from insect borne ones in several ways, firstly the control measures if one occurs (see Section 1.7). If resistance crops are grown to control soil-borne viruses the control may be more consistent without the concern of new insects reaching the crop and introducing virus, unless resistance of the crop breaks. If the same susceptible crop is grown the disease is likely to reoccur annually, and the size of patches affected is likely to expand. This is unlike insect borne viruses which require re-infestation each year from reservoir crops. Therefore, soil-borne infections may occur earlier and when wheat is at its most vulnerable. While insect borne viruses may occur in patches originating from the edges of fields, they are likely to be more spread out than soil-borne due to insect movement, perhaps making detection of them by eye easier than soil-borne viruses which could cause patches in the centre of a field. The climate is important for both soil-borne and insect vectored viruses (see Section 1.8).

The European strain of *Soil-borne wheat mosaic virus* (SBWMV)is now known as *Soil-borne cereal mosaic virus* (SBCMV), as it differs from the USA and Japanese types of SBWMV (Koenig and Huth, 2000; Kuhne, 2009). The first report of this virus was from Italy, where it was found in winter wheat (Canova, 1964); later it was found in Rome, in durum wheat (*Triticum durum*) (Rubies-Autonell and Vallega, 1987), and since then it has spread across Europe (Ratti *et al.,* 2004). In 2006 it was reported that SBCMV was becoming an increasingly worse problem in Europe and there have been reports from numerous countries, including the UK (Bass *et al*., 2006; Clover *et al*., 2001). Other countries reporting the virus include Poland, suggesting relatively long distance spread (Trzmiel *et al*., 2012). This project detected SBCMV in wheat growing in the UK in 2010 and 2012 (see Section 3.3.2). This is the second virus that wheat farmers in the UK are most aware of (farmers at the agricultural event, Cereals

2010, 2011 and 2012, personal communications). It is also the second virus (in addition to BYDV) that the virology department at Fera test for when samples of symptomatic wheat are submitted. Therefore, this is currently considered a significant threat to wheat in the UK. The virus is often found in mixed infections with *Wheat spindle streak mosaic virus* (WSSMV)(Budge *et al*., 2008).

Polymyxa graminis transmits SBCMV and can remain infective in soil for approximately a decade (Cannova, 1966; Kendall and Lomell, 1988). It was recommended by Budge *et al*. (2008) that resistance to SBCMV should be included in the criteria of wheat breeding, as planting resistant cultivars is the only possible control strategy. Budge *et al*. (2002) found that Aardvark, Charger, Claire, Cockpit and Hereward had some, or total resistance to the virus. This was likely due to them having the gene *Sbm1* and perhaps *Sbm2* (Bayles *et al*., 2007). The virus causes pale green or chlorotic mosaics and streaks on leaves and leaf sheaves and stunting may also occur. A study by Budge *et al*. (2008) found that 15/21 cultivars of wheat from the UK exhibited severe symptoms of SBCMV when planted in infected soil. Yield losses in wheat of 42 and 50% have been attributed to this virus in the UK (Budge *et al*., 2002; Clover *et al*., 2001) and up 70% in Italy (Ratti *et al*., 2004). Entire crops of susceptible wheat can be lost (Kanyuka *et al*., 2003), suggesting that the virus should be treated as a major threat to wheat in the UK. *Soil-borne cereal mosaic virus* can also be hosted by other plants such as barley (Hariri *et al*., 2007).

1.11.1.13 Wheat spindle streak mosaic virus

Wheat infected with *Wheat spindle streak mosaic virus* (WSSMV) was initially found in Kent (Martyn, 1968). Bos (1999) states that Martyn (1968) called WSSMV Wheat yellow mosaic virus and treated them as one virus. However, Budge *et al*. (2008) stated that while WSSMV occurs with SBWMV in Europe it has yet to be reported in the UK. Experiments found that while WSSMV infections could not be detected by

ELISA a UK cultivar of wheat (Cezanne) showed strong symptoms of the virus when planted in infected fields in France (Budge *et al*., 2008). *Wheat spindle streak mosaic virus* can cause mottling and mosaics in leaves, stunting, reduced tillering and reduced grain yield in wheat (Budge *et al*., 2008). *Polymyxa graminis* is the vector of the virus (Ledingham, 1939). *Wheat spindle streak mosaic virus* has previously been reported in mixed infections with SBCMV in Italy, where there was a 70% decrease in yield (Vallega and Rubies-Autonell, 1985). *Wheat spindle streak mosaic virus* has also been reported from Belgium (first report and found in wheat and rye, 32% soils tested were infected with WSSMV) (Vaianopoulos *et al.,* 2006) and Germany (since 1990, where it mainly infects rye and triticale (*Triticale hexaploide* Lart.) and is often found in mixed infections with SBCMV) (Huth, 2000; Huth *et al*., 2007). On a global scale WSSMV has been reported in Canada (Slykhuis, 1960), Michigan, USA (from where it spread to cover much of the USA) (Wiese *et al*., 1970), Zambia (37% of the 81 plants tested were infected with WWSMV) (Kapooria *et al*., 2000) and China (in a mixed infection with SBWMV causing yield losses of 25-50%) (Slykhuis, 1970). The strain of WSSMV that is found in France is very closely related to that found in Canada and the USA. There is less similarity to the German strain and even less to the Italian, although the German strain was from rye (Chen *et al*., 1999).

1.11.2 Viruses of Gramineae (not reported in wheat to date) reported in the UK, and their incidence in Europe and globally

Barley mild mosaic virus (BaMMV) was detected in fields in Cambridgeshire and Gloucestershire, UK (Adams *et al*., 1993). Symptoms include chlorotic streaks on younger leaves with some curling inwards, and the chlorosis may become a mosaic over time. There may also be necrosis and rapid death of older leaves. Newly emerging leaves do not show symptoms if the temperature is above 20°C, and infected plants will not grow when the temperature is between 5°C and 10°C. If

predicted climate change causes increased temperatures meaning the crop is exposed to temperatures above 20°C for longer periods of time, it is possible that symptoms caused by BaMMV may not be as severe. However, new strains could emerge which have higher optimum temperatures. Additionally, the vectors of the virus, *P. graminis* benefit from warmer soils therefore the level and spread of virus could increase (see Section 1.8). Yield losses due to this virus can be as high as 80%, making this a severe threat to members of the Gramineae (Hill and Evans, 1980; Huth, 1988). Winter wheat is not currently known to be a host of BaMMV and the only natural host is barley; however, several other members of the Gramineae can host the virus following mechanical inoculation including durum wheat (Ordon *et al*., 1992; Proeseler, 1988; Proeseler, 1993).

Barley mild mosaic virus is found in Europe (Chen *et al*., 1992) and examples include Spain (in mixed infections with BYDV-PAV and *Barley yellow mosaic virus* (BaYMV), causing severe damage) (Achon and Serrano, 2006) and Germany (not in the major cereal growing areas and in a variety that was thought to have a resistance gene) (Habekuss *et al*., 2008; Huth, 2000). Global reports have come from Japan (Kashiwazaki *et al*., 1990) and Korea (Choi *et al.,* 2009). It is possible that winter wheat could become infected with BaMMV because despite mechanical spray inoculation being un-successful in wheat cv. Kanzler (Ordon *et al*., 1992) sap inoculation resulted in infections in cultivars of durum wheat (Proeseler, 1993).

As mentioned, BaYMV is another virus of barley, which was first reported in the UK in 1980 (Hill and Evans, 1980). It then spread, and became more common in England and Wales (Hill and Evans, 1980; Hill and Walpole, 1989). Adams *et al*. (1987) state that BaYMV occurs in all areas of the UK where winter barley is grown. Symptoms are similar to those caused by BaMMV and include chlorotic streaks along leaves which may be systemic (Huth, 1989; Usugi, 1988). A strain of the virus has

caused yield losses of 40-80% in Japan and 50% in Europe (Proeseler *et al.*, 1988; Usugi, 1988). The virus is transmitted by *P. graminis* and can be mechanically inoculated to healthy plants (Adams *et al*., 1988; Adams *et al*., 1991). The only known host is barley (Adams *et al*., 1988), with winter cropped barley affected most severely (Plumb *et al*., 1986).

Barley yellow mosaic virus was said to be a major threat to barley production in Europe (Werner *et al.,* 2005). It was first found in Europe in 1977 and has since been reported in Turkey (first report and a relatively low percentage of samples were infected) (Koklu, 2004b), Spain (caused major crop loss) (Achon and Serrano, 2006) and Germany (widespread, two strains were detected and infected soil was predicted to remain so for fifty years) (Huth *et al*., 1984; Huth, 2000). Globally, BaYMV has been reported in Japan (where there were six strains) (Kashiwazaki *et al*., 1989) and China (first in the 1950s, then repeatedly causing major problems (Chen, 1992; Chen *et al*., 1999). The strains found in the UK, Europe and worldwide differ including their susceptible hosts. For example in China, some European cultivars are not susceptible where as Chinese types are (Chen *et al*., 1992). Suggesting introduction of some viruses to UK wheat may not cause severe damage due to natural resistance.

A final virus of the Gramineae reported in the UK is Cocksfoot cryptic virus (CCV), which was diagnosed in two cultivars of cocksfoot in Scotland (Torrance *et al*., 1994). Transmission is in a non-persistent manner by *M. persicae*, mechanical inoculation and through seed (Torrance *et al*., 1994). Currently only cocksfoot is known as a host.

1.11.3 Other viruses of wheat reported in Europe, but not currently in the UK

1.11.3.1 Barley stripe mosaic virus

McKinney and Greeley (1965) state that *Barley stripe mosaic virus* (BSMV) can cause a lethal necrosis in hosts. Transmission is efficient through seed (100%) and pollen

(Gold, 1954). In 2003, a low percentage of samples of wheat from Turkey were infected with BSMV. Symptoms observed were chlorosis, striping on leaves and stunting (however, other viruses such as BYDV-PAV were present and may have contributed to the symptoms). This was the first time BSMV had been found in wheat in Turkey, but it had been found in barley previously (Koklu, 2004b). This virus has also been found globally, for example in barley and wheat which were showing typical virus symptoms, from Tunisia. This was the first report of BSMV in cereals in Tunisia and the incidence was low (1% per field, in most cases, however, one barley field had an incidence of 10.5%) (Najar *et al*., 2000). It has also been reported in wheat in New Zealand (Lebas *et al*., 2009) and the Yemen (Kumari *et al*., 2006).

1.11.3.2 Barley yellow striate mosaic virus

This virus is also known by other names including Cereal striate mosaic virus (Matthews, 1982). It was detected in field surveys in Turkey where the incidence of the virus in any field was less than 1% (Makkouk *et al*., 1996). It was also reported in Italy and symptoms in winter wheat were chlorotic stripes or mosaics, but there was not a large economic impact (Conti, 1980). Transmission is by insect vectors, *Laodelphax striatellus* (naturally) and *J. pellucida* (in laboratory tests) (Conti, 1980).

On a worldwide scale BYSMV has been reported in Tunisia (Najar *et al*., 2000), Yemen (Kumari *et al*., 2006), Lebanon (Makkouk *et al*., 2001) and Iran (the isolate was similar to those from Italy and Morocco) (Izadpanah *et al*., 1991); in addition the strain found in Uzbekistan had similarities to Italian and Moroccan isolates (Makkouk *et al*., 2001). In Syria in 2002 the vast majority of samples of wheat that were infected with a virus were infected with BYSMV, and the isolate reacted strongly with antisera raised to BYSMV from Italy, Lebanon and Morocco; suggesting spread of the same isolate (Makkouk *et al*., 2004).

1.11.3.3 Brome mosaic virus

In Europe, *Brome mosaic virus* (BMV) was detected in wheat and barley in the Tekirdag regions of Turkey (Koklu, 2004a) and in cocksfoot with symptoms of chlorotic mottling, streaks and mosaics on leaves and stems in Lithuania (Urbanaviciene and Zizyte, 2012). Globally it was first detected in Alabama in 2003, and since then it has been found in all areas of the state where wheat is grown. The dagger nematode (*Xiphinema americanum*) was proposed to be a vector of the virus. However, it was not found in soil in which infected wheat plants were grown, although it was found in adjacent fields where potential hosts (and reservoirs) were growing. It was therefore proposed that another method of transmission is involved (Srivatsavai *et al*., 2006). Transmission was shown to be possible in a laboratory using urediospores of wheat stem rust (*Puccinia graminis*) (Erasmus *et al*., 1983).

1.11.3.4 Brome streak mosaic virus

Brome streak mosaic virus (BrSMV) was first reported in soft brome (*Bromus hordeaceus*) and barley, in which it caused chlorotic streaks on leaves, from former Yugoslavia by Milicic *et al.* (1980) and (1982). The spread was linked to the expansion of yellow nutsedge (*Cyperus esculentus*) (an invasive weed) which was proposed as a reservoir of the virus. The yield losses of wheat and barley were already of concern in Hungary, and this virus reportedly added to that concern (Takacs *et al*., 2008).

1.11.3.5 Festuca leaf streak virus

Festuca leaf streak virus (FLSV) was first detected in Denmark. Studies on the transmission of FLSV by *J. pellucida* found insects that had fed on infected giant fescue (*Festuca gigantea*) were able to transmit the virus to wheat cv. Solid, barley cv. Pallas and oat cv. Roar. Mosaicking on veins was observed as a result of the virus, and when seedlings were infected at the coleoptile stage, the first symptoms

appeared on the second or third leaf 8-16 days after introduction of insects carrying the virus (Lundsgaard, 1999).

1.11.3.6 Flame chlorosis virus

Flame chlorosis virus (FCV) was detected in the Netherlands, where cereals were the host plants (Haber *et al.,* 1995; Lapierre and Signoret, 2004). Spring crops were affected by FCV and symptoms were a chlorosis spreading from the base of the leaf, and stunting (Haber *et al*., 1990). In addition barley either produces no grain or is killed, and reduced vigour is observed in wheat, oat and triticale. Transmission is through soil and was suggested to involve a *Pythium* species as the vector (Lapierre and Signoret, 2004). Worldwide FCV has been detected in spring wheat in Canada (Haber *et al*., 1990), Peru, South Africa and Australia (Lapierre and Signoret, 2004).

1.11.3.7 Soil-borne wheat mosaic virus

Soil-borne wheat mosaic virus (SBWMV) was first found in the USA in the 1920s (McKinney, 1923), and this is where the virus is normally found. However, in 2003 Koenig and Huth (2003) reported the virus in Germany. The strain detected was related to that found in Nebraska, and was unlike SBCMV which is widely found in Europe. This was the first report of SBWMV in Germany, and indeed Europe (Huth *et al*., 2007; Koenig and Huth, 2003).

The virus is considered one of the most important in central and eastern USA, due to its ability to destroy an entire crop of susceptible wheat (Kanyuka *et al*., 2003). The virus appears to have spread across America with reports in many locations such as Alabama (Bowen *et al*., 2003), Oklahoma and Nebraska (Chen *et al*., 1997). It has also been detected in New Zealand (confirmed to be the same isolate as that from the USA) (Lebas *et al*., 2009) and Japan (it was more like *Chinese wheat mosaic virus* (CWMV), but is classed as the USA version, SBWMV) (Chen *et al*., 1993; Miyanishi *et al*., 2002). It was also found in Zambia, where the worst affected plants

were those grown on light to medium sandy-loam clay soils, around the field edges and in poorly drained soils. These conditions are conducive to *P. graminis* which is the vector of the disease (Kapooria *et al*., 2000).

1.11.3.8 Sugarcane mosaic virus

In the Trakya region of Turkey in 2004 and 2005 *Sugarcane mosaic virus* (SCMV) was detected in maize, which was the first report of its kind (Ilbagi *et al*., 2005). There were later reports of common reed (*Phragmites communis*) infected with the virus in two locations in Turkey (Ilbagi *et al*., 2006). In 2007, maize from two sites in Poland was diagnosed with SCMV, and despite being symptomless, wheat, oat and triticale were infected with the virus. The isolate had high similarity to German, Spanish, Bulgarian, Indian and Chinese isolates (Trzmiel, 2009). Transmission was by *R. padi* and *R. maidis* (Garrido *et al*., 1998). Corn suffered a 16.9% plant height decrease, 37.1% plant weight decrease and 27.8% cob weight decrease due to SCMV (Fuchs and Gruntzig, 1995). The virus was also reported in St Augustins grass (*Stenotaphrum secundatum*) from Venezuela and mechanical inoculation of the virus was possible to sorghum (*Sorghum bicolor*), maize and sugarcane (*Saccharum*spp.), but not to wheat, oat or barley (Garrido *et al*., 1998). In contrast, Abbott and Tippett (1964), suggest that wheat can be infected with SCMV; probably due to differences in strains.

1.11.3.9 Wheat dwarf virus

Wheat dwarf virus (WDV) causes disease in wheat and barley, and is found in many areas of Europe. It is a major threat as yield losses of 100% have been reported from Finland (Lemmetty and Huusela-Veistola, 2005). There are two clades of the barley strain and one of wheat. The wheat strain can infect wheat and barley, whereas the barley strain can only infect barley. Kundu *et al*. (2009) reported a new strain that could infect oats. The only known vector is the leafhopper *Psammotettix alienus* (Lindblad and Areno, 2002), and the infection level in plants was greatly dependent

on the population dynamics of the vector (Mehner *et al*., 2002). There is no evidence of this aphid existing in the UK currently. Symptoms include streaks, mottles, dwarfing and stunting (Lindsten and Vacke, 1991). *Wheat dwarf virus* and BYDV cause similar symptoms, therefore visual diagnosis is difficult and requires an experienced eye (Huth, 2000). The virus was reported in winter wheat in Sweden (Lindsten, 1970) and later, in 1997, there was an epidemic of WDV in the central region of the country. The virus was relatively uncommon but was considered to be a potentially serious threat to winter wheat. The average loss due to the disease reached 35% with the maximum loss of 90% (Lindblad and Waern, 2002). In later work samples of other Gramineae such as common wild oat (*Avena fatua*) and triticale from fields surrounding an infected wheat field were infected with WDV (Ramsell *et al*., 2008). The virus was also detected in Germany, but was not considered to be an important threat, despite the disease being worse than BYDV, which is given a higher level of importance. *Wheat dwarf virus* has also been found in Poland, the Czech Republic (Lapierre and Signoret, 2004) and Turkey (Koklu, 2004a). Analysis of the sequences of barley strains of WDV from Turkey found there has probably been recombination between a barley strain and an as yet un-described WDV-like *Mastrevirus* species to produce it (Ramsell *et al*., 2009). This suggests that the viruses have undergone evolution, supporting the theory that host virus interactions are subject to change. Worldwide, China (Wang *et al*., 2008) and Tunisia have also reported strains of the virus (Najar *et al*., 2000).

1.11.3.10 Wheat streak mosaic virus

Wheat streak mosaic virus (WSMV) is widespread around the world and it poses a serious threat to wheat production. It is vectored by *Aceria tosichella*, which are blown by wind into wheat fields; this means that gradients of disease occur from the edges of fields to the centre (Workneh *et al*., 2009). *Wheat streak mosaic virus* is also

vectored by *Aceria tulipae* (Slykhuis, 1953a) and very low levels of seed transmission have been observed in Australia (Lanoiselet *et al*., 2008). The virus can infect several plants including wheat and couch grass (Ito *et al*., 2012). In 2009 WSMV was reported to be one of the most common viruses affecting wheat in north Texas and in years when there were droughts wheat suffered increased damage. This was thought to be due to the effects the environment had on the vectors (spread and reproduction), rather than increasing the impact of the virus itself (Price *et al*., 2009). Weather conditions were thought to be responsible for the first outbreak of *A. tosichella* and WSMV in north central Washington; there had been a cool moist summer, hailstorms and a warm winter. In the Midwest, Alberta, Idaho and Montana a similar outbreak had occurred (Gillespie *et al*., 1997). There were suggestions that sheep grazing on early sown winter wheat may spread WSMV, but this was later proven to be untrue (Fahim *et al*., 2010). It does raise an interesting point, in that there may be unexpected methods of transmission. For example a viruliferous insect could be transported on an animal to a new location and continue to spread the virus.

Yield losses of 100% have been attributed to the virus (McNeil *et al*., 1996). Reports of WSMV have come from numerous countries, some of which are included here. There have been losses of $30 million in Kansas, due to WSMV. The extent of the outbreak varied from small patches, to a few fields (Wiese, 1977) and the virus is one of the biggest limiting factors for wheat production in the Texas Panhandle (Workneh *et al*., 2009). The three Argentinean isolates of WSMV are closely related to some from the American Pacific Northwest and Australia, which suggests the same lineage between them (Stenger and French, 2009). Isolates from Australia were very similar to those from the USA and Turkey (Ellis *et al*., 2003). The isolate from the USA had only been found there until this report, which suggests long distance movement. The virus probably then spread via standard distribution routes (Dwyer *et al*., 2007).

The coat proteins of Czech, French, Italian, Slovak and Turkish isolates were studied, and their relatedness suggests that there was one common ancestor that had dispersed throughout Europe (Gadiou *et al*., 2009). In 2007, wheat was diagnosed with WSMV in western Slovakia, which was the first occurrence of the virus there. The isolate was most like those from Hungary, Russia and the Czech Republic and was unlike that reported in Mexico (Kudela *et al*., 2008). The isolate found in central Europe was later reported as one of the two types in the USA, which confirms that there has been extensive spread of the virus (Robinson and Murray, 2013). The virus has also been found in New Zealand (Lebas *et al*., 2009), and the isolate had high similarity to the Turkish and American isolates but slightly less to the Australian (Dwyer *et al*., 2007).

1.11.4 Viruses of Gramineae (not reported in wheat or in the UK to date) and their incidence in Europe and globally (excluding viruses mentioned in 1.11.2)

A number of viruses that could potentially spread into wheat have been found in other Gramineae in Europe. In 2007, Hariri and Meyer (2007) reported on a virus detected in a stunted, mosaicked barley crop in 2001 in France. The virus was more closely related to the Japanese wheat mosaic virus (JWMV) than the French SBCMV, and this was the first report of JWMV outside Japan from where it originated. The symptoms are similar to SBCMV and SBWMV and the location of the strain is the key difference.

In maize, *Johnsongrass mosaic virus* (JGMV) was found in the Trakya region of Turkey in 2004 and 2005, which was the first report of the virus in Turkey in any plant (Ilbagi *et al.,* 2006). It has also been reported in Texas, USA (Shukla *et al.,* 1989), and symptoms include stunting and mosaics which cause yield loss (Teakle *et al*., 1970). *Maize dwarf mosaic virus* (MDMV) was detected in maize and common reed in the Trakya region of Turkey (Ilbagi *et al*., 2006). This was the first time this reed

was shown to be a natural host of MDMV, and that it could act as a reservoir of the virus (Ilbagi *et al*., 2006). Maize from Lower Silesia and Poland was also infected with MDMV in 2005 and 2007, and the isolate reacted positively to antisera raised against the Spanish isolate, suggesting they were related and that the virus had migrated. Sweetcorn (*Zea mays* convar. *Saccharata*) and sorghum were infected with MDMV in Hungary (Trzmiel and Jezewska, 2008). Symptoms of the virus in sorghum are systemic mosaics and necrosis (Tosic *et al.,* 1990). Corn plants were infected with MDMV in 1989 in Yugoslavia, and there were yield losses of 20-90% due to the virus (Tosic *et al*., 1990). The virus was said to be transmitted non-persistently by aphids from three subfamilies: *Aphidinae, Lachninae* and *Drepanosiphinae* (Nault and Knoke, 1981). The virus has also been reported worldwide, for example in Venezuela (*R. maidis* was the vector between sorghum plants) (Garrido *et al*., 2000). *Oat necrotic mottle virus* (ONMV) was detected in oats in Turkey and has also been reported in Canada (Gill and Westdal, 1966; Ilbagi *et al*., 2005). This virus causes a mosaic of oat and a mild or symptomless disease in other grasses. In spring-sown oat the disease causes yield loss and stunting (Gill, 1967).

1.11.5 Other viruses of wheat reported globally (excluding those in 1.11.1 and 1.11.3)

A number of viruses have been found to infect wheat in other parts of the world that have as yet not been detected in the UK or the rest of Europe. These include American wheat striate mosaic virus (AWMV), which was first found in wheat in the USA in 1953 (Slykhuis, 1953b). Feeding trials found *Endria inimica* was a vector (Jons *et al*., 1981). The first report of the virus in the Southern Great Plains was in wheat cv. Meas in Comanche County, Kansas (Seifers *et al*., 1995). Again, the vector was *E. inimica*.

Brazilian wheat spike virus (BWSV) has been reported in wheat in Brazil. It is transmitted by leafhoppers and causes young leaves to become completely chlorotic, older leaves to develop chlorotic streaks and the heads of affected plants are empty of grain (Lapierre and Signoret, 2004).

Ye *et al*. (1999) found that despite some evidence of a virus infecting wheat in China being SBWMV, it was actually a separate virus which was named *Chinese wheat mosaic virus* (CWMV). The report was supported by Diao *et al*. (1999). The impact and transmission was similar to SBCMV and SBWMV. The virus then spread across China (Yang *et al*., 2001).

Foxtail mosaic virus (FoMV) was first reported in foxtail millet and green foxtail (*Setaria viridis*) with mosaics on their leaves from the USA (Paulsen and Sill, 1969). Sorghum in Kansas was infected with an isolate of FoMV, which was able to infect barley, but did not do so readily and caused only mild symptoms. This was an example of a virus moving into a different host, which highlights the need for screening of unexpected viruses in wheat (Seifers *et al*., 1999).

The first diagnosis of High plains virus (HPV) was in corn in 1993 in the USA (Forster *et al*., 2001). Seifers *et al*. (2009) detected two isolates of HPV that were capable of infecting wheat, which caused 'significant' reductions in yield. *Aceria tosichella* Keifer was able to transmit the virus to plants including barley cv. Westford (Blunt and Brown, 2003; Forster *et al*., 2001). Forster *et al*. (2001) proposed that there was also low level seed transmission of the virus, but other studies suggest that seed transmission may occur at a much higher level (Blunt and Brown, 2003).

Indian peanut clump virus (IPCV) infects several graminaceous crops, including wheat and barley, in semi-arid and subtropical areas such as India and Pakistan, with serious economic consequences (Delfosse *et al*., 1999; Lapierre and Signoret, 2004). Seed transmission is possible in wheat (Delfosse *et al*., 1999).

Polymyxa graminis has been proposed as a vector, and infected crops such as maize and sorghum had sporosori in their roots (Doucet *et al.,* 1999).

Kumar *et al*. (2012) reported a novel virus that was infecting wheat in India; Indian wheat dwarf virus (IWDV) which was transmissible by leafhoppers and caused dwarfing. This is the first and only report of the virus to date.

Iranian wheat stripe virus (IWSV) was reported in Iran (Lapierre and Signoret 2004); host plants include wheat, barley, oat, rice, rye and sorghum. In wheat, general chlorosis, streaks and stunting are observed. Transmission is by *Unkanodes tanasijevici* (Heydarnejad and Izadpanah, 1992).

Maize rough dwarf virus (MRDV) primarily affects maize, and in extreme cases when young plants are infected no grain is produced (Grancini, 1958; Grancini, 1962). Wheat is a natural reservoir of MRDV in Argentina (Rodriguez-Pardina *et al*., 1994). The virus is transmitted by *L. striatellus* (Conti, 1966).

Maize streak virus (MSV) has been detected in South Africa, sub-saharan Africa and the Indian islands (Shepherd *et al*., 2010; van Antwerpen *et al*., 2008; Varsani *et al*., 2008). It is known as the most serious disease of maize on the continent of Africa as it poses a major threat to food security (Shepherd *et al*., 2010; Varsani *et al*., 2008). It has been suggested that many members of the Gramineae are at risk of the virus. There are numerous strains of MSV, which can infect wheat, barley, oats, rye, sugarcane and many wild, mostly annual grass species. Of all the strains only MSV-A causes such severe symptoms in maize that there are economic consequences. *Maize streak virus-A* is able to infect wheat, while MSV-B to E infect grasses but are not able to infect wheat (Varsani *et al*., 2008). *Maize streak virus* is transmitted by six leafhoppers, with the most commonly occurring being *Cicadulina Naude* and *Cicadulina storeyi*. The spread of the virus depends on the vectors, which are themselves dependent on environmental conditions (Shepherd *et al*., 2010). The

mobility and the ability of the virus to recombine with other viruses mean that this virus is relatively easily spread (Shepherd *et al*., 2010; Varsani *et al*., 2008).

Wheat in Naga Hamadi, southern Egypt was infected with Maize yellow stripe virus and had leaves showing chlorotic streaks. The leafhopper *Cicadulina chinai* is the vector of the virus. Barley and maize are also susceptible to infection by the virus, and weeds have been proposed to act as reservoirs in infection cycles (Ammar *et al*., 1989).

Mal de Rio Cuarto virus (MRCV) was known as the most important disease affecting maize in Argentina. The vector of the virus is *Delphacodes kuscheli* and *Tagosodes orizicolus,* which uses wheat, barley and triticale as breeding sites (Brentassi *et al*., 2009; Fernanda Mattio *et al*., 2008). Brentassi *et al*. (2009) state that wheat is not susceptible to the virus. In contrast, symptomatic wheat plants have been found, suggesting that they themselves were being adversely affected by the virus. Wheat from Rio Cuarto, Sampacho and La Carlota, which was thought to be infected with MRCV, was deformed and had sterile heads (Pardina *et al*., 1998).

Northern cereal mosaic virus has been detected in Japan, China and Korea (Lapierre and Signoret, 2004). The virus can infect wheat and has been reported to cause a 75% decrease in yield (Ogawa and Moichi, 1984). The main vector of the virus is *L. striatellus* (Ito and Fukushi, 1944a; Ito and Fukushi, 1944b).

In West Africa, wheat that was grown in soil that had previously contained groundnut (*Arachis hypogaea*) infected with *Peanut clump virus*, developed a systemic mosaic and was stunted. The vector was proposed to be *P. graminis* (Thouvenel *et al*., 1976; Thouvenel and Fauquet, 1981). This is an example of an unexpected host-virus interaction.

Rice black-gall dwarf virus was reported in the Fars province of Egypt from rice fields. *Laodelphax striatellus* was the vector, and was used to inoculate wheat,

barley, maize, rye and foxtail millet. Typical viral symptoms such as chlorosis were observed (Kamran *et al*., 2000).

In the central and southern areas of the Zhejiang province of China *Rice black-streaked dwarf disease* is a severe problem, which affects rice and wheat. *Laodelphax striatellus* is the vector of the virus (Wang *et al*., 2009).

Rice hoja blanca virus (RHBV) is mainly found in tropical America, which has the climate to sustain the insect vector of the virus (Web reference – DPV2). Symptoms on rice are chlorotic streaks on leaves and fewer or reduced seeds (Atkins and Adair, 1957). Wheat with typical symptoms of RHBV was reported, but RHBV was not confirmed as the causative agent, so a full diagnosis was not possible (McGuire *et al.,* 1960; Gibler *et al.,* 1961).

In 2005 in Funing, China a survey found that 84% of wheat was infected with *Rice stripe virus* (RSV) and in the same year in Jiangsu province, wheat was also diagnosed with the virus (Toriyama, 2000). *Rice stripe virus* was already common in rice, but this was the first time large amounts of wheat had been affected. Wheat showed symptoms of chlorotic stripes (Xiong *et al*., 2008). *Laodelphax striatellus* is the vector of RSV (Toriyama, 2000).

Despite initial uncertainty, it is now accepted that *Triticum mosaic virus* (TrMV) is a virus in its own right. It is the type species of the novel genus, *Poacevirus* (family, *Potyviridae*) (Tatineni *et al*., 2012). It was detected in wheat with mosaic symptoms in the High Plains, USA (Fellers *et al*., 2009) and in the Great Plains. The incidence was unknown but studies by Byamukama *et al*. (2013) show that it is present at relatively low prevalence in the Central Great Plains of the USA, certainly when compared with WSMV. The host range also includes barley and triticale (Seifers and Martin, 2009; Tatineni 2010). *Triticum mosaic virus* is often found in combination with WSMV in the Southwestern Great Plains states (Byamukama *et al*., 2013; Price

et al., 2010). *Aceria tosichella* is is the vector of the virus (Byamukama *et al*., 2013; Fellers *et al*., 2009).

In 1952, scientists in Alberta, Canada detected Wheat spot mosaic virus (WSpMV). It has also been reported from Ontario, Saskatchewan in Canada and Montana, North Dakota, Kansas, Ohio, Pennsylvania and New York (Lapierre and Signoret, 2004). The lack of other accounts of this virus suggests it either no longer exists, or has been renamed as one or more viruses.

Wheat yellow mosaic virus (WYMV) was first reported in wheat from Japan (Sawada, 1927), where it then occurred on a seasonal basis (Ohto and Naito, 1997). The virus has also been found across China in successive crops (Juanli *et al*., 1998). Chen *et al*. (2000) state that the eleven isolates of WYMV found across China have sequence variations between them, suggesting spread and then divergence (Juanli *et al*., 1998). The virus is transmitted by *P. graminis* (Chen *et al*., 2000), and WYMV can cause significant yield losses in wheat (Chen, 2005).

1.11.6 Viruses of Gramineae (not reported in wheat or the UK to date) reported globally (excluding those in 1.11.2 and 1.11.4)

Iranian maize mosaic virus (IMMV) was detected in maize in Iran (Izadpanah, 1989). The virus can infect sudan grass (*Sorghum bicolor* subsp. *Drummondii*) and maize. Symptoms include chlorotic streaks, red stripes on leaves, fine chlorosis of leaf and sheath veins and if infected early the ear may not form. It is naturally transmitted by two insects, *U. tanasijevici* and *L. striatellus* (Izadpanah and Parvin, 1979).

Maize mosaic virus (MMV) occurs in many tropical countries, including Hawaii (Kunkel, 1921) and Venezuela (Herold, 1963). In Iran maize contracts this virus via the vector *Ribautodelphax notabilis* Logvinenko (Izadpanah *et al*., 1983). There is currently no evidence that wheat is a host of MMV.

1.12 Significant viruses

Based on the review of viruses above, it is clear that there are a vast number of viruses that have already been reported in wheat, which could potentially be detected in wheat in the UK if tested for. The information about symptoms and yield loss confirms that viruses do have the potential to cause significant yield losses in wheat, and that they could realistically be contributing to the plateau in wheat which the UK is experiencing. The information about methods of transmission and the spread of reports of the viruses in different countries allows judgments to be made about which viruses pose the greatest threat; for example, a virus that has spread a lot is a more severe threat than one that has remained local. *Barley yellow dwarf virus* and SBCMV are currently the two most significant viruses of wheat in the UK, and they are widespread, as their vectors and reservoir hosts are established here. They also have the potential to cause significant yield loss, and therefore pose a threat. As mentioned previously, the farming and scientific community are aware of these viruses, which supports the fact that they are the two most well known viruses of wheat.

There are many examples in the review of unexpected viruses infecting wheat, such as Rice black gall dwarf virus. This supports the theory that a previous lack of testing may have allowed viruses of wheat to go undetected in the past. Three viruses that pose a significant threat to the UK, and could potentially be present but unreported are WDV, SBWMV and WSMV. This is because they can cause severe symptoms in wheat, and they have undergone extensive spread to date, which is likely to continue in the future by methods such as trade and travel. The UK may become a more favourable environment for vector survival in the future, thereby allowing their establishment.

1.13 Mission statement

The lack of testing and therefore lack of knowledge of prevalence of viruses in wheat is due to a historical lack of diagnostic tools. The diagnostic tools that are available now for high throughput sample preparation and testing such as qRT-PCR and pyrosequencing provide a significant opportunity to survey wheat samples for known and novel viruses. This study will use these tools in extensive screening of UK wheat, which will provide valuable information that can be used to begin to investigate the hypothesis that viruses are contributing to the plateau in the yield of wheat.

1.14 Aims

The aims of this project are to:

- Assess the incidence of known characterised viruses in UK wheat
- Investigate the possibility that currently unknown viruses are present in UK wheat
- Sequence Cynosurus mottle virus (unknown prior to the project) and develop a real time reverse transcriptase polymerase chain reaction assay
- Measure the impact of Cynosurus mottle virus on the yield of wheat

Chapter 2 – Methods

Methods used repeatedly in the project are detailed here.

2.1 CTAB extraction

Total nucleic acid was extracted by macerating the sample in CTAB grinding buffer (see Appendix 13). For plants, 300 mg of material was shaken with 2 ml CTAB grinding buffer and 10, 0.6 mm and 10, 1 cm acid washed glass beads. One millilitre of the resulting solution was placed into a 2 ml tube and incubated at 65°C for 10-15 minutes. A chloroform extraction was performed by adding 1 ml chloroform:isoamyl alcohol (24:1) and mixing to an emulsion by inverting the tube. The tube was centrifuged at maximum speed for 10 minutes. An RNA precipitation was performed by taking 800 µl of the aqueous layer in to a new tube to which 800 µl of 4M lithium chloride was added. This was incubated at 4°C overnight. The RNA was pelleted by centrifuging the tube at maximum speed for 25 minutes in a bench top centrifuge. The supernatant was poured off and the pellet re-suspended in 50 µl nuclease free water.

2.2 Total nucleic acid extraction by Kingfisher96

The machine was loaded as follows: block A- 1 ml sample and MagneSil PMPs (Promega) (50 µl for 2009 and 2010 samples and 100 µl for 2011 and 2012 samples), block B - 1ml pH 6.4 GITC 1 (Appendix 14), blocks C and D– 1ml 70% ethanol, block E - 200 µl 1 x TE buffer. Samples were further diluted in 600 µl 1 x TE buffer (2009 and 2010 samples), (2011) -520 µl DEPC treated nuclease free water 2011 and 865 µl water (2012).

2.3 Standard qRT-PCR cycling conditions

The PCR cycle was run as follows unless otherwise stated: 30 minutes at 48°C, 10 minutes at 95°C with 40 cycles of 15 seconds at 95°C and 60 seconds at 65°C. See Appendices 18 and 19 for mastermix constiuents.

2.4 ELISA

A double antibody sandwich enzyme linked immunosorbent assay (DAS ELISA) for Cynosurus mottle virus was carried out according to the instructions provided by DSMZ (the manufacturer) (DSMZ antibody number RT-0728, polyclonal antibody). A Labsystems Multiskan spectrophotometer (ThermoScientific) was used to measure extinction at 405nm. Samples were tested in duplicate. At least two negative controls were included on each plate, which were healthy wheat from the virus free glasshouse containing healthy plants only at Fera.

2.5 Mechanical inoculation

The appropriate leaf material (0.3 g) was placed in a mortar, to which 0.1 g celite (Sigma) and 3 ml mechanical inoculation buffer was added (see Appendix 20). This was ground to a paste using a pestle. The paste was gently applied to the leaves by stroking with a gloved finger. Negative control plants were inoculated with buffer and celite alone.

Chapter 3 - Annual survey of wheat for viruses

3.1 Introduction

There are a number of viruses that have previously been reported in wheat in the UK, and such viruses can cause detrimental symptoms and yield loss. It is also possible that viruses not previously reported in wheat, but present in other members of the Gramineae could also be infecting wheat in the UK (see Section 1.11). Modern diagnostic techniques are now available that provide opportunities for high through put screening of wheat samples. Such tools were lacking in the past which could be one reason why studies were not carried out. Therefore a large scale survey of wheat from the UK was carried out over four years, using real timereverse trasnsciptase polymerase chain reaction (qRT-PCR) assays for a selection of such viruses.

3.2 Materials and methods

3.2.1 Selection of viruses to test for

Twelve viruses that had been reported in the UK in the past, which were known to infect wheat or other members of the Gramineae were chosen. Selections were also based on current knowledge of symptoms, availability of sequence data for the virus (for qRT-PCR assay design) and availability of positive control material.

The viruses chosen were; *Barley mild mosaic virus* (BaMMV), *Barley yellow dwarf virus*-MAV (BYDV-MAV), *Barley yellow dwarf virus*-PAV (BYDV-PAV), *Barley yellow dwarf virus*-RPV (BYDV-RPV), *Barley yellow mosaic virus* (BaYMV), *Cocksfoot streak virus* (CSV), *Cocksfoot mottle virus (*CfMV), Cynosurus mottle virus (CnMoV), Oat chlorotic streak virus (OCSV), *Oat mosaic virus* (OMV), *Ryegrass mosaic virus* (RgMV), *Soil-borne cereal mosaic virus* (SBCMV) and *Wheat spindle streak mosaic virus* (WSSMV).

3.2.2 qRT-PCR assay design

Published qRT-PCR assays were available for BaMMV and BaYMV (Mumford *et al*., 2004). Assays were developed for the remaining viruses. Sequence data were obtained from GenBank for UK isolates of each virus, or were generated in this study (see Chapter 5 for CnMoV assay design). Briefly, primer design involved the use of MEGA 3.0 to align the sequences for each virus. Areas of good homology between isolates were selected, and sequence data loaded into Primer Express 2.0 (Applied Biosystems), specifically the Taqman probe and primer design tool. Suitable primers and probes were selected by examining suggestions by Primer Express against the earlier alignments and by BlastN searches on the GenBank website. In addition standard assay design criteria were considered (see Section 5.2.2). Primers and probes were produced by Eurofins, with all probes incorporating the quencher dye FAM and reporter dye TAMRA (see Figures 1.12 and 1.13 for explanation of the principle and analysis).

3.2.2.1 Positive control material

Where possible, fresh wheat from the glasshouses at Fera, infected with the appropriate virus was used (in the case of BYDV-MAV, BYDV-PAV and CnMoV). In the case of BaMMV, BaYMV, CfMV, OCSV, RgMV and SBCMV freeze dried plant material from Fera archives was used as the source of the virus. Freeze dried CSV and WSSMV infected plant material was obtained from DSMZ, freeze dried OMV infected plant material was obtained from the supplier ATCC and freeze dried BYDV-RPV infected plant material was obtained from Bioreba. CTAB extractions were performed according to Section 2.1.

3.2.2.2 Specificity testing of the qRT-PCR assays

Assays were tested against their target virus and the other positive control samples. Tests were performed in duplicate (see Appendix 18 and Section 2.2 for mastermix A and PCR cycling conditions).

3.2.2.3 Troubleshooting selected qRT-PCR assays

Several of the designed assays did not perform successfully, see Section 3.3.1.1.

3.2.2.3.1 Re-extraction of positive controls

Fresh samples of BYDV-MAV, BYDV-PAV, OMV and RgMV were extracted by CTAB extraction. The new extracts were re-tested with the assays for which they should not have been detected in the original specificity tests and with their own assay.

3.2.2.3.2 Sequencing products of qRT-PCR

As fresh extracts of RgMV and OMV did not solve the cross reaction problems with the CfMV and BYDV-PAV assays respectively, the products of the qRT-PCR tests for each were sequenced to investigate what was being amplified. The products were purified using a QIAquick PCR purification kit (Qiagen), according to manufacturer's instructions. Cloning was then performed using the pGEM-T easy vector system (Promega) according to the manufacturer's instructions. Clone inserts were amplified from transformant colonies by PCR using primers M13For and M13Rev. The samples were sequenced by Eurofins. The results were compared to GenBank using Blast searches, to ascertain which virus was being amplified in each case.

3.2.2.3.3 Oat mosaic virus assay

A second extract of OMV was obtained from ATCC and extracted by CTAB. It was re-tested with the OMV assay.

3.2.3 The survey

3.2.3.1 Winter wheat samples

Leaf samples were used in the case of each sample. Where multiple leaves were present in the original sample, sub-samples of each leaf were used to make the survey sample. In 2009/10 there were 716 samples; 2011, 302 samples and 2012, 338 samples. The samples were from different origins with varying information known about them including symptoms.

3.2.3.1.1 Defra winter wheat disease survey samples

Winter wheat samples at growth stage 75, from the Defra winter wheat disease survey conducted by Fera, were subsampled for this survey (621 samples-2009/2010, 296 samples-2011 and 290 samples-2012). These samples were from across England and were collected based on stratified sampling strategies based on farm size. The purpose of the Defra winter wheat disease survey study is to assess the samples for a range of diseases, not including viruses. Therefore the state of the samples in relation to viral symptoms was unknown. Sampling locations for 2009, 2010, 2011 and 2012 are shown in Figures 3.1-3.4.

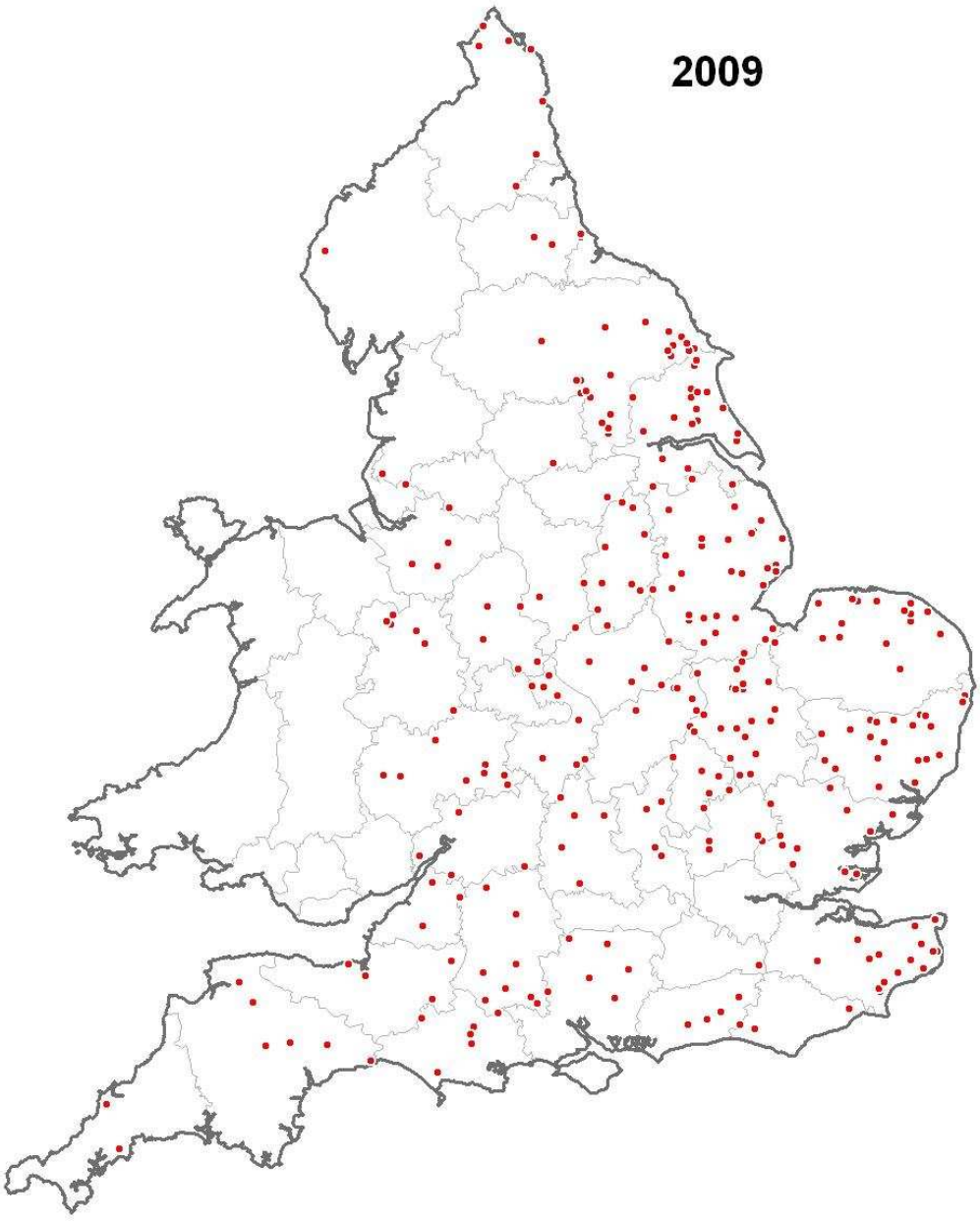


Figure 3.1. Sampling locations of the Defra winter wheat survey 2009 (Moray Taylor, Fera, personal communication).

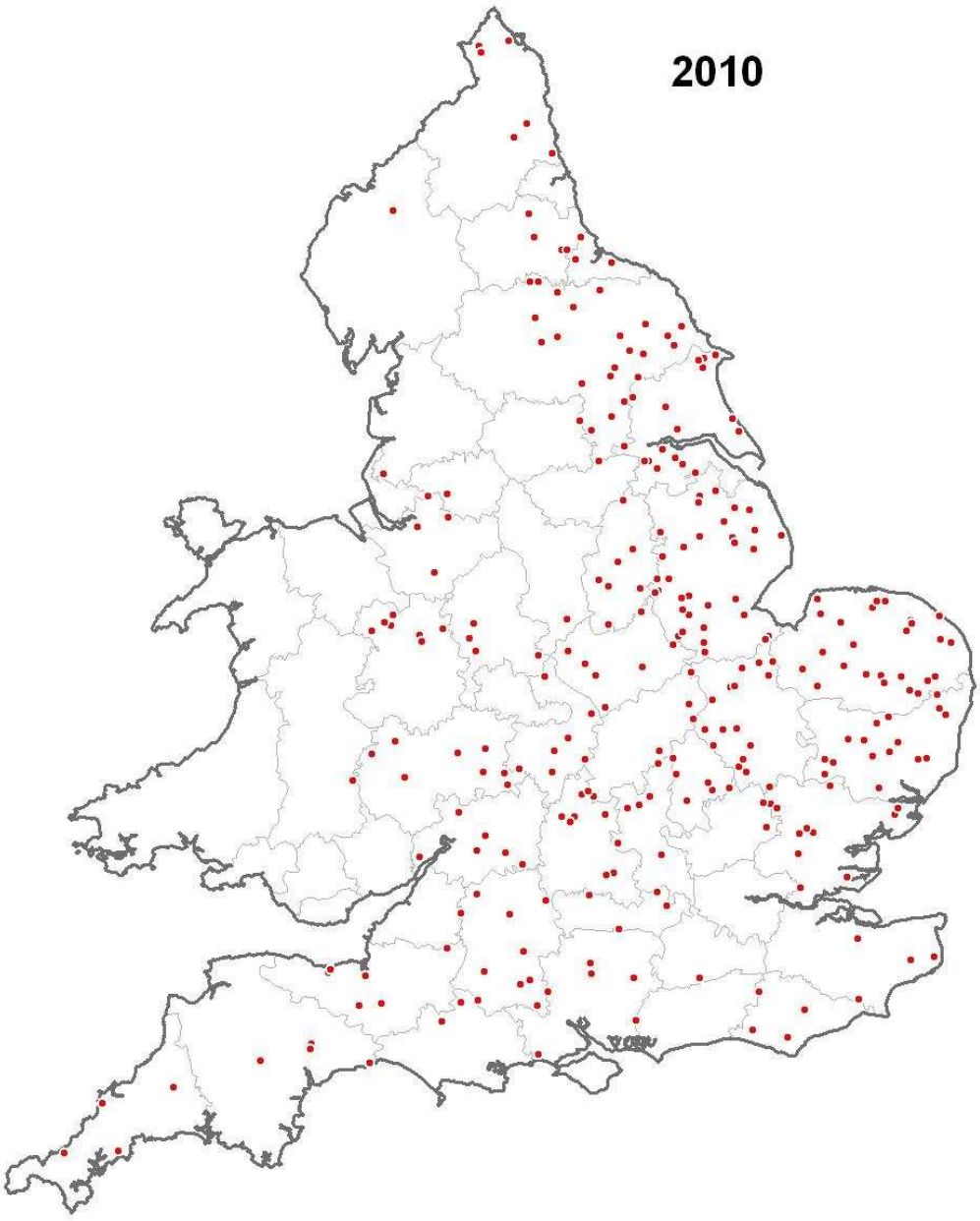


Figure 3.2. Sampling locations of the Defra winter wheat survey 2010 (Moray Taylor, Fera, personal communication).

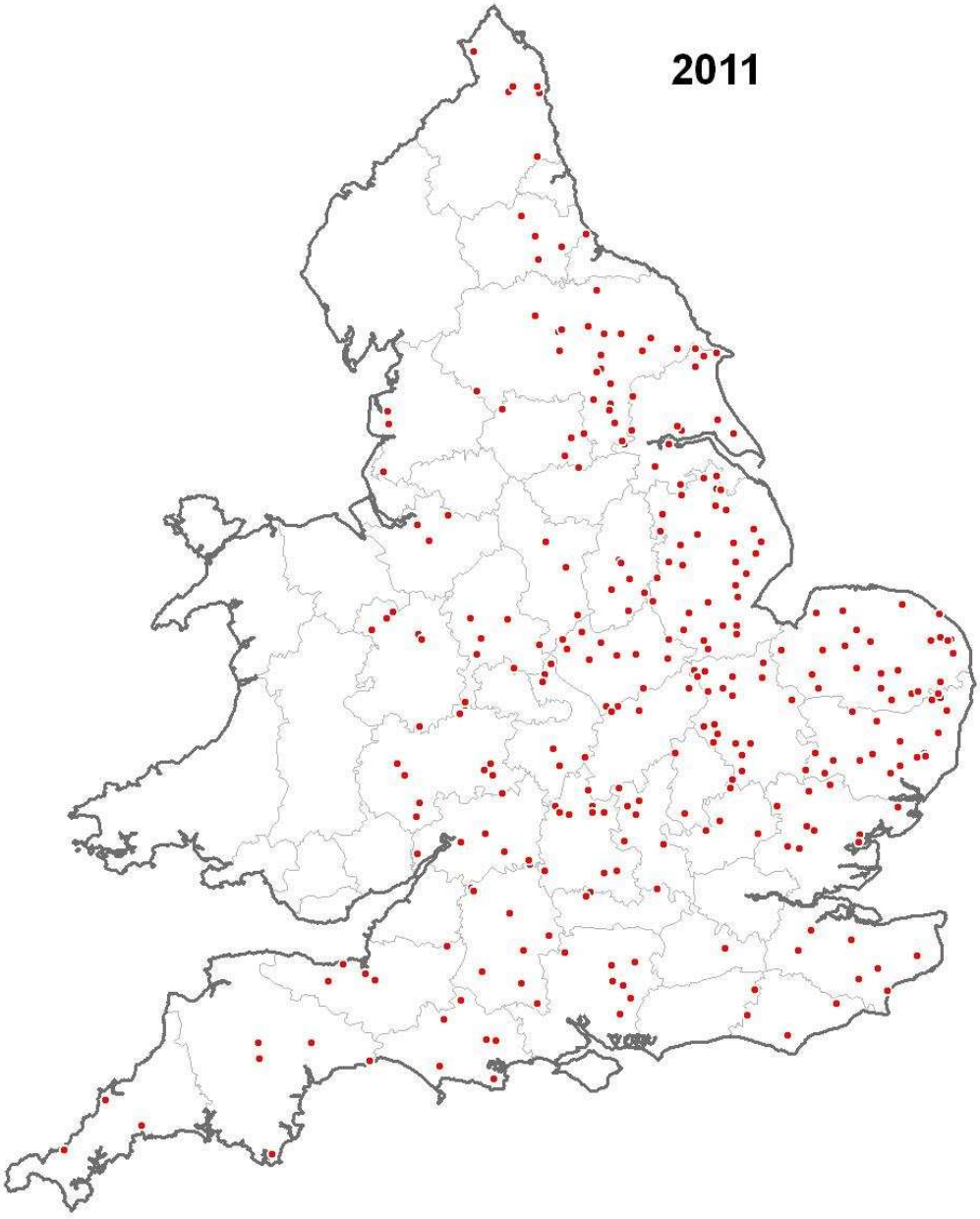


Figure 3.3. Sampling locations of the Defra winter wheat survey 2011 (Moray Taylor, Fera, personal communication).

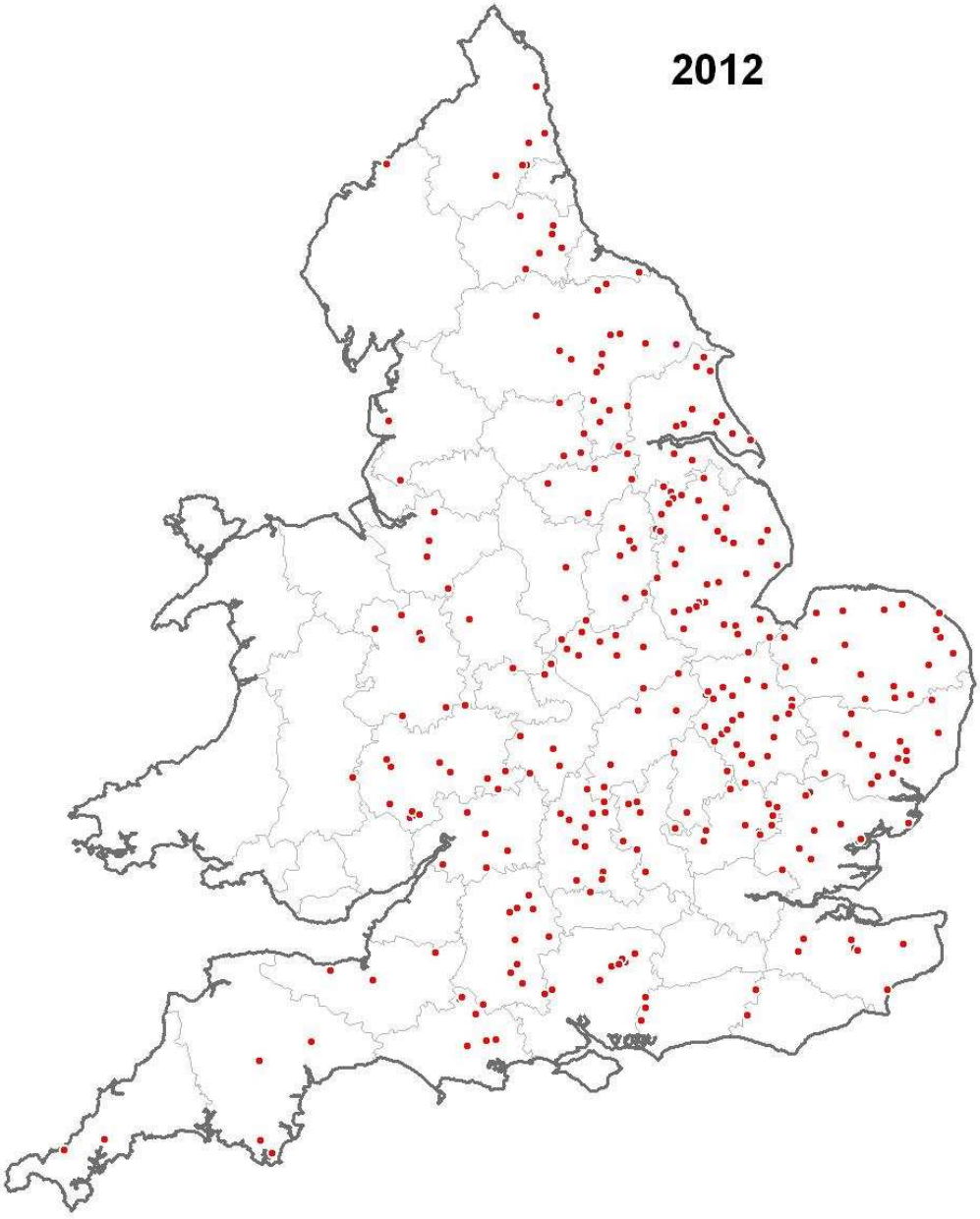


Figure 3.4. Sampling locations of the Defra winter wheat survey 2012 (Moray Taylor, Fera, personal communication).

3.2.3.1.2 Other samples

By arrangements with local farmers, samples (also at growth stage 75) were randomly collected from wheat fields in 2010 (69 samples) and 2011 (2 samples). Selection of farms was based on practicality of a visit based on distance from Blyth, Nottinghamshire (my home) and on which farms gave permission to collect samples. Sampling within the farms was based on random sampling in fields to which access was permitted. Colleagues at the University of Nottingham supplied sub-samples of wheat samples from their UK wide research samples, from a random choice from the store which provided 26 samples in 2012. In addition requests for samples at the agricultural event, Cereals, and in various farming publications such as Farmers Weekly led to samples being sent for testing (23 samples-2010, 3 samples-2011 and 6 samples-2012). Additional information was requested with the samples, such as symptoms and observations of insects in the area. Samples in the latter group were symptomatic, but other investigations into possible causes had not been able to diagnose a cause. Samples also came from the virology department at Fera; these had been sent for investigation by commercial farmers and were also symptomatic (0 samples-2010, 3 samples-2011 and 16 samples-2012). The department passed on any samples they received; therefore the increase in sample number reflects increased number of samples potentially affected by viruses each year.

3.2.3.2 Extraction of total nucleic acid from survey samples

For the 2009 and 2010 survey 0.3 g leaf material, 3 ml pH 6.4 GITC 1 (see Appendix 14), 0.3 ml TnaPP (see Section 2.5) and 150 µl Antifoam B emulsion (Sigma)) were placed in a grinding bag. For the 2011 and 2012 samples pH 6.4 GITC 2 (see Appendix 15) was used instead of GITC 1. A Homex grinder was used to macerate the plant material. One millilitre of each of the resultant solutions was used in an automated nucleic extraction using a Kingfisher 96 (ThermoScientific) (see Section 2.2).

3.2.3.3 Preparation of plates for qRT-PCR testing of survey samples

An automated liquid handling robot (Star line, Hamilton) was used to prepare qRT-PCR plates. All samples were tested for wheat phenylalanine ammonia-lyase gene (a wheat internal control gene) (Forward primer 5'-CGT TCT TGG TCG CGT TGT G-3'; reverse primer 5'-ACT CTT GAC AGC ATT CTT GAC ATT CT-3' and probe FAM 5'-CAG GCT ATC GAC CTC CGC CAC CT-3' TAMRA) (Walsh *et al*., 2005). This was to ensure the extraction of total nucleic acid had been successful. All samples were tested in duplicate with all assays shown in Tables 3.1 and 3.3 (see qRT-PCR mastermix B and qRT-PCR cycling conditions in Appendix 19 and 2.3).

Table 3.1. Details of the BaMMV and BaYMV assays

Target virus	Forward primer (5'-3')	Reverse primer (5'-3')	Probe (FAM-TAMRA) (5'-3')	Reference
BaMMV	TGA GGG TGG CAC TCT GTG TT	GCC GCA CCA TCA ACC AAT	ATG TAA TGG AAT GTG CTA TCT CGC AAC CAA CC	Mumford *et al*., 2004
BaYMV	AAA GGG AGC TGT CAC AGA GAT GA	AAA GGG AGC TGT CAC AGA GAT GA	TCT GTC CCC ATT TAT TGT TCA TGC TCG AAT	Mumford *et al*., 2004

3.2.3.4 Repeats of possible positive results

Samples that were potentially positive were tested again using the appropriate assay and the same mastermix and cycling conditions as the main survey.

3.3 Results

3.3.1 Assay development

3.3.1.1 Assay specificity tests

Blast searches of the GenBank database found that each primer and probe detected its target virus only. Table 3.2 shows the results of the assay specificity tests.

Table 3.2. The results of specificity tests for the developing assays.

Assay	Average target virus result (Ct/ΔRn)	Other positive results
BYDV-MAV	23/1.2	None
BYDV-PAV	15/1.2	Detected OMV (25/0.4)
BYDV-RPV	20/0.4	None
CfMV	16/1.0	Detected RgMV (28/0.1) and BYDV-PAV (28/0.6)
CSV	18/1.0	Detected RgMV (25/0.2)
CnMoV	5/2.3	None
OCSV	8/0.8	Detected MAV (35/0.9)
OMV	Negative	None
RgMV	17/0.6	None
SBCMV	10/1.4	None
WSSMV	19/1.25	None

3.3.1.2 Troubleshooting the assays

3.3.1.2.1 Extraction of fresh samples of RgMV, BYDV-MAV, BYDV-PAV and OMV

Tests of the CSV, OCSV, CfMV assays using freshly extracted positive controls for RgMV, BYDV-MAV and BYDV-PAV respectively showed that only the target viruses were detected in the assays (CSV assay (CSV ΔRN 0.7, Ct 24; RgMV not detected), OCSV assay (OCSV ΔRN, Ct 16; MAV not detected) and CfMV assay (CfMV ΔRn 0.6, Ct 10; BYDV-PAV not detected).However, the freshly extracted RgMV and OMV samples were detected by the CfMV and BYDV-PAV assays respectively; CfMV assay (CfMV ΔRn 0.6, Ct 10; RgMV ΔRn 0.4, Ct 29) and BYDV-PAV assay (BYDV-PAV ΔRn 0.5, Ct 18 and OMV ΔRn 0.6, Ct 23).

3.3.1.2.2 ***Sequencing qRT-PCR products***

The sequencing results of qRT-PCR products from the BYDV-PAV assay with the OMV sample and the CfMV assay with the RgMV sample showed that BYDV-PAV was present in the OMV sample and CfMV was present in the RgMV sample (data not shown).

3.3.1.2.3 Oat mosaic virus

The second extract of OMV was negative when tested with its assay (data not shown).

The results of the assay development work (assays to be used) are shown in Table 3.3.

Table 3.3. Details of the qRT-PCR assays developed for use in the winter wheat survey.

Target virus	Forward primer (5'-3')	Reverse primer (5'-3')	Probe (FAM TAMRA) (5'-3')	Reference sequence
BYDV-MAV	CCT TCA CCG CCC AAC AGA	CCT TGT ATA GCA TGT AAA ATT GGT TCA	TGA CGG GAA GGA ATT CAG GGA GAG CAC G	GU002360.1
BYDV-PAV	CAG AAG GAT CWT AYA GYG TRA ACA TT	ACC ATC CTC GTT RCC WCC WAT	CGT GCG AAG GCT TTC AAT CCG TTG	FJ687408.1
BYDV-RPV	GGA GCT TCC CAG CGA AGA T	GAG GAT ACG GAA TTG ATC TTC GTC and AAG AAT TCG GAA CTG ATC CTC ATC[1]	ATY AAC GGG TTA GAG TGG CAC CCC TC	DQ910754.1
CfMV	TTG CTG CAC ACR TCC RTG AA	TTC GAG AAC TCA TCA CAN GGR AGA	CCA YGG GCA AYG CTG TTA CGA GCG	EF422396.1
CnMoV	TTC TAT CTC GGT GAG TTC GTT CAG	GCA GGC GTC ACT TGG TAC ACT	CGA CAG CAA CCC TGA CAG CGC	Chapter 5 of this project
CSV	AGA GCT CGC GAY ACT GTG AGA	GAC AAG CTC CAC AYG TTA TCT TRA	TTT GGC AAG GGA TGT TTC CGT GCT	DQ067585.1
OCSV	GAG ACA GAC AGC AAG GTG AAG GT	CCC GCG GCA CTG GAT	TTT GTG AAA TAC GAG AAA ACC GAC CAT ACA TCC	NC 003633.1
RgMV	AAG GTG AAG AAC AGA TAK SST ATC CA	GCC ATT ATT GAC CGC AAC GT	TYG RAC CRT TCT GTC GCC ACG C	AF091234.1
SBCMV	CGC ATT GTC GAA GAT TTC CA	GCT AAG ATT GCG TCT CGG AAA A	AAT AGG CTG GTT TTG GCC GAC GAT TTG	AJ298069.1
WSSMV	GCG CGC CTA TAG TGA CGA A	GGA GGC TCC GTG TCT CAT AGC	ACT CAT CAG CGA AGG TAA ACT CGT TCC CA	X73883.1

[1]Two reverse primers were used to target the two variants of the viral genome.

3.3.2 The main survey

3.3.2.1 Wheat phenylalanine ammonia-lyase assays

Figures 3.5, 3.6 and 3.7 show the cycle threshold values of all wheat samples for the wheat phenylalanine ammonia-lyase assays (WPal). Samples which gave a Ct value of 0 failed the test.

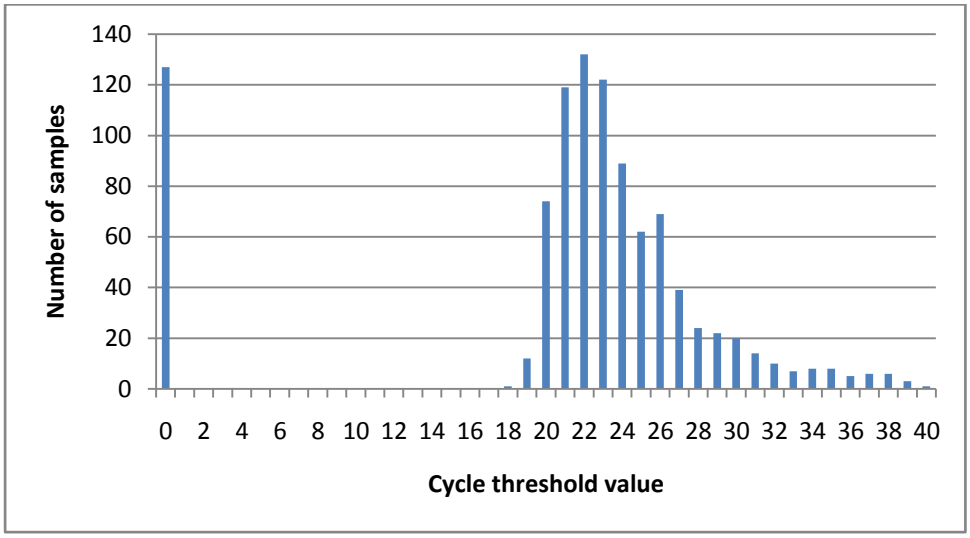


Figure 3.5. Cycle threshold values for all 2009 and 2010 wheat samples used in the survey. Note there were two corresponding values for each sample as they were tested in duplicate.

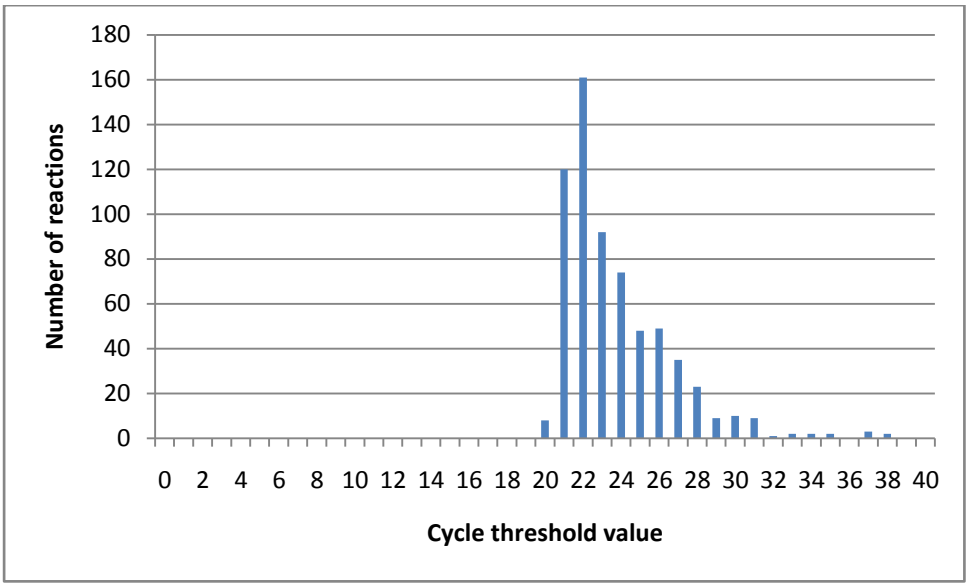


Figure 3.6. Cycle threshold values for all 2011 wheat samples used in the survey. Note there were two corresponding values for each sample as they were tested in duplicate.

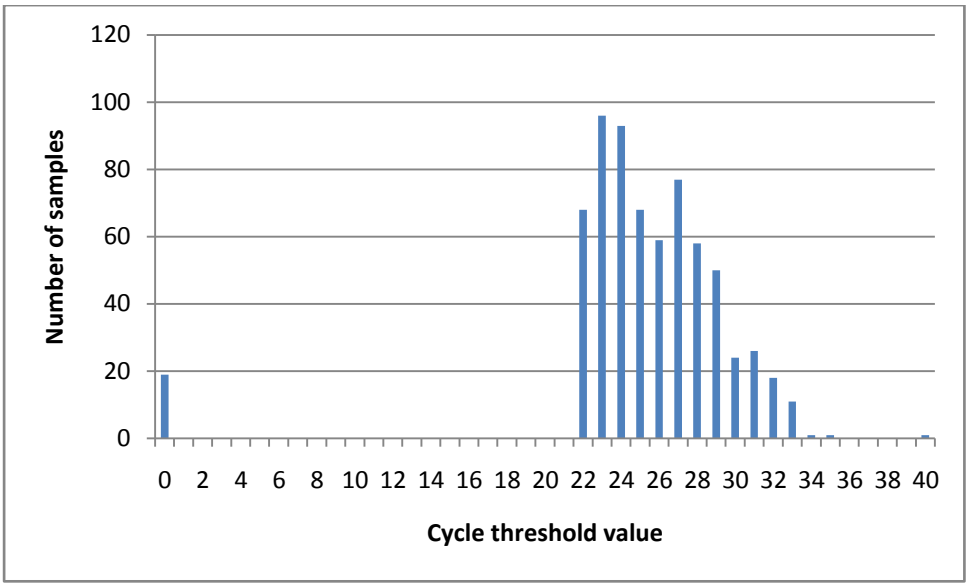


Figure 3.7. Cycle threshold values for all 2012 wheat samples used in the survey. Note there were two corresponding values for each sample as they were tested in duplicate.

3.3.2.2 Survey results

Samples that were positive in the main survey and the repeat tests are discussed below. All other samples were negative for the assays (data not shown). There were no samples which were infected with multiple viruses.

3.3.2.2.1 2009

None of the samples were positive for any of the assays.

3.3.2.2.2 2010

Barley yellow dwarf virus-MAV was detected in three samples from a wheat breeding centre in Oxfordshire. One sample was a cross of cv. Walpole and cv. Leu81024 wheat and the other two were crosses of cv. Oakley and cv. Panorama. All samples had chlorotic and red leaves. The symptoms appeared on individual plants and not in patches. The field had contained wheat for the previous two years, and barley the year before that. A Defra winter wheat disease survey sample of cv. Gladiator from East Yorkshire also tested positive for BYDV-MAV.

Soil-borne cereal mosaic virus was present in one sample of wheat cv. Viscount from Perthshire. The sample was sent in to this project by Dr Fiona Burnett, SRUC. The sample was from a stunted area of wheat within a field (covering approximately 10% of the field at the time) of continuous wheat (wheat for 20 years) which reappears and expands each year. Any possible disease was unlikely to be aphid borne as no insects were observed on the plants before or during symptoms. Additionally, other diseases such as stem base diseases had been ruled out by prior testing (Fiona Burnett, SRUC, personal communication).

3.3.2.2.3 2011

None of the samples were positive for any of the assays.

3.3.2.2.4 2012

Three samples were positive for BYDV-MAV, which were all part of the Defra winter wheat disease survey. Location and variety of wheat of the samples were Northumberland, JB Diego; Lincolnshire, Unknown; and Oxfordshire, Unknown.

Barley yellow dwarf virus-PAV was detected in twelve samples. Five samples were sent to the project by farmers who supplied additional information (see Table 3.4). The remaining seven samples were sent to the virology department of Fera and exhibited typical symptoms of viral disease, such as stunted growth, chlorosis and reddening (see Table 3.5).

Table 3.4. Details of the wheat samples that were sent in to this project, which were positive for BYDV-PAV.

Variety of wheat	Location of sample site	Symptoms observed	Area covered by affected plants	Insects present?	qRT-PCR result
KWS Santiago	Buckingham	Stunted chlorotic plants	Patches of affected plants covering 70% of four fields	None observed	29/0.1
KWS Santiago	Buckingham	Stunted chlorotic plants	Patches of affected plants covering 70% of four fields	None observed	25/0.9
Duxford	Northamptonshire	Not given	Patches in one field	Not given	26/0.35
Solstice	Leicestershire	Stunted chlorotic plants	In sandy areas of one field	Not given	20/1.7
Einstein	West Sussex	Not given	Not given	Not given	27/0.2

Table 3.5. Details about the wheat samples that were positive for BYDV-PAV, which had been sent to the virology department at Fera.

Variety of wheat	Location of sample site	qRT-PCR result (Ct/ΔRn)
Oxfordshire	Santiago	26/0.35
Herefordshire	Grafton	19/1.0
Buckinghamshire	Robigus	23/0.3
Lincolnshire	JB Diego	27/0.25
Dorset	Oakley	24/0.5
Wiltshire	JB Diego	30/0.1
Gloucestershire	Claire	19/0.7

Soil-borne cereal mosaic virus was detected in five samples. Four of the samples were symptomatic and one was part of the random Defra winter wheat disease survey survey. Details of the samples location, variety of wheat and qRT-PCR result are shown in Table 3.6.

Table 3.6. Details of the wheat samples that were positive for SBCMV.

Variety of wheat	Location of sample site	qRT-PCR result (Ct/ΔRn)
Dorset	Invicta	21/0.9
East Sussex	Unknown	18/0.9
Wiltshire	JB Diego	21/0.9
Wiltshire	JB Diego	25/0.77
Cambridgeshire * Defra winter wheat disease survey sample	JB Diego	29/0.3

Figure 3.8 shows the location of wheat samples that were positive for a virus in this study.

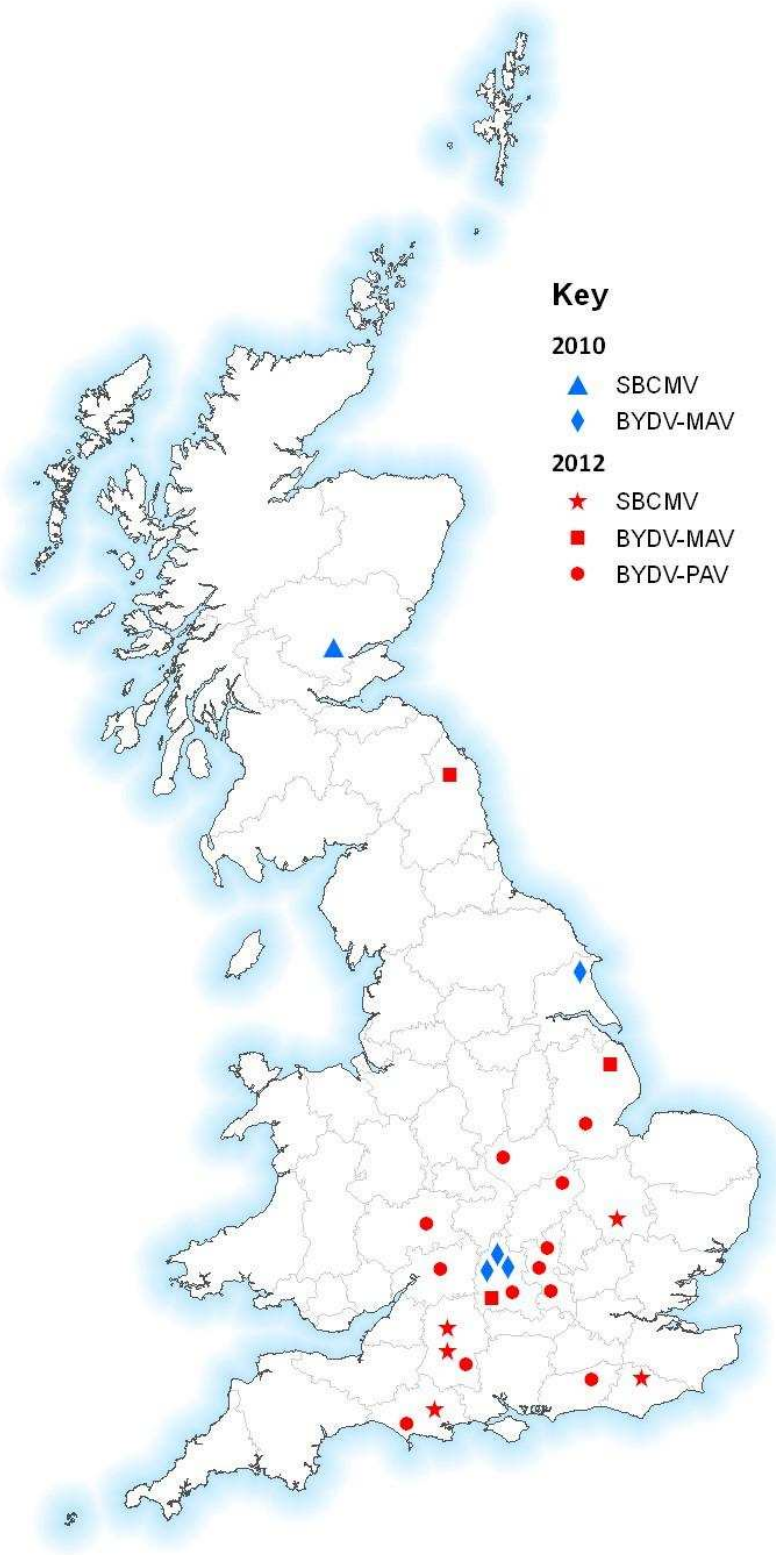


Figure 3.8. The prevalence of viruses of wheat in the 2009/2010, 2011 and 2012 surveys. Results are shown at county level, the location of the symbol within the county does not reflect the location of the site because such specific data was unavailable for the majority of samples.

3.3.2.3 Viruses found in wheat by the virology department at Fera

Figures 3.9 and 3.10 show the number of samples of commercial wheat that the virology department at Fera have diagnosed with viral infections during this PhD project.

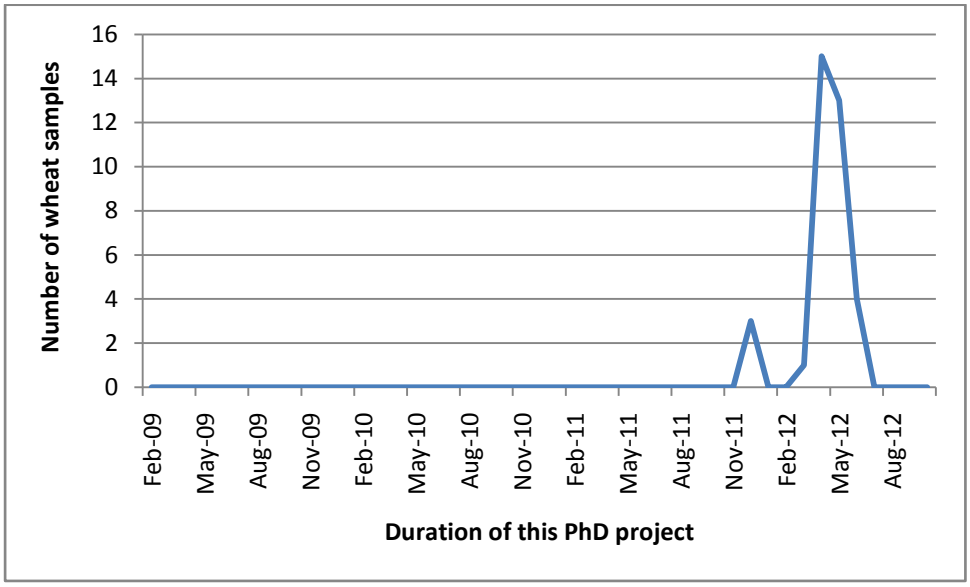


Figure 3.9. Commercial wheat samples that were diagnosed with BYDV infections by the virology department of Fera, during the duration of this PhD project.

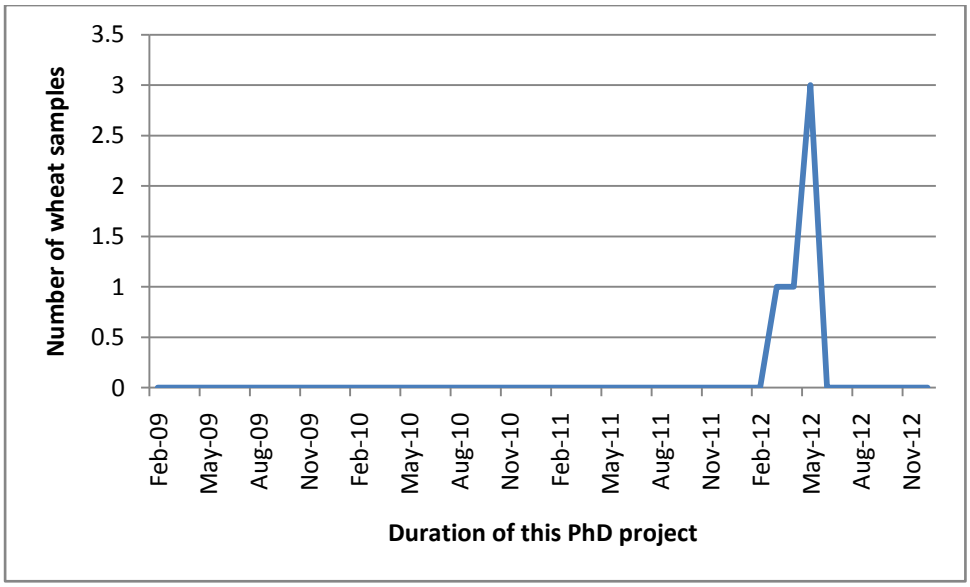


Figure 3.10. Commercial wheat samples that were diagnosed with SBCMV infections by the virology department of Fera, during the duration of this PhD project.

3.4 Discussion

3.4.1 Assay development

Blast searching of the GenBank database found that all assays should only detect their target virus. However, in the qRT-PCR specificity tests, only BYDV-MAV, BYDV-RPV, CnMoV, RgMV, SBCMV and WSSMV were species specific (see Table 3.2). As all assays were tested for cross reactivity using Blast searchs on the NCBI website, it was proposed that contamination was the cause of the cross reaction; this was the case with the CSV, OCSV, CfMV assays and the RgMV, BYDV-MAV and BYDV-PAV respectively as detection was resolved by the extraction of fresh infected samples. Contamination could have occurred during the initial extraction process; however, it is more likely that genuine dual infections occurred in the original samples. Re-extraction of fresh samples did not solve the problem for the RgMV and OMV assays, which were again detected by the CfMV and BYDV-PAV assays respectively. Sequencing the products of the qRT-PCR tests also found that contamination was the cause of OMV and RgMV being detected by the BYDV-PAV and CfMV assays respectively. The cause of the contamination is unknown, but again rather than contamination it could be that the original samples had dual infections of virus.

Two extracts of OMV were both negative when tested with the assay designed for the virus. No other samples of positive plant material could be sought either from commercial suppliers or through publishers of research into the virus. Attempts were made to re-design the assay, but this was difficult as there were only two short sequences for the virus (which were identical) on GenBank. Other diagnostic tools such as ELISA were investigated but an assay could not be sought. Methods such as TEM would not have been amenable to the high number of samples in the survey. Therefore work on the assay for this virus had to cease, and it was removed from the survey. However, should the virus be highly prevalent in wheat

samples and therefore a potential cause of the yield plateau it would have been detected by next generation sequencing performed in Chapter 4. Details of the developed assays are shown in Table 3.3. These were used in the main survey.

3.4.2 The main survey

3.4.2.1 Wheat phenylalanine ammonia-lyase assay

Figures 3.5, 3.6 and 3.7 show the results of WPal assays on all survey samples, the mode value for Ct was approximately 22 cycles for all years of the study. There were differences in the number of samples that failed between the years, shown by a value of 0. Between the 2009/2010 samples and 2011 there was a decrease in the number of failed samples, but a slight increase in 2012. It is unknown why this occurred. However, it could be due to unintentional differences in the buffers or magnetic beads used in the Kingfisher extraction. As discussed in more detail in Section 3.4.3.2 different varieties of wheat have different levels of WPal gene expression, therefore it could be that varieties with low levels of gene expression were more prevalent in 2012 (Mansoor, 2011: Steiner *et al*., 2009). It is known that stress causes increased WPal expression, therefore in 2012 in which there were more cases of virus infection than 2011 it may have been expected to see better WPal expression. Other variables such as age of the wheat, method of extraction and timings of experimental work were the same for each year, therefore these variables would not be expected to be a cause of the increased number of samples which failed with the WPal assay.

Values approaching 40 cycles should be viewed with caution, as random probe cleavage may have occurred causing the result. This theory is viewed with sceptisicm; however, the high Ct values produced by water controls observed by some researchers lead them to believe that random probe cleavage genuinely occurs (Unpublished data, Theodore Allnutt, Fera internal qRT-PCR troubleshooting

meeting). Another explanation could be that the results are genuine and be due to low copy number of the target gene in the sample

Figures 3.5, 3.6 and 3.7 were used to monitor the extraction process and ensure suitable extracts for tests with qRT-PCR assays were produced. However, all samples regardless of their result for the WPal assay, were tested for viruses detailed in the main survey. This is because there are cases in which COX assays fail but viruses which may be more stable due to their protective protein coats are detected (Ian Adams, Fera, personal communiation). Additionally, the buffers in the extraction process were biased towards RNA, therefore if a sample gave poor results for the WPal assay, it could still have good levels of any viral RNA present.The WPal assay is a standard assay used to confirm that a total nucleic acid sample is suitable for RNA virus testing by Fera. It can be used to infer the amount of RNA in the sample, but is not a direct measure of it because the assay detects DNA too.

3.4.2.2 Cut off values for positive results

To ensure possible positive results from the main survey were not ignored and because the vast majority of results were negative in most tests, any sample that gave a Ct of 40 or below for one or more of its replicates was selected to be re-tested. For positive control and repeat tests, cycle threshold cut off values were applied according to the results of their positive controls in assay development (see Table 3.2 and Section 3.3.1.2). In the majority of cases, repeating the tests for the sample that had given results with high Ct values, produced a negative second result. This could have been due to random probe cleavage at the end of the PCR cycle in the first instance, which has been proposed as a possible issue in qRT-PCR (Unpublished data, Theodore Allnutt, Fera internal qRT-PCR troubleshooting meeting).

3.4.2.3 Results of the assays for viruses

In 2009, there were no positive results for any of the viruses. However, in 2010 there were cases of BYDV-MAV; three samples were symptomatic and were sent in as a response to the request for such samples. The symptoms reported were consistent with BYDV-MAV infection (chlorosis and reddening of leaves – see Figure 1.4 and Section 1.11.1.3). The field had contained wheat for the previous two years and barley the year before that; such plants could have supported aphids which are the vectors of BYDV-MAV, and allowed overwintering. While the person who sent the sample did not observe aphids in the area at the time of sampling, information from the aphid bulletin of the Rothamsted insect survey suggest that aphids such as *R. padi* were frequently present at the nearest trapping site to the origin of the sample (origin of sample was Banbury, Oxfordshire and the nearest site was Wellesbourne). For example there were 1192 *R. padi* caught between the 5th and 11thOctober 2009, which would have been a critical time for virus infection as the wheat was at its most vulnerable as it was young (Doodsoon and Saunders, 1970; Web reference – RIS3). Therefore the lack of observations of aphids in July when the sample was taken were actually perhaps irrelevant because the critical time for aphid transmission of viruses to wheat is in the autumn. Additionally this was the time in which any remaining aphids from the barley growing in 2009, which then moved to reservoirs could have then moved to the newly planted wheat crop which would be that of the 2010 harvest. Weeds surrounding the field could also have acted as reservoirs of vector and virus. There were reports that symptoms were not observed in large areas around symptomatic plants suggesting large scale spread by insect vectors had not occurred; symptoms would have been expected at the end of the growing season had BYDV been present. In comparison to these samples, a sample from the Defra

winter wheat disease survey, which did not necessarily have symptoms, was positive in tests for the virus.

Soil-borne cereal mosaic virus was detected in one sample of symptomatic wheat from Perthshire. The information supplied with the sample was consistent with what is known about SBCMV, such as symptoms, repeat occurrence annually and aphids not being in the area so unlikely to be involved in transmission (see Sections 1.4 and 1.11.1.3), and therefore supports the conclusion that the virus was present.

In 2011, none of the samples were positive for any of the assays. However, in 2012 there were several positive samples. Three samples were positive for BYDV-MAV which were part of the random Defra winter wheat disease survey (therefore were not necessarily symptomatic). *Barley yellow dwarf virus-PAV* was detected in twelve samples, all of which were symptomatic samples.

The results of wheat testing from the virology department of Fera support the results of this study to an extent (see Figures 3.9 and 3.10). They show that there were no positive samples for BYDV (a combined test for strains is carried out) or SBCMV during 2009 and 2010, which is consistent with this survey in 2009, but not 2010 as some viruses were detected at low prevalence in this study. In December 2011 the virology department at Fera diagnosed three cases of BYDV; these samples were actually the crop of 2012, therefore the results are consistent with the results of this survey in that there were no cases of viruses in the 2011 wheat crop but there were in the 2012 crop. The highest prevalence of samples in this survey coincided with the time when the virology department recorded the highest number of cases of viruses. This was partly because of the dual testing of samples by the virology department and this study.

3.4.2.4 Climate data

The climate can have a major impact on the titre of viruses and the symptoms that they cause (Budge *et al*., 2008). For example aphids that are vectors of some viruses such as BYDV cannot survive in low temperatures and the lethal temperature (temperature at which 50% of the population cannot survive) for a grain aphid is -8°C and 0.5°C for bird cherry aphids (Web reference – HGCA3). Studies and data from the Rothamsted insect survey have shown that a 1°C increase in temperature in January and February can bring forward the date of first flight of aphids by as much as four weeks, thereby increasing the chance of an early infection with an aphid transmitted virus such as BYDV (Web reference – BBSRC) (see Section 1.8 and Figures 1.8 and 1.9). Early infections are known to cause worse symptoms due to BYDV (Kennedy and Connery, 2001). The HGCA recommend drilling wheat later towards the winter to avoid aphid infestations that can transmit viruses such as BYDV. However, if the winters become warmer aphids may survive later in the year, emerge earlier and perhaps eventually all year round, removing this as an option (Web reference – HGCA and Defra). Met office data suggests that the autumn and spring periods are becoming warmer compared to long term data, making this a possibility. However, the winter temperature has fluctuated more, but there does appear to be a slight trend towards warmer winters (see Figure 1.10).

Polymyxa graminis, the vector of SBCMV is also affected by the climate; it is most likely to infect plants in autumn when soils are wet and not frozen (Kanyuka *et al*., 2003). Warmer conditions (approximately 15°C) with cycles of wet and dry weather favour development and infection of cereal roots by *P. graminis* but high levels of rainfall have the opposite effect (Adams and Swaby, 1988; Ledingham, 1939; Legreve *et al*., 1998). Cycles of wet and dry weather are also beneficial (Adams *et al*., 1986).

To put the survey in this context, there were more cases of viruses in 2012 than other years and it is known and reported by the Met Office that the autumn and winter of 2011, and the winter, spring and summer of 2012, (when the 2012 wheat crop was in development) were warmer than average for the UK with an exceptionally warm October and November (HGCA/Rothamsted project report RD-2008-3475 2011; Web reference – Met Office 5 and 6). This allowed insects to remain at higher levels during the winter with extended flying season, greater movement and growth therefore enhancing the interaction with wheat and potentially spreading viruses (HGCA/Rothamsted project report RD-2008-3475 2011; Richard Harrington, Rothamsted insect survey, personal communication). It was also noted that insecticide resistance may have been a cause of greater *R. padi* numbers (Richard Harrington, Rothmsted insect survey, personal communication).

The warmer conditions also favour *P. graminis* and therefore SBCMV. The summer of 2012 had a lot of rainfall, receiving the highest amount of rain since 1912 (Web reference – Met Office). There were strong winds in the latter period of 2011 and early 2012, which could have dispersed aphids infected with BYDV. This could have affected both winged forms responsible for primary infections but also, wingless forms which cause secondary infections within fields increasing the chances of detecting the virus as it would be more likely to be seen by farmers or simply more prevalent in the field, and therefore more likely included in random sampling for the Defra winter wheat survey (van Emden and Harrington, 2007).

A similar climate in the UK occurred during the growth of the 2010 wheat crop, when there were also higher levels of BYDV and SBCMV than other years such as 2011 (Web reference – Met Office 7). Predictions from a HGCA/Rothamsted project were that the colder winter at the end of 2009 and the start of 2010 would cause late aphid flight, but that reduced numbers of natural enemies would mean

higher numbers of aphids could occur, the predictions were proven correct (HGCA/Rothamsted project report RD-2008-3475 2010). This could have contributed to the detection of BYDV in 2010.

In contrast to the growing seasons of the 2010 and 2012 wheat, the autumn of 2010 when the 2011 wheat crop was planted was below average in terms of temperature. This trend continued through the winter 2011, with an exceptionally cold December (Web reference - Met Office 8 and 5; HGCA/Rothamsted project report RD-2008-3475 2011). This meant that active stages of insects could not survive in such high numbers throughout the winter, so there were later migrations and lower numbers, therefore not infecting wheat in such high numbers early in the season when the plants were most vulnerable (HGCA/Rothamsted project report RD-2008-3475 2011). However, despite predictions of greater aphid numbers due to a milder spring and the lack of survival of natural enemies this did not result in cases of viral infections in wheat from transmission events. Perhaps this was because the wheat may have had mature plant resistance by the time aphid numbers had risen (HGCA/Rothamsted project report RD-2008-3475 2011; Lindblad and Sigvald, 2004). The growing period of the 2011 wheat was generally drier than previous years and in the spring it was the driest since 1910 (Web reference – Met Office). This meant that conditions for soil-borne viruses, such as *P. graminis,* were not ideal. Similar conditions were observed for the 2009 wheat crop, including snow at the end of 2008, which would have limited overwintering active insect vector population numbers as temperature fell below lethal temperatures for aphids (Web reference – HGCA 3; Met Office 7 and 9). Soil borne viruses and their vector *P. graminis* are not in optimum conditions during periods when the ground is frozen (Kanyuka *et al*., 2003).

Therefore, the climate conditions are probably contributory to the higher levels of viruses that were found in wheat in 2012, compared to other years, such as

2011. This also suggests that if climates are warmer and wetter (in cycles) in the future, there may a higher prevalence of wheat viruses.

Increased temperatures not only affect vectors of viruses but the viruses themselves (see Section 1.8). While this is important in terms of symptoms, it also increases the viral titre, thus the chance of detecting a virus by qRT-PCR (Dahal *et al*., 1998; Hull, 2004).

3.4.2.5 Location of viruses

Figure 3.8 plots the results of the survey on a map and shows that the greatest prevalence of viruses in wheat is in southern England. This could be because most symptomatic samples of wheat were sent from that region, but accordingly that leads to the conclusion that symptomatic wheat was not observed in northern England to such an extent. The area of land in hectares on which wheat is grown is substantially greater in the south of the UK than the north. In 2011, the proportions were as follows: 1,885,000 ha – south and 469,000 ha – north (Yorkshire and north) (Web reference – HGCA4). The Defra winter wheat disease survey samples that were the main contributor to this work are collected in a stratified manner to represent these proportions in England, but the samples from other sources may be biased towards the south because more wheat is grown in the south (see Figures 3.1-3.4). As the majority of the samples were collected according to a stratified plan, the higher prevalence of viruses in the south is likely to be because more wheat is grown there. Another reason for a greater number of viruses in the south of the UK is that insects which act as vectors are more prevalent in the warmer south and the process of transmission of viruses by these and by soil-borne vectors such as *P. graminis* benefits from these warmer conditions (see Section 1.8) (Adams and Swaby, 1988; Ledingham, 1939; Legreve *et al*., 1998; Lucio-Zaveleta *et al*., 2001; Smyrnioudis *et al*., 2001). For example, the average annual maximum temperature between 1981 and

2010 was 11.4°C in Kinbrace, northern Scotland and 14.3°C in Everton, Southern England (Web reference – Met Office 2 and Met Office 3). Evidence from the Rothamsted insect survey shows that between the 24th and 30th of September 2012 110 *R. padi* were caught at Gogarbank, Southern Scotland but 225 at Starcross, Southern England (Web reference – RIS2). This is a critical time for viral infections of wheat as it is at its most vulnerable (Doodson and Saunders, 1970).

Tables 3.4, 3.5 and 3.6 show that JB Diego was the variety of wheat that had the highest number of viral infections. This is a concern because it was the highest selling variety of winter wheat in 2012, with 12% of the market share. It is also on the HGCA recommended list for 2013/2014 (Web reference Fwi; HGCA7). Data from the Plant Disease and Protection team at Fera shows that the proportion of wheat grown in the UK that is JB Diego has increased from 2009 to 2012 (0.7% to 12 %) and as the proportion of wheat samples that were positive for a virus which were JB Diego was 27%, this suggests that the reason for JB Diego having more cases of viral infections is because it is more susceptible, and not because there is simply a higher proportion of it grown than other cultivars. It could be the case that JB Diego is more symptomatic when infected with a virus than other cultivars, therefore it was more visible to those who sent samples into the survey. However, the only sample from the Defra winter wheat survey for which samples are collected at random, which was positive for a virus was from a sample of JB Diego, this suggests that it is more prone to infections of viruses. With regard to JB Diego, resistance to diseases such as rust are a focal point, but there is no mention of viruses in wheat variety profiles and in addition the breeder of JB Diego (Saatzucht Josef Breun) confirmed that to their knowledge the variety has no resistance to viruses and the seed marketer Senova stated that JB Diego is known to be susceptible to SBCMV and that BYDV resistance status is unknown (Ludwig Ramgraber, Saatzucht Josef Breun, personal communication; Tom

Yewbrey, Senova, personal communication; Web reference – Fwi). This is a concern because if the trend of growing JB Diego continues and increases, along with the number of viruses and spread by vectors due to climate change, there could be even more severe yield losses in the future.

3.4.2.6 The viruses which were not found in this survey

The viruses that were selected for testing in this study were chosen because they had either been found in wheat or other members of the Gramineae in the UK in the past. Viruses such as RgMV or CnMoV, which had not been reported in wheat, were included because a lack of testing could have been the reason for them not having been reported. However, this survey suggests that such viruses are not present in wheat in the UK, and therefore not responsible for the plateau in the yield of wheat. It is known that certain viruses compete both within plant and insect hosts, with differences in success for example BYDV PAV is known to outcompete BYDV MAV due to greater efficiency of transmission and perhaps a molecular basis including more efficient transcription factors for replication of the viral genome (Power, 1996). Therefore it is possible that in the samples in which viruses were detected, there may have originally been multiple infections but only one virus was detected as it was the most successful and at higher titre. It is also possible that insect vectors in the area would have transmitted greater numbers of certain viruses such as BYDV-MAV but BYDV-PAV prevented it from occurring. Potentially other viruses which were not tested for were more successful than those which were, for example, a currently unknown virus the likes of which were potentially detected in this project (see Chapter 4).

3.4.3 Discussion of methods

3.4.3.1 Specificity testing of assays

In order to test the specificity of the assays that were being developed, Blast searches of the GenBank database were carried out. Therefore, despite only some viruses being tested for in physical tests, all viruses on GenBank were screened in theory. The physical tests highlighted the requirement for careful extraction of positive controls and ideally to know that only the target virus is present in the sample, so that contaminants and dual infections were not a problem.

3.4.3.2 Extraction method

The extraction method was refined each year in an attempt to produce the highest quality extracts. It does not appear that this caused a major bias to the study because there were not considerably more positive samples as the study progressed. The higher number of virus infected samples in 2012 is probably because more samples from the virology department of Fera were tested, and these were symptomatic samples and not because of a better extraction method.

Wheat phenyalanine-ammonia lyase testing was used to suggest if nucleic acid extraction had been successful and therefore the sample was suitable for use. The assay is routinely used at Fera for this purpose. However, there are issues with using the assay. Firstly the level of expression of the WPal gene varies between genotypes of wheat, and importantly has been seen to increase in response to stress such as fungal disease and aphid infestation (Mansoor, 2011: Steiner *et al*., 2009). Therefore the assay may not have been appropriate for all varieties of wheat used, but it is currently not known which varieties have high levels or gene expression and which do not. This could be an area for further study by testing different varieties of wheat grown in parallel by qRT-PCR and using Ct values as an indicator of the levels of the gene in each variety, or by studying gene expression using next generation

sequencing (Varshney *et al.,* 2009). The fact that the gene is upregulated in response to stress suggests it may be so if a virus is present, thus adding a bias to the virus infected samples. It is also possible that the age and condition of a wheat sample could affect the WPal assay results; however, in these studies these variables inparticular the first should have been approximately constant as all samples were taken as growth stage 75.

Rather than using a WPal assay to test the samples for a successful nucleic acid extraction, a more direct test would be to test for an RNA virus (another plant virus which was unrelated to the targets of the assay) which was added to all samples in sap prior to extraction. Alternatively an RNA specific assay for a gene such as NADH could be used (Chen, 2010). As positive results were obtained for some samples it suggests that despite poor WPal results in some cases the extraction method was suitable for use.

3.4.3.3 Check for inhibitors in wheat samples

None of the 2011 wheat samples tested positive for any of the viruses. This was a genuine result and not due to inhibitors of PCR being present in the samples, as such samples did not prevent other consistently positive samples for the COX assay from giving positive results when they were added to the samples (data not shown).

3.4.3.4 Choice of diagnostic method

Real time reverse transcriptase polymerase chain reaction was chosen for this study for several reasons. The first was that it could be used to test a large number of samples efficiently using robotics for extraction and plate production. In addition, qRT-PCR machines that can tests 190 samples at once were available. This was in preference to other methods such as ELISA, for which such machinery was not available. In addition, the procedures involved do not have the long incubation periods including overnight steps required for an ELISA test. Real time reverse

transcriptase polymerase chain reaction was used in preference to conventional PCR because it eliminated the need for gel electrophoresis, which with the large number of samples involved would have been time consuming. There were also two qRT-PCR assays available at Fera for two of the target viruses and none for ELISA, so the decision was made to continue with this method. It may have been beneficial to test some samples which were positive using qRT-PCR for a virus with the corresponding ELISA test, or other method because a positive result using two different methods would have provided a more reliable result. Conversly, some samples which were negative when tested by qRT-PCR could have been tested using ELISA to confirm that the virus was not present. This would be in agreement with protocols from EPPO (European and Mediterranean Plant Protection Organization) which requires confirmation by either repetition of the method or by a different method (Web reference – EPPO).

3.4.3.5 Samples

The majority of samples were from England and were supplied by the Defra winter wheat disease survey. Requests for samples in publications and at Cereals, which targeted UK farmers but also some visitors from overseas, did lead to a sample from outside England being sent in. This was from Perthshire, Scotland and it was infected with SBCMV. Future studies with access to samples from the other areas of the UK would be interesting, particularly Scotland, which historically had been assumed to have fewer cases of viruses due to the colder climate. It may be that soil-borne viruses are more prevalent in this area than insect transmitted viruses due to the climate; however, with rising temperatures this may change in the future.

The Defra winter wheat disease survey samples were collected at the end of the growing season, which is the standard method used annually, decided upon by the project manager. This may have allowed any viruses within the wheat to build to

high titres, thus aiding detection. Conversly, the wheat defence system could have become effective for example by the production of small interfering RNA (siRNA) (Kreuze and Cuellar, 2011). This would mean that the target RNA was cleaved into smaller fragments which may have reduced the chance of detection by qRT-PCR if the cleavage was in the region of the primer and probe site. The leaves of the wheat plants were sampled, in accordance with previous studies of this type (Budge *et al*., 2008). Additionally, a virus would likely have become systemic in the growing season. For example, in experiments in which viruliferous aphids were exposed to one leaf of a wheat plant (using a plastic cage) for different periods of time before being excised and used as feeding material for new virus free aphids in the prescene of a healthy barley plant, infections were confirmed in the barley plants using visual symptoms after just twelve hours (Jensen, 1973). Additionally, S*oil-borne wheat mosaic virus* in wheat moves between cells via plasmodesmata until it reaches the phloem, where long distance movement occurs, thereby distributing the virus in the roots, stem and leaves (Verchot *et al*., 2001). The virology department at Fera rountiely test for soil-borne viruses of wheat such as SBCMV by sampling the leaves. Therefore it seems likely that any viruses present in the wheat samples would have been detected by sampling the leaves. It is possible that the wheat samples tested had levels of resistance to viruses, as is the case with some varieties of wheat which have genes such as *Sbm1* and *Sbm2* which limit replication and movement of virus particles within the SBCMV resistant plants (Bayles *et al*., 2007). This would have meant that such viruses were not detectable by qRT-PCR. Resistance to insect transmitted virus such as BYDV is not included in breeding strategies because according to the breeder DSV wheat, there is competition from the chemical industry which strives to develop products to control the insect vectors (Michael Koch, DSV Wheat Ltd, personal communication). Therefore this is unlikely to be a major cause for the lack of BYDV

detection unless unknown resistance occurred. However, Field (2013) states that while chemicals such as pyrethroids while were effectively controlling vectors of BYDV such as *S. avenae* up to the year 2011, it appears resistance is developing, raising concerns for 2014 and the future (Web reference – Field). Therefore this could explain the lack of BYDV in the earlier years of the project with more cases being detected in 2012.

While the specific information about each sample in the Defra winter wheat survey with regard to chemical applications is unavailable due to confidentiality it is likely that the vast majority of samples received some form of insecticide. In addition the majority of samples from other sources received some form of insecticide. This suggests that insects, which are vectors of many viral diseases such as BYDV were under some level of control therefore reducing the level of spread of viruses, contributing to the low number of viruses found with insect transmitted viruses in the study.

3.5 Conclusion

A large scale survey of wheat for twelve viruses, which had been found in wheat or other Gramineae in the UK, was carried out successfully. The viruses that were found were BYDV-MAV, BYDV-PAV and SBCMV. It is not surprising that these viruses were found as they have been found in the UK in the past (see Section 1.11.1.3 and 1.11.1.12), are the only viruses of wheat that a selection of farmers at the Cereals industry event whom I spoke with were aware of, and they are also the only viruses in wheat for which the virology department of Fera commonly test. All three viruses can cause considerable yield loss, for example up to 50% due to SBCMV in the UK (Clover *et al*., 1999a), and are therefore a threat. Following a study in 2008, Budge *et al.* (2008) strongly recommended that resistance to SBCMV be incorporated into breeding strategies. This has now become the case for some breeders such as DSV,

United Kingdom Ltd and Limagrain who use the genes *Sbm1* and *Sbm2* which have been identified as having a role in resistance to SBCMV, for selection of new varieties (Bayles *et al*., 2007; Matthew Kerton, DSV United Kingdom Ltd and Edward Flatman, Limagrain, personal communications). This study confirms that SBCMV is present in the UK, and supports the recommendations made by Budge *et al*. (2008) especially as climatic conditions may become more favourable for the spread of the virus in the future. The relatively low prevalence and geographic spread of BYDV and SBCMV does not suggest that these viruses are a major contributor to the current plateau in the yield of wheat in the UK. During the period of testing of this survey the prevalence of viruses has increased. If this trend continues it would pose an increasing threat to the yield of UK winter wheat. Overall it is likely that a combination of unfavourable weather conditions, good control of insect vectors and breeding for SBCMV resistance caused there to be such low numbers of samples in which viruses were detected. However, it is possible that the 'wrong' viruses were tested for in this study and that other viruses, which were not tested for are responsible for the plateau. These viruses may include novel, currently unknown viruses (hence they could not be tested for using qRT-PCR), these were tested for in Chapter 4.

Chapter 4 - Using next-generation sequencing technology to search for novel wheat viruses

4.1 Introduction

It is proposed that novel, currently unknown viruses could have infected wheat and therefore be responsible for the plateau in the yield of wheat (see Section 1.11). This was suggested because an extensive screen of wheat in the UK for native viruses did not reveal that they were prevalent at high levels (see Section 3.5), therefore these are unlikely to be causing the plateau.

While target designed applications such as PCR, qRT-PCR, ELISA, and TEM are valuable for diagnosis of certain viruses, they are inherently biased to their target and require prior knowledge of it. Next generation sequencing technologies, such as pyrosequencing used here, provide an opportunity to look for novel and as yet unknown viruses, with the advantage that any viruses present are equally likely to appear in results, as compared to TEM where the most easily identifiable are detected, leaving some potentially overlooked (Adams *et al*., 2009).

One hundred and twenty samples comprising of 48 wheat, 38 natural weeds, 24 mown perimeter samples and the contents of 10 insect traps (hereafter referred to as wheat, weeds, mown and insect samples respectively) were investigated using next-generation 454 pyrosequencing technology.

The weeds and mown plants were sampled in addition to wheat because they could have been acting as reservoirs of viruses, which could eventually pass to wheat. Insects are known to be vectors for numerous virus diseases of plants (see Section 1.11), therefore screening them could reveal viruses that may already be in the wheat crop or could transfer to it.

RNA samples were used because the vast majority of viruses that infect plants have RNA genomes (Roossinck *et al*., 2010). The method chosen was that of Cox-Foster *et al*. (2007) and Margulies *et al*. (2005). It involved the production of cDNA from RNA using random primers. Using cDNA rather than genomic DNA had the advantage that only the active host genes that are transcribed, ribosomes, viruses, viroids and the RNA stages of actively replicating DNA viruses would have been sequenced, while avoiding the large amount of un-transcribed genomic DNA in the samples (Adams *et al*., 2009). Therefore RNA, and to an extent DNA viruses could be detected if present.

4.2 Materials and methods

4.2.1 Sample collection

One hundred and twenty samples (48 wheat, 38 weeds, 24 mown and 10 insect) were collected from in and around an organic wheat field (Wakelyns Agroforestry, Metfield Lane, Fressingfield, Eye, Suffolk IP21 5SD), in July 2011. Samples were collected from all regions of the field to give a complete representation of the area (see Figure 4.8).

The majority of the main field was planted with a mix of Hereward, Solstice and Spark wheat (mixed before sowing). The synthetic hexaploid, Einkorn, Alkor and Col-122 wheat plants were interspersed and were sampled in the same way as the majority of the wheat samples. Two wheat samples were collected from each row, with the distance apart varying to allow maximum coverage of the field. The type of wheat, positioning of it, and the management of the weeds (types were those naturally occurring) was decided by the site owner before discussions about this work began.

The perimeter of the field was divided into twelve regions and weeds were collected based on what was encountered first on reaching the area, with the aim of

sampling as many species of plant as possible. The HGCA Encyclopaedia of Arable Weeds (Web reference – Encyclopaedia), internet searches and advice from colleagues at Fera were used to identify the weeds.

Pit and pan insect traps were used at each sampling point to target a range of insects. Pit traps are likely to catch ground dwelling insects and pan traps (which were set just under the level of the top of the wheat plants) primarily would catch flying insects.The duration of the collection period was one week. The insect traps contained 200 ml 0.5 M EDTA. The decision to use this solution was made following the study detailed in Section 4.2.2.

4.2.2 Investigating storage regimes for insect traps for the preservation of insect and viral RNA

4.2.2.1 Introduction

Insect mRNA and RNA viruses are unstable; therefore experiments to monitor their presence require extensive planning to manage their preservation. The aim of this study was to determine which solutions (if any) should be put into pan and pit insect traps before being set, to facilitate good recovery of polymerase chain reaction (PCR) amplifiable RNA. The outcome would be used to inform the methods used in the main study in Chapter 4.

RNA folds into complex structures that are vital for it to perform its biological functions. It is known that the solvent the RNA is in contributes to the electrostatic charges that influence the stability of the RNA (Misra and Draper, 2000).

As insects and nematodes are vectors of viruses of wheat, traps were to be set to capture them for use in next generation sequencing (see Section 1.11; Benkovics *et al*., 2010; Westwood and Stevens, 2010). The model of BYDV-PAV which is transmitted by *S. avenae* was chosen because stocks of BYDV-PAV infected aphids were readily available at Fera, and this virus was relevant and likely to be present in

field samples as it had been detected in wheat in the UK previously (Tanguy and Dedryver, 2009).

4.2.2.2 Establishing a set of BYDV-PAV infected insects and natural variation (baseline) testing

Sitobion avenae were fed on wheat plants that were infected with BYDV-PAV (infection confirmed by CTAB extractions of plant material (see Section 2.1 and Appendix 13) (data not shown) and qRT-PCR testing with an assay for BYDV-PAV (see Table 3.3). To maintain the virus, new plants were introduced every week alongside the existing ones (old and dead plants were removed every four weeks). This cycling had begun several years previously so that there were therefore a range of life stages of *S. avenae* present. Aphids that were at late instar stage or later were randomly selected for use in the experiments. It is suggested that size, sex, age, aptera or alate stages could affect the viral load and ability to transmit viruses of the individual aphid, however the aim here was to study RNA recoverability in general from insects; therefore, a mix was used (Froissart *et al*., 2010; Parizoto *et al*., 2013; Larissa Collins, Fera, personal communication). The person in charge of maintaining the system stated that it took two weeks for new born *S. avenae* to reach late instar which coupled with the fact that *S. avenae* can acquire BYDV-PAV in just 30 minutes means that all aphids selected were very likely to be carrying the virus (Gray *et al*., 1999; Stephen Forde, Fera, personal communication). This was because while a range of life stages were required it was deemed necessary for these experiments that all aphids should contain virus, to be able to monitor storage. For each reaction Mastermix A and the standard PCR cycle was used (see Appendix 18 and Section 2.3)

Total nucleic acid was extracted from five lots of 10 fresh *S. avenae* using Chelex 100 (Biorad). The 10 insects were placed in a 0.5 ml tube, sterile nuclease free water (50 µl) was added to each tube and the contents were ground using a sterile

micropestle for 20 seconds. Chelex suspension (50 µl) (25 µl Chelex resin and 25 µl nuclease free water) was added to each tube before vortexing briefly, incubating at 95°Cfor 5 minutes and centrifuging in a table top centrifuge at maximum speed for 5 minutes. The top layer was removed and transferred to a sterile tube - this was the extract. The five samples were tested using qRT-PCR assays for the 18S rRNA gene ((Applied Biosystems) an insect internal control gene) and BYDV-PAV.

4.2.2.3 Testing storage regimes

Storage regimes trialled were: dry (no solution); DEPC treated nuclease free water; 100% acetone; 100% hexane; 100% ethanol; 100% methanol; CTAB (see Appendix 13); phosphate buffered saline (PBS) (see Appendix 17); Solution A (10mM trisaminomethane, 10 mM EDTA and 0.05% sodium dodecyl sulphate); 0.5 M EDTA; RNA later (Applied Biosystems) (an aqueous, non toxic solution which quickly permeates tissues to preserve them - no further details available from manufacturer) and RNA stabiliser (Qiagen) (unknown composition).

4.2.2.4 Rationale for regime choices

Table 4.1 shows the rationale behind the choices of regimes trialled in this study.

Table 4.1. The rationale behind the choices of the storage regimes for insects traps tested for their ability to preserve RNA.

Storage regime	Rationale for inclusion in the study
Dry and water	Negative controls, to see if more complex storage regimes are required. Used in Stevens *et al*. (2011).
Acetone	Known to kill insects and for being relatively environmentally safe (Pourmirza, 2006)
Hexane	Used to extract oil and grease contaminants from soil and water (Web reference - TemaNord)
Ethanol	Considered the ideal 'killing and preserving' medium for insects for later molecular work (Web reference – Ars)
Methanol	Kills insects and is relatively harmless in the environment (Ogunleye and Adefemi, 2007).
CTAB	Used in many downstream nucleic acid extractions, introduction during collection would streamline the method
PBS	A general laboratory buffer for plant diagnostics and used in Stevens *et al*. (2011).
Solution A	Recommended by Theodore Allnutt, Fera, personal communication
EDTA	A chelating agent - collects and removes RNAses from nucleic acid samples
RNA later and RNA stabiliser	Ready to use commercial options

Three repeats of each regime were set up in autoclaved 100 ml beakers, with liquid regimes containing 30 ml of each solution. Fresh batches of 10 *S. avenae* (infected with BYDV-PAV) were added to each beaker. A fine mesh was secured over the beakers. Beakers were placed outside, with half the beaker buried in the ground, amongst wheat cv. Einstein plants that were at growth stage 75 (see Figure 4.1). Liquid level data was collected after three and seven days at which point the beakers were removed to the laboratory. Rainfall data were collected during the experimental period. Chelex extractions of nucleic acids (detailed previously) were then performed on the *S. avenae* in all beakers separately. Real time reverse transcription polymerase chain reaction assays for 18S rRNA and BYDV-PAV were used for all samples.

Figure 4.1. An example of test beakers in the field, during the trial of storage regimes for recovery of RNA from insects.

4.2.2.5 Statistical analysis

A multivariate analysis of variance (MANOVA) was run on the Ct and ΔRn values produced from the BYDV-PAV and 18S rRNA assays which were used to test the insects from each beaker. This was to investigate whether there was evidence of an overall effect of the regime when looking at all four variables together. Analysis of variance (ANOVA) tests were then run on each individual data set (BYDV-PAV and 18S rRNA, Ct and ΔRn values). This was to examine for which of these variables the regimes differed as well as which (if any) regime was found to be significantly better than the others for those variables.

4.2.2.6 Establishing a set of BYDV-PAV infected insects and natural variation (baseline) tests

The results of the 18S rRNA assays for the five lots of insects ranged from Ct 14-18 and ΔRn 0.6-0.7. For the BYDV-PAV assays the results ranged from Ct 21-24 and ΔRn 0.85-1.

4.2.2.7 Testing storage regimes

After three days in the field acetone, hexane, ethanol and methanol had evaporated completely, while the other solutions had decreased to an extent. By the end of the experiment, all beakers contained liquid. For those mentioned as having completely evaporated and the dry regime, this would have been due to the rainfall of approximately 4.8 ml during the period (see Figure 4.3). The liquid in the beakers containing water remained at approximately the same level throughout. PBS and EDTA remained at a relatively high level until three days had passed, and by seven days there was likely a mixture of the original solution and rainwater (see Figure 4.2).

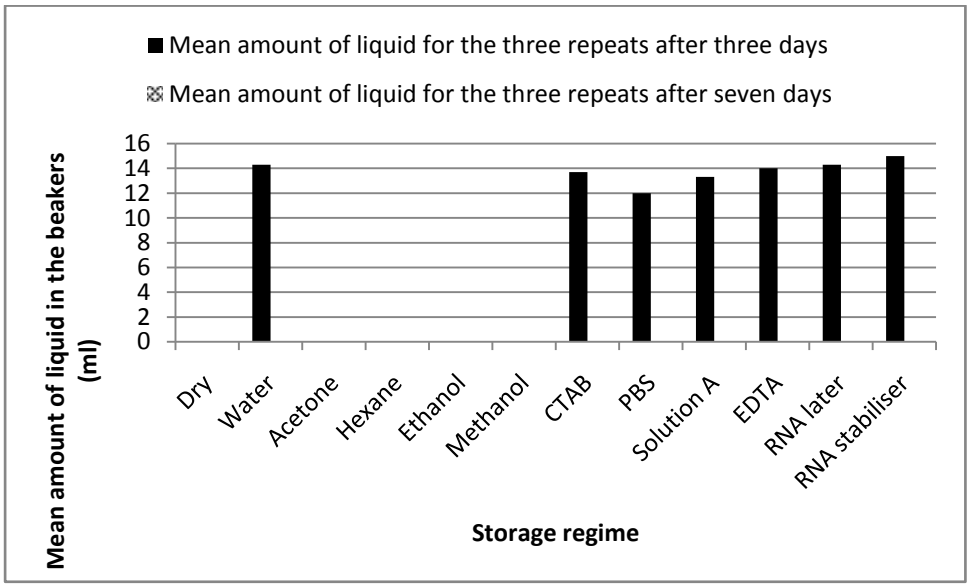


Figure 4.2. Mean amount of liquid remaining in the beakers for each storage regime, after three days (black bars) and seven days (hatched bars) in the field.

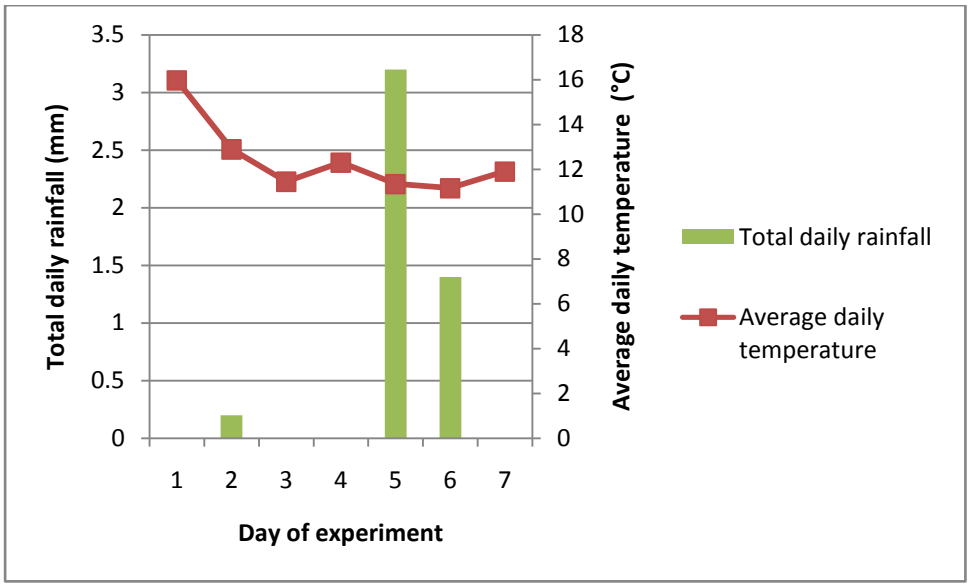


Figure 4.3. The total daily rainfall and the average daily temperature during the experimental period.

Figures 4.4-4.7 show the results of qRT-PCR assays for BYDV-PAV and 18s rRNA in terms of cycle threshold and ΔRn values, using samples from all storage regimes after one week in the field.

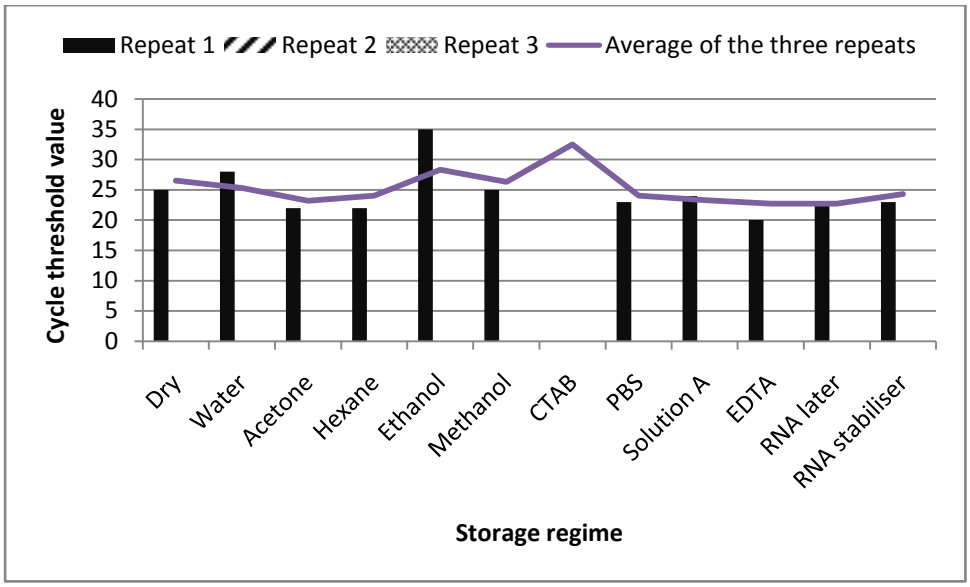


Figure 4.4. Cycle threshold values for a qRT-PCR assay for BYDV-PAV for the three replicates of each storage regime tested (bars) and average values for each regime (line).

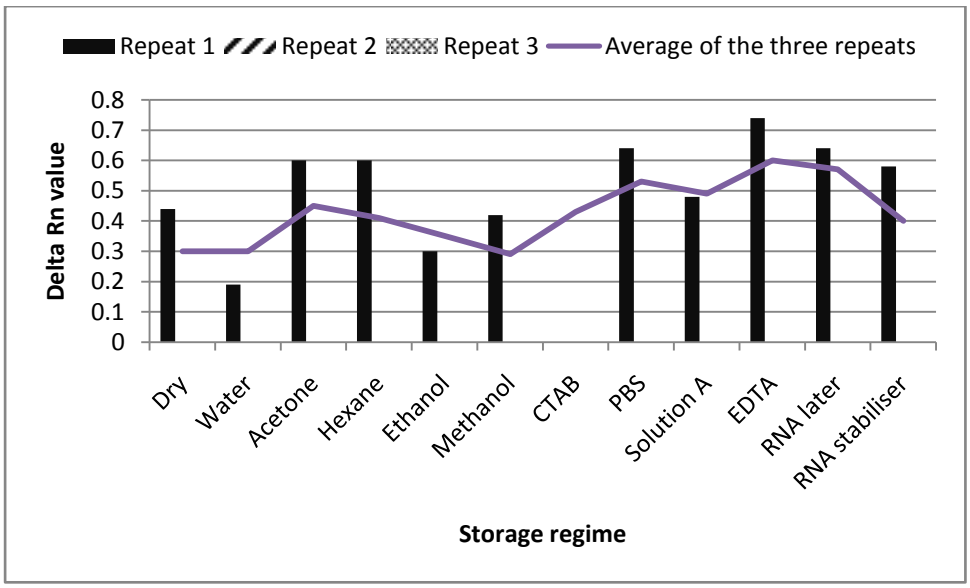


Figure 4.5. Delta Rn values for qRT-PCR assay for BYDV-PAV for the three replicates of each storage regime tested (bars) and average values for each regime (line).

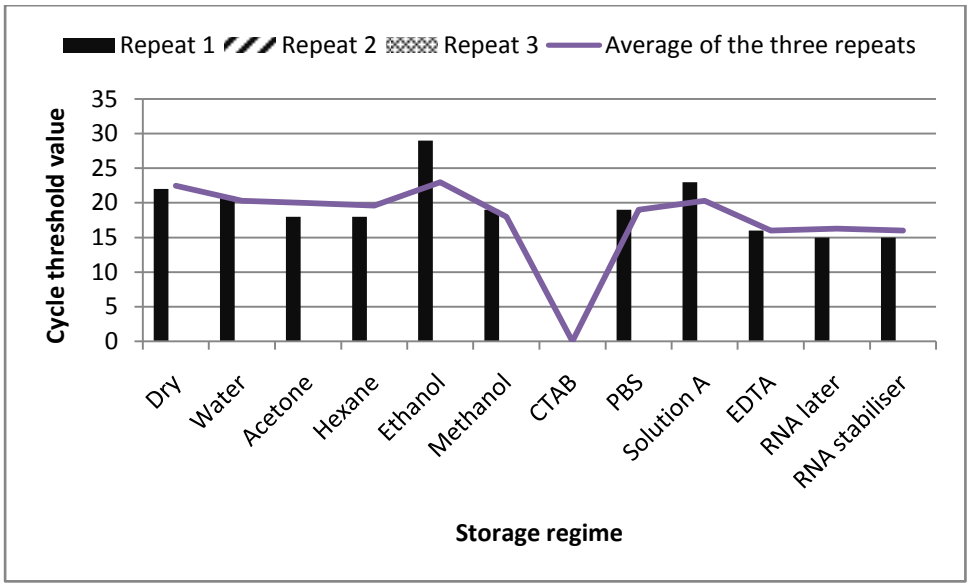


Figure 4.6. Cycle threshold values for a qRT-PCR assay for the 18S rRNA gene for the three replicates of each storage regime tested (bars) and average values for each regime (line).

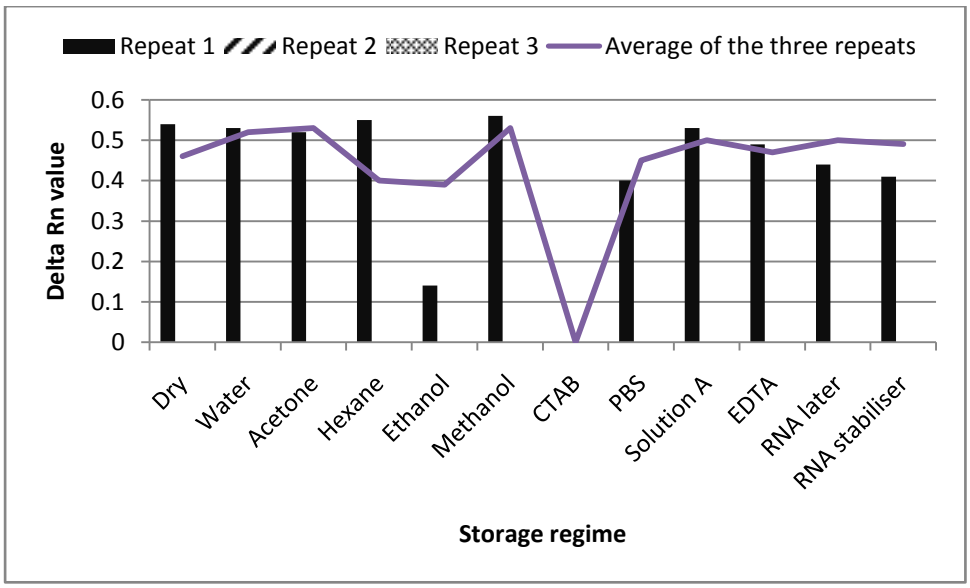


Figure 4.7. Delta Rn values for qRT-PCR assay for the 18S rRNA gene for the three replicates of each storage regime tested (bars) and average values for each regime (line).

4.2.2.8 Statistical analysis

The results of the MANOVA suggested that there was an effect of the regime used when looking at all four variables together (variables being BYDV-PAV and 18S rRNA, Ct and ΔRn values) ($F = 1.58$; df = 40,70, $p = 0.046$). Further ANOVAs showed that while there was no evidence of any significant difference between the solutions for either of the ΔRn results (BYDV-PAV – $p = 0.20$; 18S rRNA - $p = 0.63$), there were significant differences between the Ct results (BYDV-PAV –$p = 0.003$; 18S rRNA –$p = 0.01$).

4.2.2.9 Discussion of results

Initial experiments to investigate whether there was natural variation in the levels of 18S rRNA and BYDV-PAV which could be recovered and detected in the qRT-PCR assays, showed that there was limited variation, because all five sets of insects produced similar results. Therefore the results of further testing of storage regimes were likely to be due to the differences in storage regimes, but if the differences between storage regimes were within approximately 3 Ct values or 0.1 ΔRn, it could be that natural variation was contributing to the result. This fact was used when analysing data to interpret whether differences in regimes were due to the regime or natural variation.

It is important to select a storage regime that remains in the trap for as long as possible, so that it can preserve the contents of the trap. There was loss of all storage solutions due to evaporation during the experiment. The temperature was variable during the experiment and after day four the temperature was lower than at the beginning of the experiment, possibly lowering evaporation rates. However, all temperatures would have been high enough for solutions such as ethanol to evaporate. Water remained at the highest level (45% of the starting volume) with PBS and EDTA also remaining at high levels (18.3% and 15% respectively). There was

dilution of all samples by rainwater (see Figures 4.2 and 4.3). Figure 4.2 shows that acetone, hexane, ethanol and methanol were not present in the beakers after three days, due to evaportation. However liquid was present when levels were assessed after seven days, Figure 4.3 shows that the rainfall on days 5 and 6 was the source of the liquid. The rain which fell on day 2 is likely to have evaporated before the liquid level assessment on day three as there was a small amount of liquid and the temperature was relatively high on day 2.

Barley yellow dwarf virus-PAV from insects stored in RNA later was consistently detected earlier than those in all other storage regimes, as the cycle threshold values were the lowest. BYDV-PAV from insects stored in acetone and EDTA were also detected relatively early in the PCR cycle (mean Ct value 23 for both solutions). In addition these top three regimes gave results within natural variation limits, suggesting limited differences between them due to regime (see Figure 4.4). The greatest amount of amplification (delta Rn) of BYDV-PAV was in insects that were stored in 0.5 M EDTA, as there was the greatest change in Rn value compared to other storage regimes. This also suggested that EDTA storage enabled a nucleic acid extract with the least inhibitors of PCR to be produced. RNA later and PBS also led to high levels of amplification of BYDV-PAV. The differences in the top three were also within natural variation limits set as a result of the baseline testing (see Figure 4.5).

The 18S rRNA was consistently detected earlier in insects which had been stored in 0.5 M EDTA than any other storage regime, as all replicates had lower cycle threshold values. RNA later and RNA stabiliser also allowed early detection of the gene and was within the natural variation limit in relation to EDTA (see Figure 4.6). The greatest delta Rn when tested with the 18S rRNA assay was in insects that were stored in 100% acetone and 100% methanol, as there was the greatest change in Rn value compared to other storage regimes. DEPC treated nuclease free water also led

to high levels of amplification of the gene. The top three regimes were within natural variation limits of each other (see Figure 4.7).

It is clear that no storage regime was the best for both assays (see Figures 4.4-4.7). However, 0.5 M EDTA was the best solution in terms of delta Rn of BYDV-PAV and cycle threshold of 18S rRNA, as well as being the third best solution in terms of cycle threshold value for BYDV-PAV. While not being one of the best for delta Rn values for the 18S rRNA gene, it did provide satisfactory results; it also remained at relatively high levels in the beakers during the trial, which is important for the insects to be able to be preserved throughout the collection period.

The statistical analyses that were carried suggested that while none of the regimes were significantly better than the others, there were significant overall differences in Ct values between the regimes. Figures 4.4-4.7 show that of all the regimes 0.5 M EDTA had the lowest Ct values for both assays and the highest ΔRn value for 18S rRNA (although ΔRn values were not significantly affected by regime). Therefore, in conclusion 0.5 M EDTA is the best storage regime to use overall.

4.2.2.10 Other considerations

There are considerations other than performance when selecting a solution to use in a natural experimental setting. The solution must not be toxic to the environment including animals, plants and humans and disposal must be practical and safe. Used at 0.5 M EDTA is suitable for use in such a setting when used responsibly and it is routinely used in many household products such as shampoo (Sigma Aldrich). Insect traps can be large and require large quantities of storage solution, therefore financial cost must be considered, and 0.5 M EDTA is a relatively cheap solution, certainly when compared the other storage regimes trialled such as RNA later.

4.2.2.11 Discussion of methods

It is possible that there are other storage regimes which would preserve the insects and their RNA better than those compared in this study. For example 'Solution 21' which was used in the Rothamsted insect survey to preserve the contents of suction traps. However, this solution is no longer used in the survey and tests into its usefulness in preserving RNA were never carried out (Richard Harrington, Rothamsted insect Survey, personal communication). Anstead *et al*. (2008) remarked that 'Solution 21' did not preserve DNA in a suitable state for use in PCR. Therefore it is thought that due to the less stable nature of RNA compared to DNA it is unlikely that it would be successful in preserving it in a suitable state for PCR amplification (Richard Harrington, Rothamsted insect survey, personal communication).

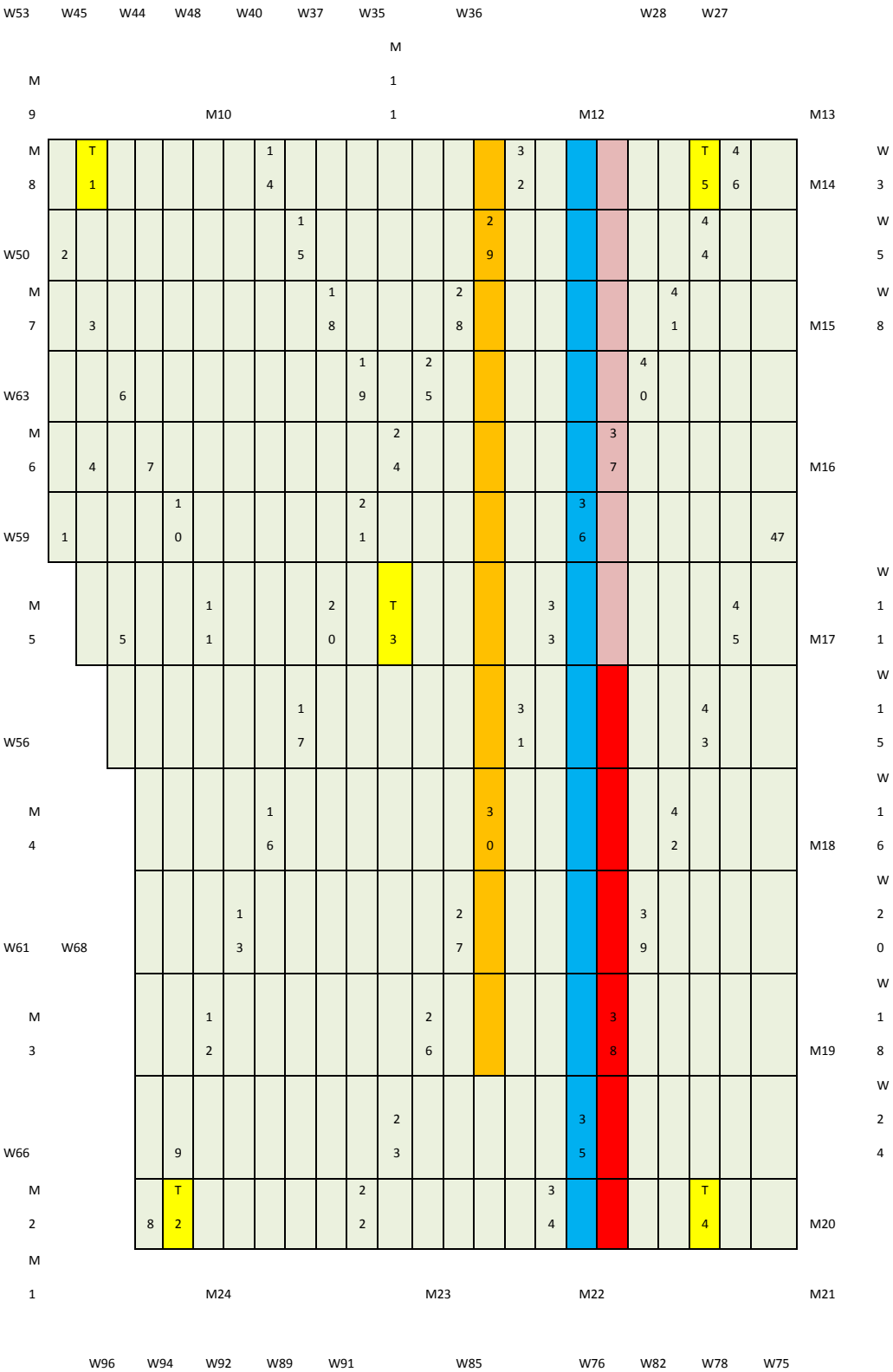
Rainwater entered the beakers during the study, This was a natural occurrence which is very likely to happen during outdoor insect trap studies; therefore this was not considered to be a problem; in fact, studies in a laboratory under more controlled conditions or covering the area rather than it being open to the air would have produced results that were less applicable to the outside environment. However future trials could repeat this experiment during different periods of weather and perhaps under shelter to observe how the results may change. The wheat crop which was the subject of the main experiment was due to be harvested at the end of August 2011. Therefore, these preliminary experiments had to be done prior to that, meaning they were done in July. This also meant that the experiment could be run only once, including the initial natural variation baseline tests which were also run once. However, it would be beneficial to repeat the experiments in the future to provide repetition and therefore more accurate results with conclusons that one can be more confident in. However, this study which

involved a short piece of preliminary work, provided a conclusion which was suitable for use as proven by the recovery of viruses from insects (see Section 4.3.3).

4.2.2.11 Conclusion

In conclusion, considering all factors, 0.5 M EDTA was the best storage solution to use in pit traps positioned between wheat plants at growth stage 75-100, during August 2011 in a natural environment in the UK in an experiment lasting for 7 days. This allows good recovery of insect and viral RNA that can then be amplified by PCR. This result was used to inform the main experiment in this chapter.

Figure 4.8. Sampling map for the study. The field was 60m in length and 48m in width (at the top). The length of the squares corresponds to 4.6m and the width to 2m, and they represent the rows of crops. Wheat samples are shown as numbers alone. Mown samples are shown as M(number). Weed samples are shown as W(number). Insect trap points (pit and pan trap together) are shown as T(number) and are highlighted in yellow. A synthetic hexaploid wheatis shown as Einkorn wheat is shown as Alkor wheat is shown as Col-122 wheat is shown as *Weed sample identification:* 3- scented mayweed (*Matricaria chamomilla*), 5- rough stalked meadow grass (*Poa trivialis*), 8-timothy, 11- redshank (*Persicaria maculosa*), 15- common hemp (*Galeopsis tetrahit*), 16-blackgrass (*Alopecurus Myosuroides Hunds*), 18- field forget-me-not (*Myosotis arvensis (L.) Hill*), 20- hedge mustard (*Sisymbrium officinale*), 24- scarlet pimpernel (*Anagallis arvensis*), 27- common nettle (*Galeopsis tetrahit*), 28- clover (*Trifolium repens*), 33- yorkshire fog (*Holcus lanatus L. Occurrence*), 36- buttercup (*Ranunculus repens*), 37- greater plantain (*Plantago major*), 40- yorkshire fog,44- dove's-foot cranesbill (*Geranium molle*), 45- blackgrass, 48- annual meadow grass (*Poa annua L. Occurrence*), 50- cow parsley (*Anthriscus sylvestris*), 53- creeping thistle (*Cirsium arvense*), 56- broad-leaved dock (*Rumex obtusifolius*), 61- linseed (*Linum usitatissimum*), 63- common nettle (*Urtica dioica)*, 66- smooth meadow grass (*Poa pratensis*), 68- scented mayweed, 75- hedge mustard, 76- field forget-me-not, 78- yorkshire fog, 82- pale persicaria (*Persicana lapaythifolia*), 83- stickyweed (*Galium Aparine*), 85- cocksfoot, 89- smooth sow thistle (*Sonchus oleraceus L. Occurrence*), 94- loose silky bent (*Apera spicaventi*) and 96- crested dog's-tail.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M10
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M12
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M20
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1
M24
M23
M22
M21
W96 W94 W92 W89 W91 W85 W76 W82 W78 W75

4.2.3 Sample preparation

4.2.3.1 Extraction of RNA

Total nucleic acid was extracted from all samples using the standard CTAB extraction method (see Section 2.1 and Appendix 13). For plants, 300 mg of sample was shaken in a bead beater (Sigma Aldrich) with 2 ml CTAB grinding buffer and 10, 0.6 mm and 10, 1 cm acid washed glass beads. For insects, a 5 ml tube was filled with the strained contents of a trap (specific insects unidentified, see Section 4.4.7.12), 3 ml CTAB grinding buffer and one 1.5 cm diameter stainless steel ball bearing was added before shaking in the paint shaker. Some samples that failed the later quantification tests were extracted again using an RNeasy plant mini kit with QIAshredder following manufacturer's instructions (Qiagen).

The extract was passed through an RNeasy column with on-column DNase digestion (Qiagen, following manufacturer's instructions). Quantification was carried out using a spectrophotometer (Nanodrop ND-1000, ThermoScientific) and a fluorometer (Qubit machine and Qubit-iT^{TM} RNA HS kit, Invitrogen). Samples above 5 µg were used in the next steps of preparation, as this is the recommended lower limit (Rose Souza-Richards, the University of Nottingham, personal communication).

4.2.3.2 Production of cDNA

Complementary DNA was prepared for each RNA extract by adding up to 5 µg of sample extract to 0.5 µl (100 µM) Foster Rand primer (5'-GTTTCCCAGTAGGTCTCNNNNNNNN-3'), 0.5 µl (100 µM) Foster Tag dT primer (5'-GTTTCCCAGTAGGTCTCTTTTTTTTTTTTTT-3'), 1 µl dNTPs (10mM) and making up to a total of 13 µl with DEPC treated nuclease free water.

The mixture was incubated at 65°C for 5 minutes, followed by ice for 1 minute. Four microlitres of first strand buffer (Invitrogen), 1 µl DTT (Invitrogen) and 1µl Superscript (Invitrogen) were added. The following incubation steps were then

performed - 25°C for 5 minutes, 50°C for 90 minutes, 70°C for 10 minutes and 4°C for ∞. On removal, 2.5 units of RNaseH (Fermentas) were added followed by incubation at 37°C for 20 minutes.

Polymerase chain reaction amplification was done by mixing the following: 10 µl first strand cDNA, 5 µl standard Advantage II buffer (Clontech), 1 µl (10 mM) dNTP, 1 µl Advantage II Taq (Clontech), 2 µl primers (from stock: 10 mM Foster MID 1-12 and 1 mM Foster Tag Rand) (samples were split into 10 batches of 12, within each batch each sample was tagged with a different multiplex identified (MID) (see Table 4.2). The samples were randomly allocated to a batch. Batches were used because of financial and time constraints, in addition to pyrosequencer availability which meant that it was not possible to have a single sample per run of the pyrosequencer, therefore a compromise of 12 samples per run was reached)) and 31 µl water.

Table 4.2. Sequences of the MIDs used to identify samples when mixed together in batches.

MID	Sequence (5'-3')
1	ACACGACGACT
2	ACACGTAGTAT
3	ACACTACTCGT
4	ACGACACGTAT
5	ACGAGTAGACT
6	ACGCGTCTAGT
7	ACGTACACACT
8	ACGTACTGTGT
9	ACGTAGATCGT
10	ACTACGTCTCT
11	ACTATACGAGT
12	ACTCGCGTCGT

Polymerase chain reaction cycling as follows was performed: 94°C for 10 minutes, then 5 cycles of (94°C for 30 seconds, 25°C for 30 seconds and 72°C for 90 seconds)

followed by 40 cycles of 94°C for 30 seconds, 55°C for 30 seconds an 72°C for 90 seconds, 72°C for 10 minutes and 4°C for ∞.

Quantification was done with a Qubit machine and Qubit-iT^{TM} dsDNA BR kit (Invitrogen). Equal amounts (µg) of each sample were transferred to a QIAquick column to give a total amount of 3 µg (Qiagen) for PCR purification before eluting in 20 µl TE buffer, following manufacturer's instructions. The double stranded cDNA was used according to manufacturer's protocols to produce a cDNA library (454-Roche GS FLX Titanium Series (October 2009) cDNA Rapid Library Preparation Method Manual from manual Section 3.2.4) followed by emulsion PCR according to manufacturer's protocols (454-Roche GS FLX Titanium Series (October 2009) emPCR Method Manual – Lib-L SV). Each batch of samples was sequenced on 1/8th of a picotitre plate by a 454 GS-FLX+ (Roche) according to manufacturer's protocols (Roche, Sequencing Method Manual for the GS FLX+ Instrument (August 2011) (Web reference – 454)).

4.2.4 Sequence analysis

Filtered (<20 quality and <100 bp in length data was removed) fastq data was split by MID; MID sequences were removed and reads were labelled individually. The data was then split according to group (wheat, insect, mown or weed).

Data within each group was assembled into contigs using Newbler v 2.6 (Roche). Contigs and single sequences were compared to a local download of the NCBI GenBank database using BlastN and BlastX. Possible taxonomy of results was assigned and organised using MEGAN 4.70.4 (Huson *et al*., 2007).

Plant or insects viruses were of interest for this study and were investigated further by looking at their Blast results. As well as nodes labelled as virus, the roots of the trees were also examined because this is where sequences with distant relationships to each other were assigned. This was of interest because mis-

labelling of entries to GenBank from expressed sequence tag screens of plants which happen to have been infected with virus can cause viral sequences to be identified as both plant and viral in origin therefore they cannot be separated to a greater level than the root, meaning they are deposited there. The homology, based on identity and length of the homologous sequence was examined, to assess the quality of the similarity to a virus. The length of the read/contig in terms of nucleotides was considered in relation to the length of any homologous regions to viruses, bearing in mind that homology to proteins would be reduced by a third after translation. The genus of homologous viruses was analysed and compared to other samples. In such cases where there were relationships between the genera, further investigation by Blast X searches to suggest locations of reads/contigs within their homologous proteins and tentative genomic structures were carried out. Meta data such as host range, history of the sampling field and whether the virus had been reported in the UK was also used to analyse data. In addition, known conserved domains of the amino acids from plant viruses, such as those of the RNA dependent RNA polymerase were highlighted when encountered (Koonin, 1991).

4.3 Results

4.3.1 Sequence analysis

Statistics for the data for all batches are summarised in Table 4.3. The data were then re-organised into groups for wheat, insect, mown and weeds for further analysis. Figure 4.9 shows the number of reads produced for each sample, according to MID and batch.

Table 4.3. Summary of statistics about the data produced from the 454 pyrosequencer for each batch of samples

Batch	Number of reads produced for the batch	Number of reads which passed quality control	Range of length of reads	Average length of reads	Number of reads without a MID
1	50,997	42,615	40-600	100	6,881
2	57,248	51,992	40-500	95	3,997
3	100,759	95,377	40-500	100	6,345
4	88,230	83,935	40-500	100	5,995
5	4,416	4,176	40-520	75	572
6	48,490	47,654	40-600	100, 230 and 410	8,493
7	42,015	41,366	40-590	110, 250 and 380	5,655
8	65,617	49,988	100-600	100	17,341
9	82,511	70,472	100-600	100	11,333
10	77,631	63,452	100-650	100 and 400	9,180

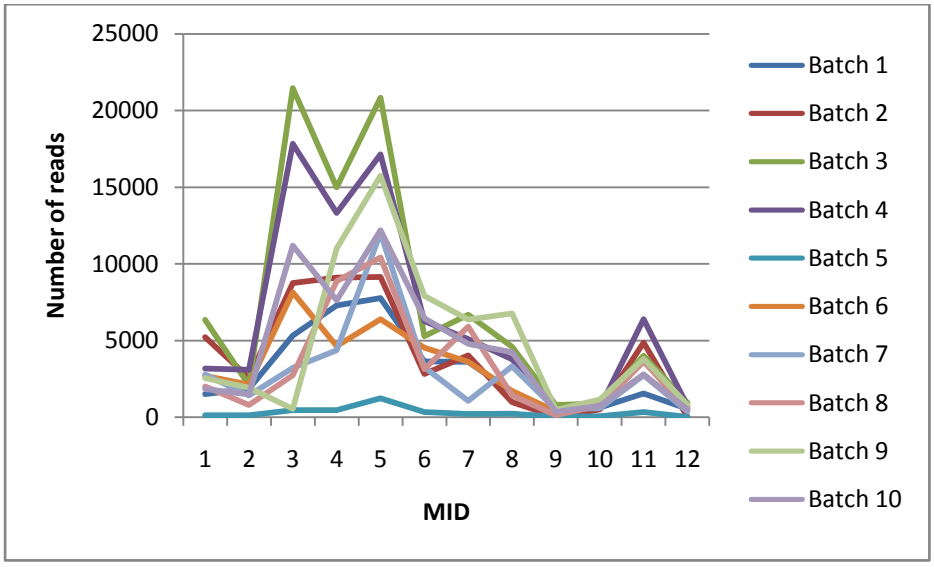

Figure 4.9. The number of reads produced for each sample (which were tagged with MIDs), for all ten batches of samples.

4.3.2 Wheat results

A total of 132,617 reads were analysed by Newbler v 2.6, and 973 contigs and 32,365 singletons were produced. MEGAN was used to assign possible taxonomy to the Blast N and Blast X results (see Figures 4.10 and 4.11). Table 4.4 summarises significant results.

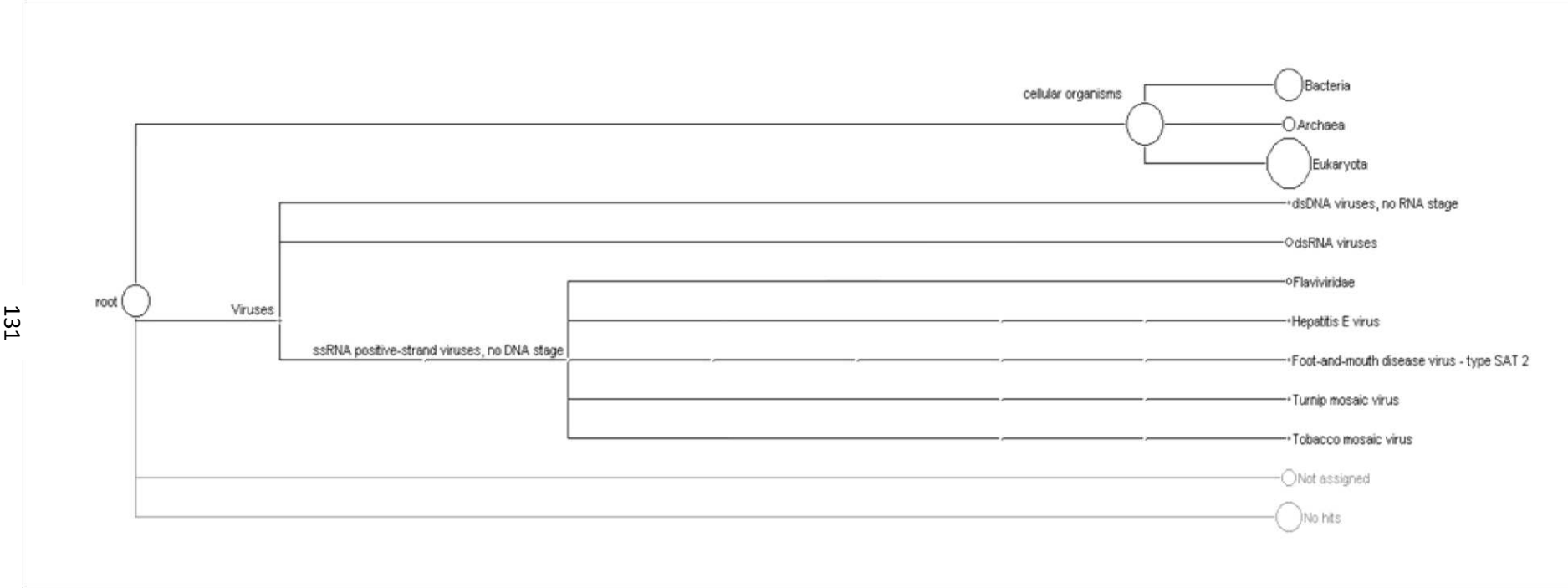

Figure 4.10. MEGAN output of the BlastN results of wheat samples.

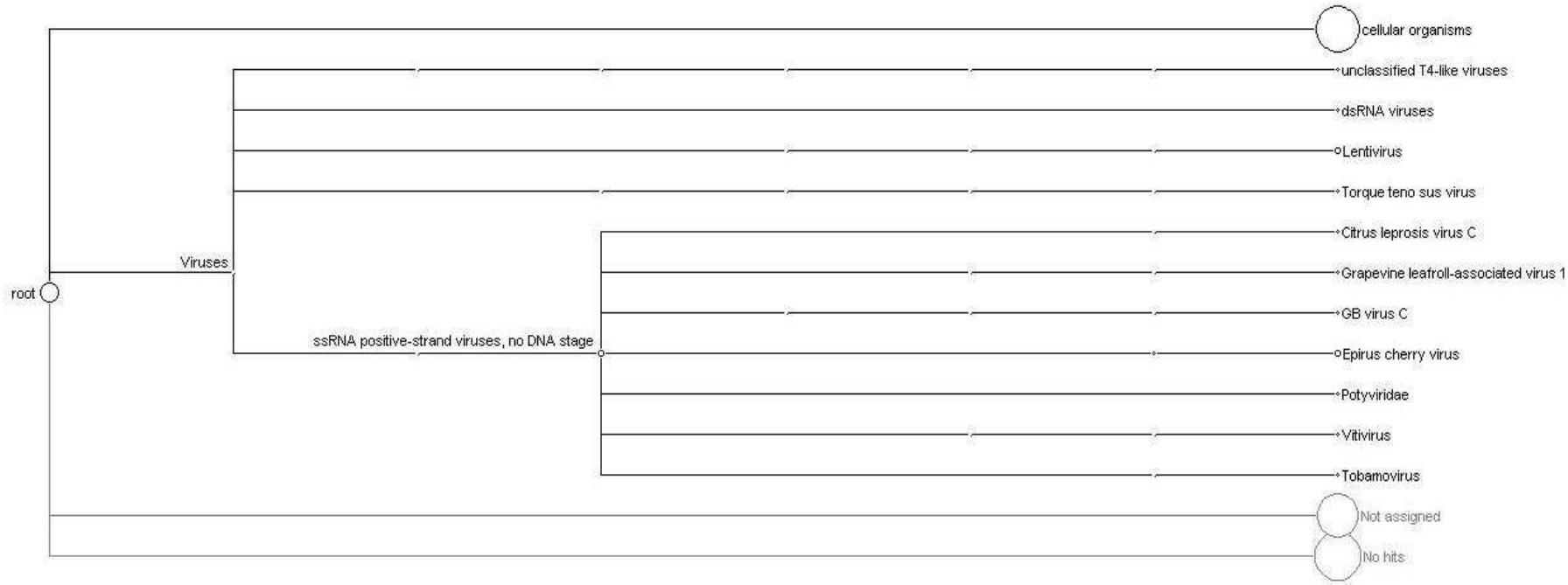

Figure 4.11. MEGAN output of the BlastX results of wheat samples.

Table 4.4. Summary of reads from wheat samples that had homology to plant viruses in the GenBank database. Standard font is used for nucleotide homology and bold font for amino acid homology. * Probable contamination.

Sample	Number of reads/contigs (length in nucleotides)	Homologous virus from GenBank	Genus of homologous virus	Identities and score
Wheat 24 *	2 reads (201 and 231)	*Tobacco mosaic virus* genome (variant 1) (V01408.1) and virus movement and coat protein of *Tobacco mosaic virus* (AF273221.1) **RNA polymerase of *Tobacco mosaic virus* (gb AAF80605.1|AF273221 3)**	*Tobamovirus*	192/201 (94%), score 302 and 216/231 (94%), score 333 **39/44 (89%), score 72.4).**
Wheat 24	2 reads (different to above, in a mixed contig with 8 other reads) (1484)	***Freesia mosaic virus* CI (Ref YP 003620393.1)**	*Potyvirus*	**31/83 (37%), score 56.6**
Wheat 16	1 read (51)	*Turnip mosaic virus* (AB252140.1)	*Potyvirus*	35/41 (85%), score 48.2
Wheat 19	4 reads (112)	**Putative RNA dependent RNA polymerase (RdRp) of *Epirus cherry virus* (gb ACF16357.1)**	*Ourmiavirus*	**14/22 (64%), score 35.4**
Wheat 1 (x1), 4 (x4), 11, (2), 24 (x5), 27 (x8), 33 (x1), 40 (x1) and no MID (x4)	2 reads as sole contributors to a contig (contig1) (185)	**Putative RNA dependent RNA polymerase; RdRp of *Citrus leprosis virus C* (ref YP 654568.1)**	*Cilevirus*	**18/43 (42%), score 36.2**
Wheat 10	1 read (321)	**125 kDa replicase of *Brugmansia mild mottle virus* (YP 001974324.1)**	*Tobamovirus*	**24/47 (51%), score 44.3**
Wheat 15	1 read (468)	**Helicase of *Mint vein banding-associated virus* (AAS57938.3)**	*Closterovirus*	**19/46 (41%), score 38.9**
Wheat 36 (a)	1 read (296)	**Putative second envelope polyprotein of *GB virus C* (AAC58133.1)**	*Hepacivirus*	**15/36 (42%), score 35.0**
Wheat 36 (b)	1 read (different to above) (456)	**Replicase of *Grapevine virus B* (ABU62819.1)**	*Trichovirus*	**27/71 (38%), score 47.0**
Wheat 36 (x3), wheat 15 (x1), wheat 10 (x1), wheat 21 (x1) and No MID (x5)	1 contig (contig2) (1080) (reads from Wheat 36,15 and 10 different to above)	**RdRp of *Olive leaf yellowing-associated virus* (emb CAD29306.1)**	*Closterovirus*	**37/118 (31%), score 68.2**
No MID	1 read	**Partial helicase of *Grapevine leafroll-associated virus* 1 (gb AAF22737.1|AF195822 1)**	*Closterovirus*	**30/118 (25%), score 51.2**

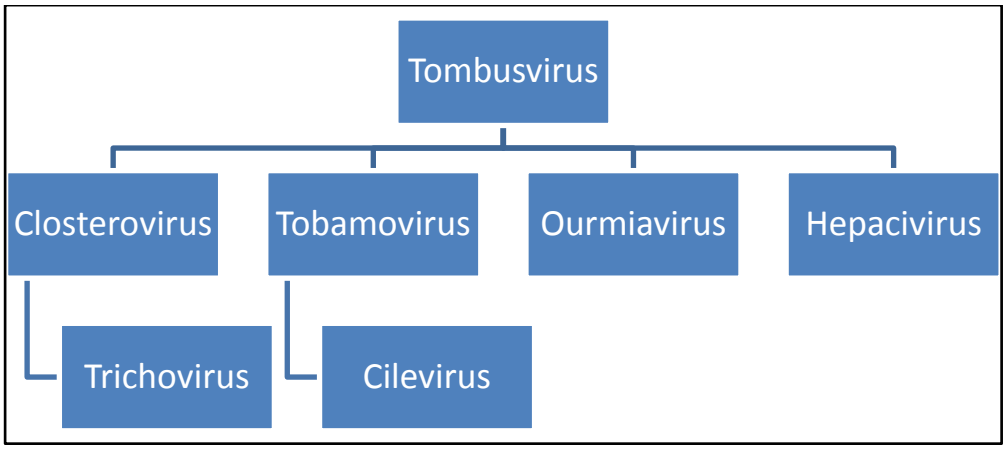

Figure 4.12. Relationships between the genera which wheat samples had homology with.

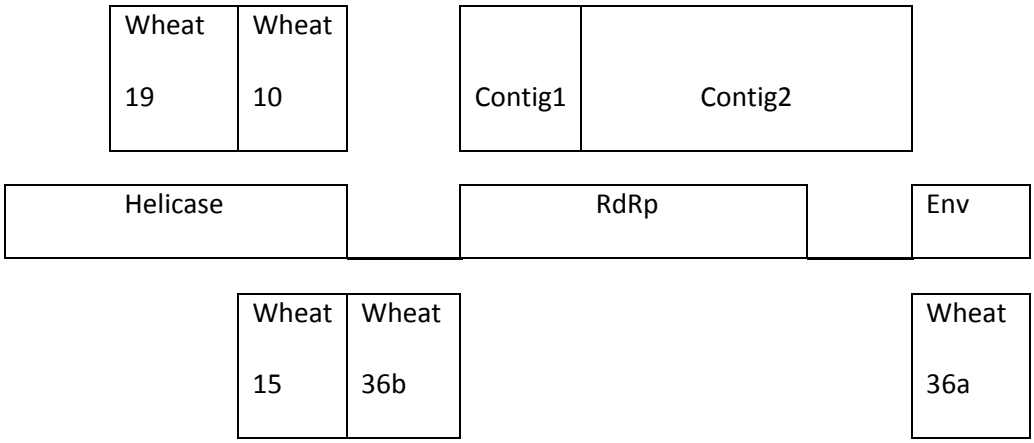

Figure 4.13. Tentative genome organisation of a novel virus detected in wheat. The helicase, RNA dependent RNA polymerase (RdRp) and putative second envelope protein (Env) are shown. See Table 4.4, for key to samples and contigs.

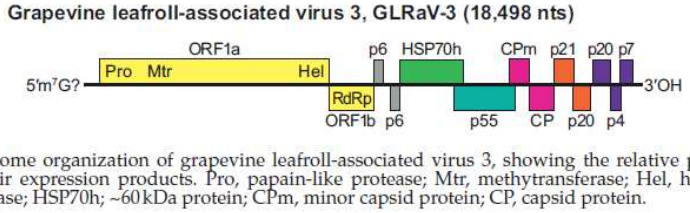

Figure 4: Genome organization of grapevine leafroll-associated virus 3, showing the relative position of the ORFs and their expression products. Pro, papain-like protease; Mtr, methytransferase; Hel, helicase; RdRp, RNA polymerase; HSP70h; ~60 kDa protein; CPm, minor capsid protein; CP, capsid protein.

Figure 4.14. The genome organisation of the type species of the *closteroviruses* (King *et al*., 2012).

4.3.3 Insect results

A total of 37,800 reads were analysed by Newbler v 2.6, and 111 contigs and 7,902 singletons were produced. MEGAN was used to assign possible taxonomy to the Blast N and Blast X results (see Figures 4.15 and 4.16). Table 4.5 summarises the significant results.

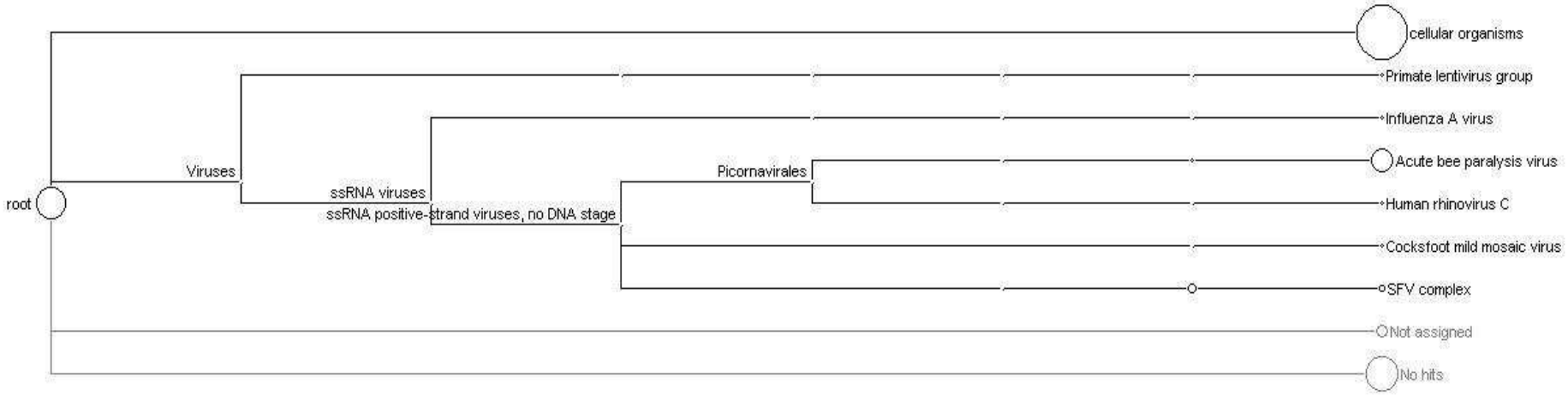

Figure 4.15. MEGAN output of the BlastN results of insect samples.

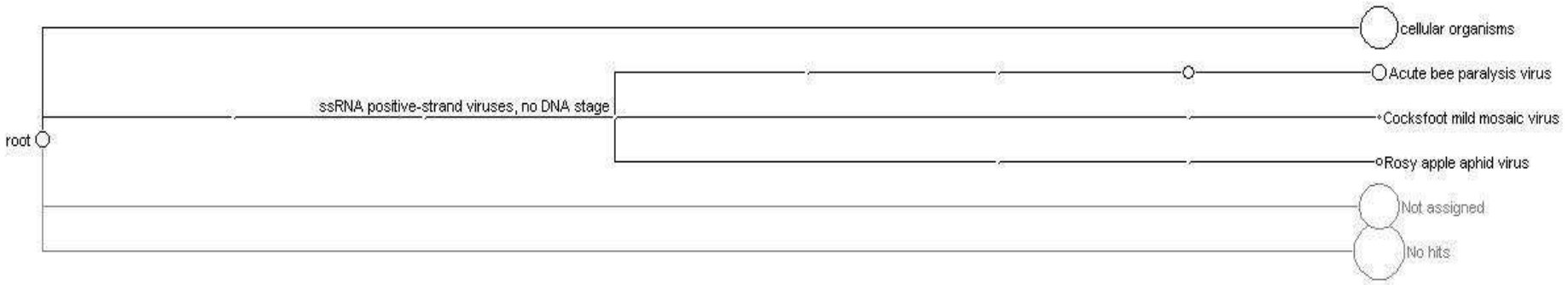

Figure 4.16. MEGAN output of the BlastX results of insect samples.

Table 4.5. Summary of reads from insect samples with homology to viruses in the GenBank database. Standard font is used for nucleotide homology and bold font for amino acid homology.

Sample	Number of reads/contigs (length in nucleotides)	Homologous viruses from GenBank	Genus of homologous virus	Identities and score
Pit 1	1 read (315)	*Cocksfoot mild mosaic virus* (gb EU081018.1) and **p106 of *Cocksfoot mild mosaic virus* (gb ABW74550.1)**	*Sobemovirus*	131/163 (80%), score 150 and **50/85 (59%), score 90.5**
Pit 1	1 contig made of 4 reads (154)	**P1 protein of *Acyrthosiphon pisum virus* (gb AAC58718.1) and the polyprotein of *Rosy apple aphid virus* (gb ABB89048.1)**	*Caudoviridae* (order)	**19/48 (40%), score 42.4 and 17/48 (35%), score 40.0**
Pan 1, 2 and 3	Three mixed contigs (see Table 4.6)	*Acute bee paralysis virus* (see Table 4.7)	*Aparavirus*	See Table 4.6
Pan 2	2 reads (491)	**Polyprotein of *Rosy apple aphid virus* (gb ABB89048.1)**	Unassigned	**35/104 (34%), score 60.1 and 29/82 (35%), score 57.0**

Table 4.6. Proportions of each sample in the mixed contigs with homology to *Acute bee paralysis virus.*

Contig (length in nucleotides)	Pan 1	Pan 2	Pan 3
00008 (407)	0	8	16
00009 (387)	17	34	71
00010 (372)	3	6	9

Table 4.7. Homology of Contigs 00008/9 and 00010 to viruses in the GenBank database. Normal font represents BlastN (nucleotides) and bold font BlastX (amino acids).

Contig	Homologous virus	Identity	Score
00008	Polish isolate of *Acute bee paralysis virus* (gb AF486073.2)	359/377 (95%)	589
00008	**Replicase of *Acute bee paralysis virus* (gb AAN63803.2)**	**112/131 (85%)**	**219**
00009	Polish isolate of *Acute bee paralysis virus* (gb AF486073.2)	374/386 (97%)	643
00009	**Replicase polyprotein of *Acute bee paralysis virus (*gb AAN63804.2|AF486073_1)**	**126/129 (98%)**	**273**
00010	Polish isolate of *Acute bee paralysis virus* (gb AF486073.2)	358/370 (97%)	614
00010	**Capsid protein *Acute bee paralysis virus* (gb AAO74622.1)**	**50/50 (100%)**	**107**

4.3.4 Mown area results

A total of 71,449 reads were analysed by Newbler v 2.6, and 194 contigs and 10,920 singletons were produced. MEGAN was used to assign possible taxonomy to the Blast N and Blast X results (see Figures 4.17 and 4.18). Table 4.8 summarises the significant results.

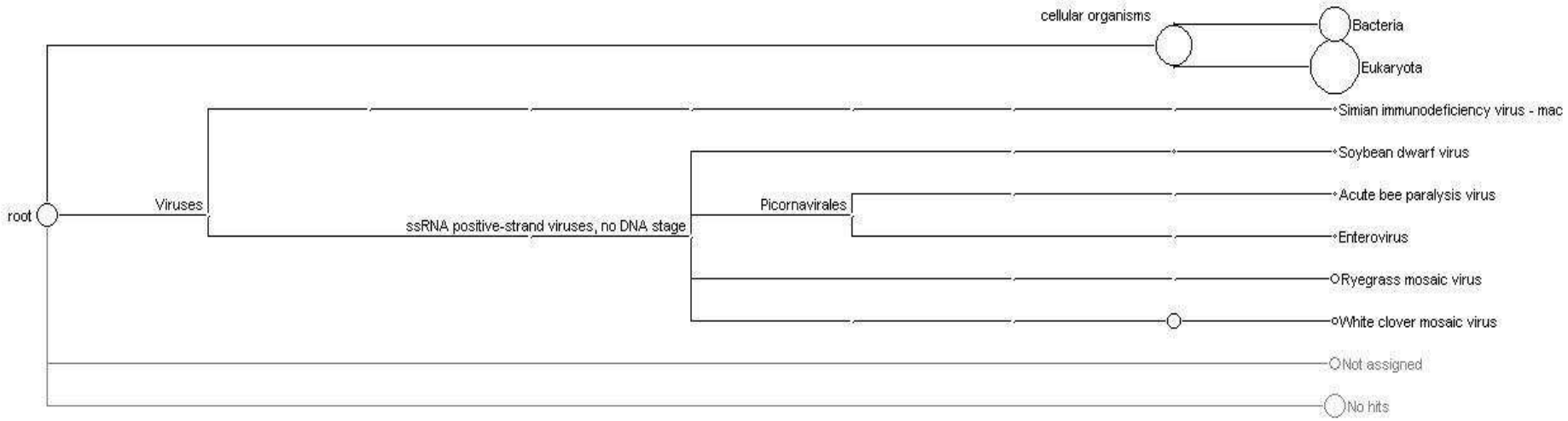

Figure 4.17. MEGAN output of the BlastN results of the mown area samples.

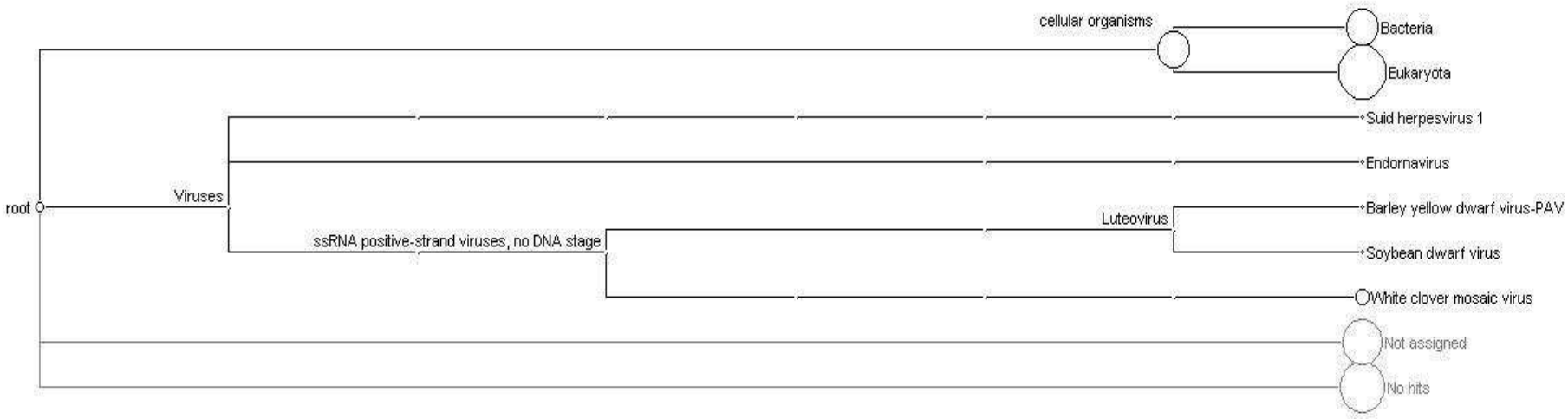

Figure 4.18. MEGAN output of the BlastX results of mown area samples.

Table 4.8. Summary of reads from mown area samples with homology to viruses in the GenBank database. Standard font is used for nucleotide homology and bold font for amino acid homology.

Sample	Number of reads/contigs	Homologous viruses from GenBank	Genus of homologous virus	Identities and score
Mown 15	1 read (89)	Complete genome of *Soybean dwarf virus* (dbj AB038147.1) and **replicase of *Soybean dwarf virus* (dbj BAB62824.1)**	*Luteovirus*	84/89 (94%), score 138 and **29/29 (100%), score 60.8**
15	1 read (72)	Virus coat/nuclear inclusion polyprotein gene of *Ryegrass mosaic virus* (gb U27383.1|RMU27383)	*Rymovirus*	60/61 (98%), score 105
15	Multiple reads and contigs	See *White clover mosaic virus* (below)	*Potexvirus*	
13	I read (145)	Complete genome of*Acute bee paralysis virus* (gb AF150629.1|AF150629)	*Aparavirus*	80/84 (95%), score127
16	1 read (160)	**Aphid transmission protein of *Barley yellow dwarf virus* PAV (gb ABY73558.2)**	*Luteovirus*	**33/35 (94%), score 74.3**
16	4 reads (example 96)	Complete genome of *Ryegrass mosaic virus* (emb Y09854.10)	*Rymovirus*	74/79 (94%), score 116

4.3.4.1 White clover mosaic virus

A mixed contig (6/8 reads were from Mown 15,) (173 nucleotides long) had nucleotide homology to the capsid protein gene of *White clover mosaic virus* isolate 12/13 (gb DQ784572.1) (Identities = 79/83 (95%), score 132).

Two reads from Mown 15 (different to those in the contig) (228 and 72 nucleotides long respectively) had nucleotide homology to *White clover mosaic virus* (WC1MV) RNA (emb X06728.1) (Identities = 194/207 (94%), score 306) and (Identities = 41/42 (98%), score 68.0).

One of these reads, plus ten different ones from Mown 15, had amino acid homology to various regions of *White clover mosaic virus*; for example, the triple

gene block of protein 3 of *White clover mosaic virus* (refNP 620718.1) (25/25 (100%), score 58.2).

4.3.5 Weed results

A total of 158,175 reads were analysed by Newbler v 2.6, and 1,235 contigs and 42,854 singletons were produced. MEGAN was used to assign possible taxonomy to the Blast N and Blast X results (see Figures 4.19 and 4.20). Table 4.9 summarises the significant results.

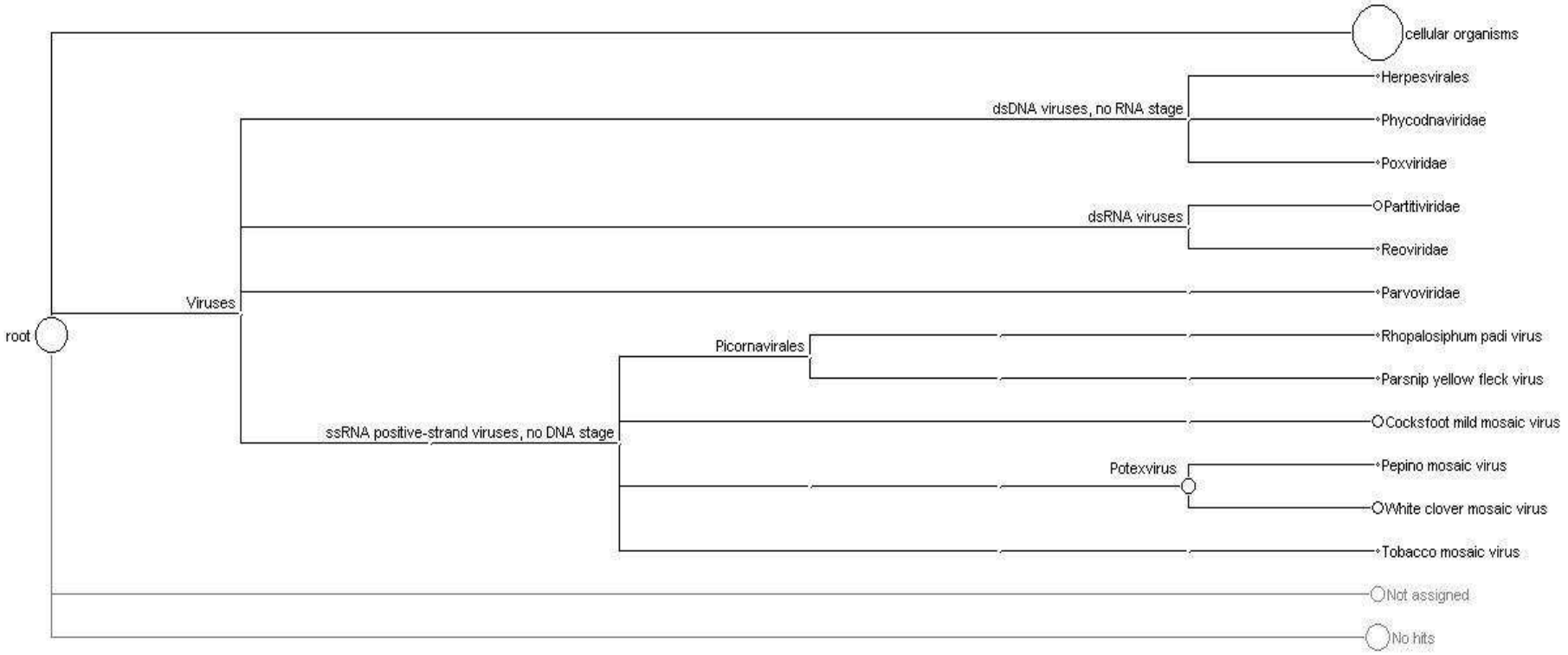

Figure 4.19. MEGAN output of the BlastN results of the weed samples.

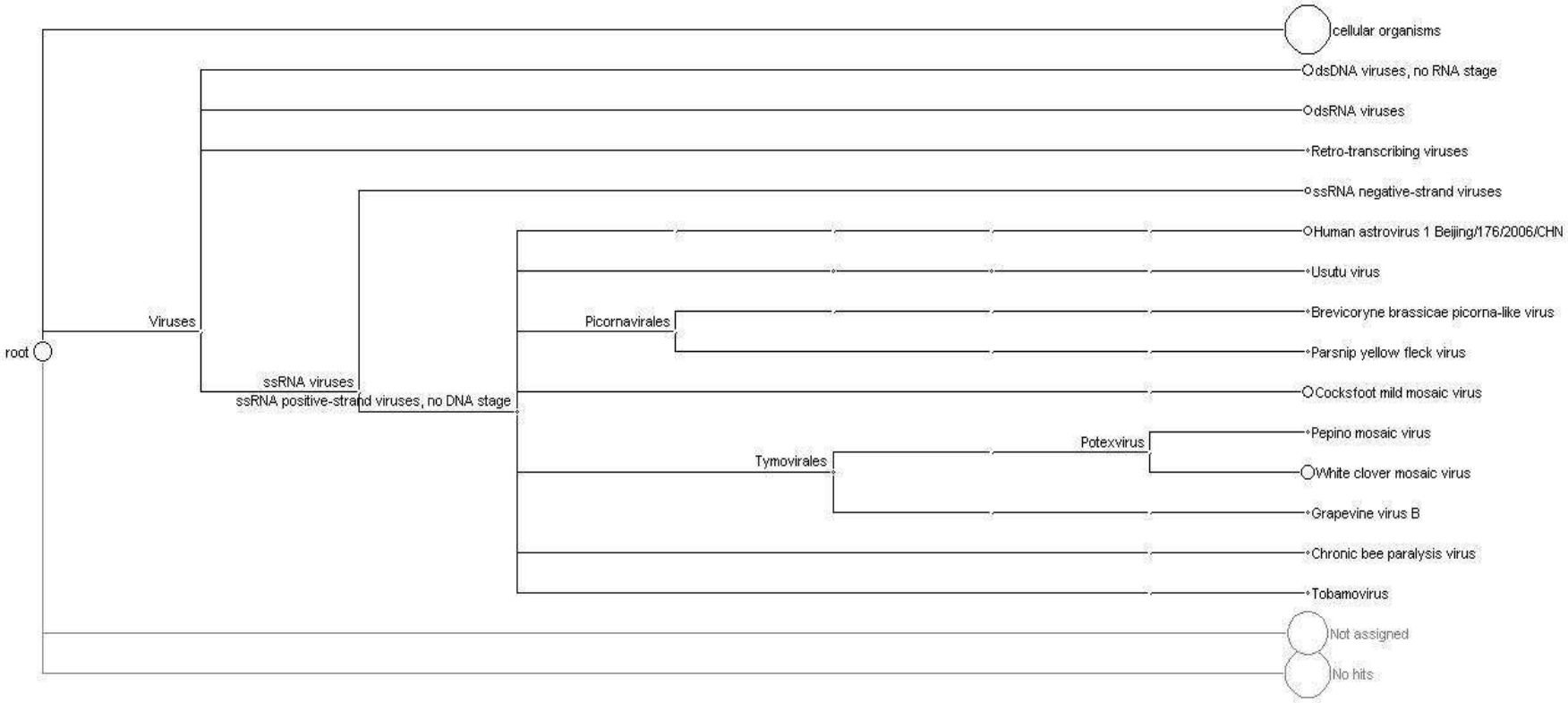

Figure 4.20. MEGAN output of the BlastX results of the weed samples.

Table 4.9. Summary of reads from weed samples with homology to viruses in the GenBank database. Standard font is used for nucleotide homology and bold font for amino acid homology. * Probable contamination.

Sample	Number of reads/contigs (length in nucleotides)	Homologous viruses from GenBank	Genus of homologous virus	Identities and score
92	4 reads (example read 363)	*Cannabis cryptic virus* isolate Fedora17 putative RNA polymerase gene (gb JN196536.1) **and putative RNA polymerase of *Cannabis cryptic virus* (gb AET80948.1)**	*Alpha or betacryptovirus*	Example - 263/358 (73%), score 199 and **59/184 (70%), score 137**
92	1 read (different to above) (469)	*Raphanus sativus cryptic virus* 1 segment dsRNA 1 (gb AY949985.2) and **RNA-dependent RNA polymerase of *Raphanus sativus cryptic virus* 1 (gb AAX51289.2)**	*Alphacryptovirus*	347/462 (75%), score 304 and **94/128 (73%), score 203**
16	1 read (502)	*Rhopalosiphum padi virus* complete genome (gb AF022937.1 and AF022937)	*Picornavirus*	466/475 (98%), score 810
50	1 read (367)	*Parsnip yellow fleck virus* gene for polyprotein, complete (dbj D14066.1|PYFPOLYP) and **the RNA-dependent RNA polymerase of *Parsnip yellow fleck virus* (ref NP 734447.1)**	*Sequivirus*	302/366 (83%), score 365 and **71/74 (96%), score 113**
50	1 read (different to the above read) (142)	**Putative replicase of *Grapevine virus B* (ref NP 619654.1)**	*Trichovirus*	**17/36 (47%), score 40.0**
45	1 contig made of 7 reads solely from this sample(195)	Complete genome of *Cocksfoot mild mosaic virus* isolate Scotland (gb EU081018.1) **the p106 of *Cocksfoot mild mosaic virus* (ref YP 002117834.1)**	*Sobemovirus*	147/195 (75%), score 136 and **54/64 (84%), score 119**
45	1 contig made of 12 different reads to those above (285)	**p6.8 of *Cocksfoot mild mosaic virus* (gb ABW74552.1)**	*Sobemovirus*	**31/31 (100%), score 68.9**
28	62 reads in a contig (272)	*White clover mosaic virus* isolate 12/13 capsid protein gene (gb DQ784572.1) and **the capsid protein of *White clover mosaic virus* (gb ABG88080.1)**	*Potexvirus*	237/249 (95%), score 396 and **62/62 (100%), score 125**
28	20 reads in a mixed contig (2 were also in the above contig, 2 reads from a different sample were also in the contig) (391)	**RNA replication protein of *White clover mosaic virus* (ref NP 620715.1)**	*Potexvirus*	**124/129 (96%), score 242**
85	1 (269)	*Tobacco mosaic virus* genome (variant 1) (emb V01408.1) and **replication protein *Tobacco mosaic virus* (emb CCH64147.1)**	*Tobamovirus*	267/268 (99%), score 479 and **89/89 (100%), score 158**
85	1 (different to above) (370)	**Hypothetical protein CBPV s1gp1 *Chronic bee paralysis virus* (ref YP 001911136.1)**	Unclassified	**34/105 (32%), score 67.8**
NoMID*	2 reads	*Pepino mosaic virus*	*Potexvirus*	

4.4 Discussion

4.4.1 Wheat discussion

4.4.1.1 A potentially novel virus of wheat

Potentially, the most significant result from this study is the detection of a possible novel virus in multiple samples of wheat. Evidence suggests that wheat 10, 15, 19 and 36 and those contributing to contigs 1 and 2 were infected with a virus, but it is unlikely that it was with the specific viruses they had homology to (see Table 4.4). This is because there was low amino acid homology to the viruses, in terms of identity and length of the homologous area. In addition, the majority of the read/contig was not homologous to the suggested virus in all cases. It seems more likely that one virus not listed in GenBank, which had similarity to the homologous viruses was present in the samples. The homologous viruses are in the genera *Cilevirus, Trichovirus, Hepacivirus, Closterovirus, Ourmiavirus* and *Tobamovirus*, which share similarities and ancestry suggesting that a novel virus that is similar to these viruses was present (see Figure 4.12 where some of the relationships are illustrated). *Tombusviruses* appear to link the other genera but this does not necessarily mean that the potentially novel virus is of that genus. At this stage all of the sequence data from the genera is tentatively considered; however, it may be that only that from more closely related genera such as *Trichoviruses* and *Closteroviruses* would be included in a final novel genome (King *et al*., 2012). The reads that contributed to contigs 1 and 2 obviously had similarity to each other, which suggests they may have contained the same virus. Further evidence of a novel virus common to all is that there were examples of single read homology with some samples that also had reads in contig 2. In an alignment of the contig with *Olive leaf yellowing-associated virus* (OLYaV) there is a conserved GDD motif, common to the RdRp of plant viruses, which adds confidence to this result being homologous to a genuine virus (see Appendix 2)

(Koonin, 1991). Contig 1 does not have this motif because its region of homology to the RdRp is different (see Figure 4.13). Figure 4.13 shows the tentative organisation of the novel genome based on wheat samples 10, 15, 19, 21, 36, contig 1 and contig 2. The region covered by the single reads from Wheat 10 and 15 is the same; however, the sequences of the two do not have good homology (data not shown). Therefore, there are two different sequences, which may mean two different novel viruses were present or that one of the reads should not be included in this novel genome as it is from something completely different. The location of the second envelope protein is currently unknown; therefore it is placed arbitrarily. Viruses in the genera discussed do not tend to have an envelope so this feature may not be genuinely part of this novel virus (King *et al*., 2012). The organisation is similar to those of some of the genera such as *Trichovirus*, *Closterovirus* and *Cilevirus* (see Figure 4.14) (King *et al*., 2012), which adds confidence to the result. For example the helicase and RdRp are in relatively the same positions. The reads and contigs did not cover the other areas of the genome shown in Figure 4.14, but further sequencing may allow detection and assembly using a reference such as that shown in the Figure 4.14 and other type species for the various genera. The other contributors to the genome do not overlap and have enabled the tentative genome to be developed. At this stage it appears that randomly sequenced fragments of the genome have been produced and it has been possible to begin assembly in to the tentative genome. During sampling no symptoms marking the plants as unusual or symptomatic of a viral infection were noted on the samples involved here. This suggests that if a virus is present it may be a cryptic virus. Such a situation occurred in the case of a watermelon (*Citrullus lanatus* Thunb.) found by next generation sequencing to contain a novel cryptic virus which was likely a *Partitivirus* (Sela *et al*., 2013). Kreuze *et al.* (2009) also found a novel cryptic virus in sweet potato (*Ipomoea batatas*) which

caused significant impact on yield, highlighting the significant advantage that next generation sequencing provides over targeted diagnostics methods, or using diagnostics on symptomatic samples only.

4.4.1.2 Wheat 24

At least one virus was present in Wheat 24, because there was very good nucleotide homology to *Tobacco mosaic virus* (TMV) for two reads from the sample (see Table 4.4). The entire length of the reads was homologous to TMV at the nucleotide level, which suggests the virus was genuinely present. There was also good homology at the amino acid level between the same two reads and TMV (see Appendix 1). The bases are identical apart from a short region; this could be due to sequencing error or genuine sequence differences. According to King *et al*. (2012), strains of TMV have less than 10% nucleotide differences; therefore, this sample appears to contain a strain of TMV. This finding is unexpected because TMV has been reported in the UK in plants, but is not known to infect wheat (Web reference – Pvo1). It is highly likely that TMV was not present in the original sample but was introduced as contaminiation during sequencing preparation in the laboratory because a colleague was working with TMV in the laboratory at the same time. The virus is known for its stability and ease of contamination (Creager, 1999; Val Harju, Fera, personal communication). If a genuine case of TMV infection had occurred the number of homologous reads would be expected to be much higher than just two reads (432 bases) (see Table 4.4). This is because TMV is known to be a very high titre virus (Creager, 1991). A sample in which a genuine TMV infection had occurred, inestigated by a colleague produced 140 reads (46,172), this highlights the difference. Therefore further work was not carried out on this sample.

Two different reads from Wheat 24 were part of a mixed contig (there were 8 other contributors to the contig from 5 different samples) that had limited

homology to *Freesia mosaic virus* (FMV) and other *Potyviruses* (data not shown but exemplified by FMV) (see Table 4.4). Because of the nature of the contig no definite conclusions can be drawn from it, but it suggests that a virus that had limited homology to *Potyviruses* was also present in wheat 24. Only a very small proportion of the contig was homologous to FMV, indicating limited shared homology. The area of homology between the two reads and *Potyviruses* and those reads with homology to TMV is not the same, and as these are in different families it seems likely that two separate viruses could be present in Wheat 24.

4.4.1.3 Wheat 16

One read from Wheat 16 had relatively good homology to *Turnip mosaic virus* (TuMV) at the nucleotide level (see Table 4.4). Different species of *Potyvirus* have less than 76% sequence identity over their whole genome, therefore from this small section of homology it appears that TuMV was present specifically. There is no evidence to suggest TuMV can infect wheat, and it primarily infects dicotyledonous plants. Therefore it was unlikely to genuinely be present. However, the lack of reports of the virus in wheat could be due to a lack of testing. The virus has been reported in the UK in the past, so it is possible that it was in the field and has infected wheat (Pallett *et al.*, 2008). It is also possible that another novel virus, with some similarity to TuMV was detected.

Depsite the low number of homologous reads to viruses from this sample, and other following samples from wheat, weeds, insects and the mown area they have not been classed as contamination in the same way as TMV in Wheat 24. This is because the homologous viruses were not known to be present in the laboratory at the same time as the samples were prepared, further the viruses are not known to be especially prone to contamination as TMV is (Creager, 1991). Therefore they are still considered to be potential detections of genuine viruses present in the original

samples. It is possible that the reason for the low number of homologous reads was that the viruses are low titre in the host. There could be further issues which limited the number of homologus reads (see 4.4.7.3).

4.4.2 Insect discussion

4.4.2.1 Plant viruses

4.4.2.1.1 Pit 1

A virus was present in Pit 1 and it is likely that it was *Cocksfoot mild mosaic virus* (CfMMV) or a related virus. This is because of the relatively high levels of both nucleotide and amino acid homology to the virus (see Table 4.5). However, the alignment between amino acids of the read and CfMMV (see Appendix 3) shows that there are a number of bases that do not match, suggesting this exact virus is not present. Also, only approximately half of the read had homology to CfMMV. *Cocksfoot mild mosaic virus* is a tentative member of the *Panicovirus* genus according to King *et al*. (2012), for which there is no species demarcation data available. However, according to other sources CfMMV is a *Sobemovirus*, for which sequence similarity must be approximately 75% over the complete genome to be considered the same species, thus suggesting a different species of *Sobemovirus* may be present. *Cocksfoot mild mosaic virus* is transmitted by aphids such as *M. persicae* and coleopteran; in theory a similar virus may be transmitted in the same way (Anstead *et al*., 2008; Chamberlain and Catherall, 1976). It is highly likely that *M. persicae* was present in the pit trap as it is a common pest of UK crops. If CfMMV was confirmed this would be an interesting result, since CfMMV was apparently found in Weed 45 (see Section 4.4.4.1). Therefore an insect vector and host cycle has possibly been sampled. Figure 4.8 shows that Pit 1 and Weed 45 were in close proximity which supports this statement. The lack of results of this virus in other samples may be due

to vectors travelling only short distances or alternatively infected samples may simply have been missed.

4.4.2.2 Insect viruses

Some insect viruses may be able to infect plants. Singh *et al*. (2010) reported that RNA viruses of bees such as *Black queen cell virus* have been found in the pollen of flowering plants such as clovers. Therefore, the following insect viruses could potentially infect wheat and may be important for yield loss.

4.4.2.2.1 Rosy apple aphid virus and Acyrthosiphum pisum virus

There was relatively low homology of several reads from Pit 1 and Pan 2 to insect viruses (see Table 4.5). In the case of Pit 1 the majority of the read was homologous to the viruses. This suggests that one or more viruses were present that had a limited relationship to *Rosy apple aphid virus* (RAAV) and *Acyrthosiphum pisum virus* (APV).The fact that there were multiple reads, which in the case of Pit 1 formed a contig, adds confidence to a virus genuinely being present in the samples.

4.4.2.2.2 Acute bee paralysis virus

Multiple reads from pans 1, 2 and 3 were assembled into contigs by Newbler that had high nucleotide and amino acid homology to *Acute bee paralysis virus* (ABPV) (see Tables 4.5, 4.6 and 4.7 and Appendices 4 and 5). The contigs were almost completely homologous to ABPV. The honey bee is known to be a host of ABPV in the UK (Web reference - Fera), and was likely to have been in the pan traps. It is also possible that other insects infected with the virus were present in the traps. The fact that such a virus was found in the pan traps that were in the air and not the pit traps that were in the ground suggest a flying insect or insects were carrying the virus. There is currently no evidence to suggest that ABPV can infect wheat.

4.4.3 Mown discussion

There is evidence that in areas of plants that are disturbed by machinery, such as lawnmowers, there are greater levels of spread of mechanically transmissible viruses, because of the physical movement of virus infected material and wounding of plants (Upstone, 1969; Web reference – Pvo3). Therefore, it was expected that there would be high levels of viruses in these samples. However, while analysis has shown that there were possible viruses present, the area does not seem to have a significantly greater number of possible viruses than weeds that are not mown. This could be due to several reasons, including that vast numbers of mechanically transmitted viruses simply were not present in this field.

4.4.3.1 Mown 15

Potentially, *Soybean dwarf virus* (SDV)*, Ryegrass mosaic virus* (RgMV) *and White clover mosaic virus* (WCMV) were all present in sample Mown 15 (see Table 4.8 and Appendix 6). There is evidence that all of these viruses have been found in the types of plants that could have been in the mown area. For example WCMV and SDV are hosted by clover, which was planted in the field in 2004, 2006 and 2007 and RgMV is able to infect a range of plants that may have been present in the mown area and importantly are related to wheat; such as italian ryegrass (Slkyhuis, 1958) and brome grass (Mulligan, 1960). It is possible that more than one type of plant was present in the sample, which may explain why clover and grasses appear to be the hosts that are most likely, meaning different viruses were present in their different hosts. Table 4.8 shows that there were very high levels of identity at nucleotide and amino acid level between samples and their homologous viruses; however, the regions of homology were not very long. In all cases, in particular SDV and RgMV, almost the entire read was homologous to the virus. There are currently no reports of these viruses in wheat.

4.4.3.2 Mown 16

Ryegrass mosaic virus was also detected in Mown 16 (see Table 4.8). As there were four reads, with homology to different areas of RgMV, it suggests the virus was present, as a large region of it was covered by the homology. This result also adds confidence to the detection of RgMV in Mown 15, due to their close proximity in the field, hence possible transmission route (see Figure 4.8). There was also good homology between a read from Mown 16 and the aphid transmission protein of *Barley yellow dwarf virus* PAV (BYDV-PAV) (see Table 4.8). There was a good level of amino acid identity but not over a very long region. This is an interesting result as BYDV-PAV is known to infect wheat (see Section 1.11.1.3) and may serve as evidence of surrounding plants acting as hosts and possibly reservoirs of the virus when wheat is not present.

4.4.3.3 Mown 13

It seems likely that ABPV was present in sample Mown 13 because despite there only being one homologous read, the homology at the nucleotide level was good. It is interesting to note the location of Mown 13 in relation to the insect sampling sites where pans 1, 2 and 3 were positioned (see Figure 4.8). They are all at the top of the field, suggesting the viruses transmitted by insects were mainly in that region. It is now known why this could be, but perhaps the 'edge effect' played a role as insects entered the crop from the edge of the field and spread a limited distance.

4.4.4 Weeds discussion

4.4.4.1 Weeds 28 and 45

It is certain that Weed 28 had WCMV and Weed 45 had CfMMV, because of the high number of reads and contigs from each that had high levels of identity to nucleotides or amino acids of their respective viruses over a relatively long region of sequence (see Table 4.9 and Appendices 7, 8, 9 and 10). In the case of WCMV the first contig

was almost completely homologous to WCMV. The second contig contained two other samples, which may have been the cause of only part of the contig being homologous to WCMV. In relation to CfMMV, the first contig was completely homologous with CfMMV at the nucleotide level; however, the second was only partially homologous to the p6.8 of CfMMV, which was 81 amino acids long. It is possible that the rest of the contig was in the opposite direction to the rest of the p6.8. Further tests to confirm the presence of the viruses using qRT-PCR assays may not be necessary in these cases, because of the good homology. There is no evidence suggesting that CfMMV has been detected in blackgrass (Weed 45) to date, but it has been found in other members of the Gramineae (Torrance and Harrison, 1981). In the case of Weed 28, which was clover, it is known to be a host of WCMV (Pierce, 1935).

4.4.4.2 Weed 16, 50 and 85

Despite there being only a single read from samples 16, 50 and 85 that had homology to a *Rhopalosiphum padi virus* (RPV)*, Parsnip yellow fleck virus* (PYFV) and TMV respectively, it seems likely that the viruses were genuinely present (see Table 4.9). This is because the level of homology was high and over long regions of sequence (especially Weed 16). There was almost complete homology between the respective viruses and the reads from samples in all cases, which is convincing and suggests that the read length limited the homology observed. In the case of Weed 50, which was cow parsley (*Anthriscus sylvestris*), there is evidence that the Anthriscus serotype of PYFV infects this plant, which supports this result (Davis and Raid, 2002). In addition, Appendix 11 shows the comparison of the amino acids of the read to the RdRp of PYFV (note the FLKR conserved domain, which is characteristic of *pico, coma* and *nepoviruses* which are all in the *Secoviridae* family, as is PYFV) (Koonin, 1991). There was also homology to the replicase of *Grapevine virus B* (GBV), a Clustal alignment in

MEGA 4.1 showed good homology of the putative replicase of GBV to the complete genome of PYFV (sp Q05057.1) (data not shown). Therefore this was probably homology of the same virus being highlighted in connection with a different virus.

Weed 16 was blackgrass, which had high nucleotide homology over a very long region of sequence to RPV. This virus is part of a group of viruses that infects insects (Moon *et al*., 1998). It seems likely that an aphid or other small insect that was carrying the virus was present in the weed sample. However, it is possible that the insect virus had been passed to the plant, as has been discussed in earlier analyses.

Weed 85 was cocksfoot, in which TMV was detected. As was the case with the detection of TMV in wheat 24 it is highly likely that this result was due to contamination in the laboratory.

4.4.4.3 Weed 92

There were several reads from couch grass that had nucleotide and amino acid homology to *Partitiviridae* (see Table 4.9). The area of homology was very long but the level of identity was not high enough to conclude that the specific viruses listed were present. However, the vast majority of the length of the reads was homologous to the virus, which suggests a genuine likeness to the virus. Appendix 12 shows the alignment of *Raphanus sativus cryptic virus* (RSCV) *1* and Weed 92, in which there is a GDD motif that is characteristic of the RdRp of plant viruses (Koonin, 1991). Both viruses are described as unclassified members of the *Partitiviridae*. Based on the allocation of other viruses to genera such as RSCV2 and 3 as potential members of the *Alphacryptoviruses*, it seems probable that RSCV 1 would be in the same genus and that Cannabis cryptic virus (CCV) would either be in the same genus or in that of the *Betacryptoviruses*. Species demarcation is based partly on the size of DNA segments produced; therefore the complete sequence of the virus present would

need to be obtained to be able to make this judgement. Based on the level of identity, it seems likely that a different strain or species of the viruses was present in the sample. As there is a chance that the virus present was a cryptic virus, it should be noted that these viruses cause no, or few symptoms but can cause considerable yield loss (Hull, 2004; Kreuze *et al*. 2009). Also, because different viruses can have synergistic interactions, and in some cases helper viruses are required for symptomatic infections, this virus could be important should it occur in plants with other such viruses. In addition, *Patitiviridae* are seed transmitted, thereby causing systemic infections. Future testing could therefore sample any area of the plant, but seeds may have a lower titre of virus than the rest of the plant (Hull, 2004). This is an interesting result because couch grass is a member of the Gramineae, as is wheat, therefore making an infection in wheat more likely. Further work using Koch's postulates concluded that a virus was not genuinely present in this sample (data not shown). It may have been that the virus degraded in the nucleic acid extracts and green material before testing, hence this result. Further study of samples from the field may detect the virus again.

4.4.5 Summary of viruses that were potentially present in samples

It is possible that there are numerous viruses present in natural weeds, managed weeds and insect samples. It is interesting that the same viruses do not appear to have been detected in wheat samples in this study. This may suggest that these potentially novel viruses do not infect wheat; however, it may be that a greater number of wheat samples require sequencing in order to find these viruses.

A number of viruses may have been present in the samples that have not been reported in their specific host before. This does not necessarily mean that they cannot infect the host, but is due to a lack of testing. There are also examples that could be completely novel viruses, in cases where there was homology to a known

plant virus, but the evidence was not convincing enough to identify a specific virus. Therefore, these could potentially be the first reports of such viruses in their specific hosts. It is possible that some of the viruses which were potentially detected cause asymptomatic infections of wheat and do not have a major impact on plant healthy of yield, for example *Oat mosaic virus* and certain cultivars of wheat (Lapierre and Signoret, 2004). Therefore it would be important to conduct studies to investigate impact of any potential viruses (see Section 4.4.6.1).

Some viruses were found that did not come as a surprise due to reports of them in the UK in similar hosts in the past. For example, BYDV-PAV, which is currently known to infect wheat in the UK (see Sections 1.11.1.3 and 3.5). This virus can cause significant yield loss of wheat, but was not present at high enough levels to be contributing majorly to the plateau in the yield of wheat, which is likely to be because the weather conditions during the wheat growing season 2011/12 were not favourable for the vector (see Section 3.4.2.4). *Cocksfoot mild mosaic virus* is another virus that has been reported in the UK in the past (Torrance and Harrison, 1981). Studies into the impacts of the Scottish isolate and others from Europe found that wheat could only be infected by the Scottish isolate, and that only local infections with no visible symptoms were observed. This virus was potentially found in both a known insect vector and another plant, but none of the wheat samples. This suggests that the virus does not readily infect wheat, and that the virus is not a major threat to wheat in the UK; however, yield studies would be necessary to confirm this. Finally, RgMV has not been found in wheat in the UK before; however, there is evidence from Eagling (1992) that an Australian isolate could infect wheat although symptoms were not observed. The results of a large scale survey of wheat (see Chapter 2, Sections 4 and 5) also suggest this virus does not infect UK wheat.

4.4.6 Further investigations

4.4.6.1 Confirmation that a virus is present

Further investigation is required for all samples that potentially were infected with viruses to be able to state if any possible viruses that were detected were genuine and importantly if they could be responsible, in part, for the plateau in the yield of wheat. Unfortunately, time constraints meant that further work was not possible in the case of each potential finding of a virus. This was because the preparation of nucleic acid extracts and sequencing took twelve months for all batches to be completed. This was because batches had to be run individually and were placed in a queue along with many other samples to be processed for Fera staff and customers. Additionally there were issues of machine failure rendering the machine unuseable for long periods of time. These issues are disadvantages of the pyrosequencer at the moment, but these should be resolved in time as initial machinery issues are resolved (the 454 pyrosequencer was relatively new to the market at the time) and lower costs may mean purchasing additional machines to reduce waiting times. The vast amounts of data produced require bioinformatic analysis which was also very time consuming. For example, it required long periods of time to allow the computer to perform processes such as Blast searching (for example overnight). Additionally this exact type of analysis had not been done before, so it took time to develop the bioinformatics and computer scripts required.

However, as mentioned a sample was studied further. This was *Elymus repens* labelled Weed 92. This was chosen simply because the result was available first in terms of batches and it had relatively high homology to plant viruses. Studies included mechanical inoculation to a range of standard indicator plants regularly used at Fera (*Nicotiana benthamiana, Nicotiana occidentalis, Nicotiana hesperis* and *Chenopodium quinoa*), *Elymus repens,* wheat (cv. Gladiator, Scout, Beluga, Solstice,

JB Diego and Einstein (necessary because different isolates of viruses can have different impacts on different varieties of wheat such that specific tests are needed rather) and barley (cv. Sequel and Saffron). Transmission electron microscopy of inoculated plants was also carried out (with comparison to healthy control plants). A qRT-PCR assay for the sequence homologous to a virus in Weed 92 was developed (tested for specificity by Blast searches and physical testing with the positive control viruses used in Chapter 3), however it could not detect the target in the extract that was sequenced. Re-extractions from the original material were done by CTAB and RNeasy, but the qRT-PCR assay could not detect the target again. Had the aasay been successful it would have been used to test all 120 pyroseuenced samples to study prevalence of the virus in the field. Data is not shown because none of the experiments suggested that a virus was present in the original sample in the survey and mechanical inoculation was not successful in producing symptoms or particles visible by the TEM. Insect inoculation was not attempted, therefore it is possible that transmission may have been possible by that method. It was concluded that the pyrosequencing result was not due to a genuine infection by a virus, but perhaps due to random homology to plant viruses. This highlights that while pyrosequencing can be successful in detecting viruses, it may also produce 'red herrings'. Therefore Koch's postulates are crucial for confirmation of results. However, it is possible that the virus had degraded in the original extract and the sample.

The most important result for further study is that highlighted in the wheat results section. The further investigation steps in this case would include designing primers at the ends of the reads and contigs to cross gaps and attempt to extend into the next sequences, which would show if these sequences are part of the same genome. The use of bioinformatics would enable assembly and potentially lead to a novel genome being developed. Information about the organisation of the genera of

homologous viruses could be used to guide the assembly. Assigning the novel virus taxonomically would be based on a number of factors such as the vectors of the virus, serological reactions and cytopathological features induced in plants. Once a genus was assigned, the confirmation of a new species would be based on similar criteria and also sequence similarity (King *et al*., 2012). If a novel virus was found, testing all the samples in this study for it would be interesting as it may be present in other samples. For example, Weed 50 also had homology to the replicase of a GBV; however, the region covered was not homologous to that covered by Wheat 10 and 36, which also had homology to GVB. However, they could be from different regions of the genome, which would explain this.

A sample such as Weed 28 or Weed 45, for which there was convincing evidence that a specific virus was present, would require little further investigation for confirmation. For example a qRT-PCR test for the suspected virus followed by sequencing may be sufficient. The main focus would be to investigate the effects of the viruses on a range of wheat varieties. In contrast, some samples which had low levels of homology and/or low numbers of reads, would require more investigation to confirm if a virus was present and what it was first. This second group is more likely to include novel viruses of wheat.

Koch's Postulates and the modern derivatives such as those stated by Fredericks and Relman (1996) should be referred to to confirm a virus is present and which it was. With that in mind the following steps could be carried out:

1. Develop or use an existing qRT-PCR for the virus which was potentially present and sequence the products. Repeat with healthy controls. This would confirm whether that virus is present in the potentially infected sample but not healthy plants. Other investigation steps and sequencing would differentiate between strains of the virus.

2. Re-sequence the original sample and/or fresh plants from the same area for examples that have low depth of sequence. This would give greater confidence in the result. Sequencing a fresh sample would also rule out any results that were introduced as contamination.
3. Inoculate a fresh host plant with sap from the original sample and include a healthy control, to look for symptoms associated with the virus in the former but not the latter (if a specific virus was thought to be present) and other possible symptoms. This step would show if a pathogen was present, and sequencing would help confirm exactly which. This would also confirm that an infectious agent was replicating. Finally it would broaden knowledge of what may be a novel virus.
4. Inoculate a range of common indicator plants with sap from the original sample. Using published literature as a reference when analysing symptoms will give an indication about which virus is present.
5. Examine a prepared grid of the original sample and a sample from a repeatedly inoculated plant, under a TEM, looking for possible virus particles. Knowledge about the particles of the potential virus may be useful, but must not cause a bias that could cause other relevant particles to be ignored.
6. Collection of further samples from the area where the host of a genuine virus was found and testing for the virus again to assess the prevalence in other plants.

4.4.6.2 Investigating the impact of viruses on wheat

7. Inoculate a selection of varieties of wheat with sap from the original sample and any resulting plants that are thought to have the virus, to look for symptoms. Allowing the wheat to grow for a long period of time after inoculation would show whether the virus has an impact on yield.

8. If a potential virus is found to be genuine and to cause symptoms in wheat, designing a qRT-PCR assay for it and testing samples from this study and future wheat samples would be necessary.

4.4.7 Discussion of methods

In the last five years the use of next generation sequencing technology such as pyrosequencing has increased, being used for several purposes not limited to plant pathology. Infact its use in human medicine is what has allowed it to be developed so rapidly, because there is greater funding available than in plant sciences (Siqueira *et al*., 2012). For example Finkbeiner *et al*. (2009) used the technique to identify a novel virus which causes gastroenteritis. It had previously been the case that the etiology of outbreaks of the disease could not be determined even after extensive testing. The opportunity for massively parallel sequencing has been used to study genetic mutations which may be causes of human disease (Nemeth *et al*., 2013). More relevant to plant pathology and inparticular the method used in connection with next generation sequencing in this chapter were the studies of Coetzee *et al*. (2010) and Thapa *et al*. (2010) who used the technique to investigate the range of viruses present within a vineyard and prairie grasses respectively, the so called 'virome'. The former highlighted that error prone RNA virus replication results in quasispecies which may not be detected by target led diagnostics if they are too dissimilar from the target, however next generation sequencing would likely detect these. Other studies found novel viruses in single plants under investigation, (therefore different types of studies to this chapter) such as grapevine, *Liatris spicata* and sweet potato by Al Rwahnih *et al.* (2009), Adams *et al*. (2009) and Kreuze *et al*. (2009) respectively. Additionally, if a range of viruses which were interacting within a plant were present they would be detected, whereas if target led methods were used only those actually tested for would be such that the etiology of disease would be missed (Coetzee *et al*.,

2010). The method of pyrosequencing has the advantage of not requiring prior knowledge of potential pathogens in order to detect them, because sequence specific primers are not required (Adams *et al*., 2009; Kreuze *et al*., 2009). Therefore the technique has had a significant impact on plant virology in terms of diagnostics and is said to be revolutionary (Prabha *et al*., 2013). The tool offers significant opportunities to investigate the currently unknown and undetectable viruses present in a vast range of sample types.

Next generation sequencing studies use the same overall method in that samples of nucleic acid are extracted, preprared for sequencing and results compared to GenBank to suggest what is present. While the studies of Adams *et al*. (2009), Coetzee *et al*. (2010) and Thapa *et al*. (2010) were used to develop some of the method used here, such as sample preparation, many aspects of the project had to be developed as literature did not provide suitable information. For example development of an insect storage solution and a sampling strategy (see Section 4.2.2). The general bioinformatics method was similar to published studies such as Adams *et al*. (2009), Coetzee *et al*. (2010) and Thapa *et al*. (2010) in that the data is compared to GenBank by Blast searching to search for comparable sequences suggesting what may be present in the samples. However, specific scripts were written to perform parts of the analysis in this project, to make the vast amount of data manageable. Other studies have used siRNA produced as part of the plants defence system during RNA silencing, as the target for sequencing (De Serio *et al*., 2009; Kreuze *et al*., 2009). However, this requires laborious laboratory preparation, results in large number of very small (21-24 nucleotide) sequences which then require extensive bioinformatics and due to the very nature of siRNA could result in the sequencing of a virus against which the plant was successfuly defending itself by performing RNA silencing. Additionally, it depends on the plant having begun

defending itself by RNA silencing. Therefore it was not used in this study, rather dsRNA which is the hallmark of viral infection was used (Roossinck *et al*., 2010).

4.4.7.1 Amount of sequence data

There were differences in the amount of sequence data generated for each batch of samples (see Figure 4.9). For example, batch 5 was a particularly poor batch in terms of number of reads generated. Unfortunately financial and time constraints prevented repetition of the pyrosequencing of batch 5. The concentration of batch 5 prior to library preparation was not the lowest of all the batches and checks during library preparation and emulsion PCR would have shown a problem in concentration of sample. It is therefore unknow why the amount of sequence data generated was lower for batch 5 than other batches. However, it is possible that the technical difficulties experienced by the 454 pyrosequencer contributed to this issue. In the future, such issues should be resolved by the manufacturer, as this technology is still relatively new and despite being products of large renowned companies there are a number of issues with the machinery. For example collection tubes which are too long, resulting in them bending in the machine and not collecting reagents (Ian Adams, Fera, personal communication).

There was also a difference in the number of reads produced for each sample, over a large range (see Figure 4.9). The specific MID used had an influence on the number of reads produced for the samples. Equal amounts of each sample were used to prepare the library (calculated according to their individual concentrations), therefore any difference occurred after that point. In future work, MIDs 9, 10 and 12 should not be used, and MIDs 3 and 5 when using more than two samples (but MIDs 3 and 5 should be used preferentially when using two samples). It is possible that dimerization or unexpected PCR amplification artefacts may be the cause of some MIDs performing worse than others (Web reference – GS-FLX). Re-

sequencing the samples that produced low numbers of reads, likely due to the MID they were linked with could be done, using one of the more successful MIDs.

Longer regions of sequence, for example whole genomes, can be obtained using pyrosequencing following assembly into contigs, but greater depth of sequence is required, achieved by using larger proportions of the sequencing plate for each sample (Monger *et al*., 2010). For example in Chapter 5 1/8th of a plate was used to sequences Cynorusus mottle virus, while samples in this chapter had a twelth less of the plate (due to financial constraints). Therefore dedicating a greater proportion of the plate to the sample would increased the amount of sequence data generated.

Despite the amount of sequence data of interest for individual samples being too little for conclusions to be drawn, overall there was a huge amount of data which required analysis, which is the case with next generation sequencing and is perhaps its biggest disadvantage (Stobbe *et al*., 2012). However computer software is able to make the process easier and efforts to streamline this further have begun and will continue to be so in the future, to make a practical diagnostic tool (Stobbe *et al*., 2012).

4.4.7.2 Foster sequencing has a bias

It is known that the type of sequencing used in this study can be biased towards some areas of genomes, meaning some areas have little or no depth of coverage. The reasons for this are not completely known, but a PCR bias has been suggested. In addition the regions favoured are difficult to predict (Ian Adams, Fera, personal communication). This makes the method less suitable when whole genome sequences are required, but for the purposes of this study in which fragments could be used for identification, the method was suitable. However, if a read was produced for a virus for which only part of the genome was published on GenBank it may have been missed in Blast searching and therefore not included in the analysis by MEGAN.

Other sample preparation methods such as the cDNA Rapid Library Preparation Method (Roche), which was used in part for this work, could be used instead of the Foster method. The method was not used here due to financial constraints and because in preliminary work it produced far less sequence data or failed when tested in parallel with the method used here (data not shown).

The Foster method is also known to be very prone to contamination, because of the random primers that are used and their tendency to amplify anything including themselves. This leads to wasted sequence data and can cause artificial results. Despite stringent measures to prevent contamination it appears that *Pepino mosaic virus* from the weed sample results (see Table 4.9) and *Tobacco mosaic virus* from Wheat 24 (see Table 4.4) were likely to have been produced due to contamination because a different region of the plate was used to sequence a sample of *Pepino mosaic virus* and *Tobacco mosaic virus* was being prepared simultaneously in the laborartory. In order to avoid this in the future, plates made solely of samples from the survey could be used, therefore any possible viruses would have been likely to have been from at least one of the samples in the survey. Further to this, plates made solely from one sample would be beneficial, but financially costly and time consuming. However, it is also possible that contamination can occur at other points in the sample preparation process, as may have been the case with another read without a MID from the wheat sample results (see Table 4.4). The lack of a MID highlighted this result as potentially erroneous, therefore MIDs and their analysis are an important control measure in this work. It is also important to ensure that sample preparation is carried out using high standard laborartory practices. However, despite this contamination can unforutnatlely still occur as the process is so sensitive.

In order to be confident that a possible diagnosis of a virus is real and not contamination it would therefore be necessary to re-extract from the original sample

and re-sequence to compare the data. It may also be useful to return to the site of sampling to collect other samples to search for the virus, thereby eliminating contamination in the laboratory.

Other methods of sample preparation have been used, for example that by Kreuze *et al*. (2009), However, for the reasons discussed above, that method was not used in this project, although it would likely have been less contamination prone because the need for amplification of fragments would be less since the levels of siRNA would be higher than fragments of dsRNA used in this project, as a result of processing of whole viruses by the plant.

4.4.7.3 Low number of reads per sample which had homology to a virus

There were low numbers of reads, often just one, for the majority of samples that had homology to existing viruses. This meant that there was limited confidence in the results and full conclusions could not be drawn. In order to increase the number of reads for the samples several actions could be taken in further work.

Further purification of samples to ensure the ratio of virus to other components would be beneficial because more of the sequence data of interest would be generated compared to that which is not, such as host material. The standard CTAB extraction method plus RNeasy did not provide an extract of Weed 85, which was of a high enough RNA concentration, therefore a total RNA purification (Qiagen) was carried out. This sample produced sequence data that had good homology to plant viruses. It is possible that the different method of preparation compared to the majority of samples allowed viruses within the sample to be sequenced which otherwise might have been missed. However, other samples that were extracted in the standard way, such as Mown 15, also produced sequence data with homology to plant viruses. The standard CTAB method followed by RNeasy was chosen for extraction because it had been proven to be the best combination for the

majority of samples when RNA was required, but for samples which could not successfully be extracted in this way QIAquick extractions were preferential (Ian Adams, Fera, unpublished data). Commercial kits such as QIAquick are able to remove inhibitors of PCR, such as secondary metabolites which are present in some types of plant. Therefore, for samples about which less is know, such as weeds, it is not unexpected that such a method is required, compared to wheat samples which have been proven to be a suitable matrix for the CTAB extractions (Web reference – Qiagen). In the first instance all samples, including weeds were extracted using CTAB and RNeasy as this had the potential to be successful and was cheaper than using QIAquick kits.

As mentioned, each sample could be sequenced on a larger region of a plate, which would provide a larger number of reads per sample and may provide complete genomes (see Chapter 5; Monger *et al*., 2010). In this study, due to financial constraints, each sample was sequenced along with eleven other samples on one eighth of a plate. There are limitations on the amount of sequence that can be generated from one eighth of a plate, based on the reagents, beads and wells on the plate. As has been the case to date, and will continue to be so in the future, the cost of sequencing is decreasing making dedicating more of a plate for each sample a more realistic option.

Finally, more sub-samples could be taken from different parts of each plant, with each being sequenced separately to give more sequence data about the sample.

4.4.7.4 Length of reads and contigs

Table 4.3 shows the average and range of lengths of reads. The average length of read was 100bp in most cases, because reads below 100bp were removed in quality filtering; therefore, many reads were removed. The cut off of 100bp is to avoid low quality reads; however, it is possible that short but genuine data which was from a

virus was lost. Future bioinformatics tools may be able to resolve this issue by examining all reads regardless of quality and sorting them accordingly.

Tables 4.4, 4.5, 4.8 and 4.9 detail the length of reads/contigs that were homologous to a virus and the length of the homologous region. In some cases the entire read/contig was homologous to the virus. This gave a high level of confidence in the result, but it is possible that the length of the read limited the score because a longer read would have been homologous to a larger region. For example, the first contig from Weed 28/45 that was completely homologous to CfMMV.

4.4.7.5 Nebulisation

Gas nebulisation, as detailed in Roche's protocol, was performed in sample preparation. However, as the lengths of reads in the first batches were shorter than expected, later batches did not have this step in an attempt to extend read length. However, this did not significantly improve the numbers of reads which passed quality control (see Table 4.3).

4.4.7.6 Issues with the pyrosequencer

There are known problems with the 454 pyrosequencer including the inability to distinguish between bases within homopolymers, when there are three or more identical bases consecutively. In some cases a fourth identical base is falsely added (Monger *et al*., 2010; Siqueira *et al*., 2012). This fault could cause a frame shift which, dependent on the position and length of a read, may affect the ability of homologous viruses to be found by Blast searches, thus missing results. Different pyrosequencing machines and chemistries may be better able to deal with these issues, for example the MiSeq (Illumina). During the analysis of results studying the read and looking for homopolymers was done to compensate for this, and either prevent results being discarded or improving the match of sample and virus.

4.4.7.7 Reads without a MID

Reads were produced that did not have MIDs (see Tables 4.4 and 4.9), and therefore assignment to a sample was not possible. In addition to contamination that was discussed previously, it is possible that nebulisation sheared some MIDs from the sequences; however, the latter batches that did not have nebulisation had more reads without MIDs, which contradicts this theory. The reads without MIDs meant sequencing resources were wasted, and therefore potentially important data lost. Steps to minimise them in future work would be beneficial. An alternative approach to using MIDs would be to sequence all samples without MIDs and then design a qRT-PCR assay to any sequences of interest from results and test all samples to identify which sample the data were from.

4.4.7.8 Newbler produced mixed contigs

In several cases contigs were produced by Newbler that were made of reads from different samples. In this study, groups, for example wheat, were assembled together, as individual assembly would have taken an incredibly long time with the existing software. For this analysis mixed contigs had to be treated with caution and could not be used to make definite conclusions without further work. However, they were of use because they could provide evidence that a virus was present in multiple samples, but perhaps at low titre in individual samples. In this respect they highlighted potentially significant results such as that in wheat, further work to confirm that it is not a chimera would be required.

4.4.7.9 Sequence data which could not be assigned to a group by MEGAN

Large amounts of reads and contigs were not homologous to anything on the GenBank database according to Blast and were assigned to the 'no hits' or 'unassigned' groups. It is possible that viruses were present in these groups that were so different to anything currently known and included in the GenBank database that

they were not recognised as viruses. This study has shown that relatively low levels of similarity only are required for detection of a virus. Roossinck *et al*. (2010), who did a similar survey using prairie grass, concluded that many of these unassigned reads were likely to be due to viruses, because the host genes were known and on GenBank, and novel genes of them without homologues were rare. This seems likely to an extent; however, other unknown components such as bacteria could also be present in the group. A large scale, worldwide database of such sequences could eventually lead to similar sequences being identified, which would begin the process of identification of the novel sequences.

4.4.7.10 Number of samples

The financial and time constraints on this project meant that only a certain number of samples could be sequenced. Figure 4.8 shows the sampling plan that was chosen to represent the maximum area of the field. Increasing the number of samples from the site would allow a more thorough search for viruses and allow greater confidence in conclusions. Soil-borne viruses occur in patches, which can vary in size from just a few plants to an entire field (Christine Henry, Fera, personal communication). It is possible that due to the distance between samples, regions of soil-borne virus were missed. Insects, such as aphids can travel over a large range of distances, from around a leaf, to across seas (van Emden and Harrington, 2007). Therefore it is also possible that insect transmitted viruses were missed. Sampling inbetween the samples would give a more thorough representation and be more likely to sample any viruses present. It appears that CfMMV was found in a weed and an insect from a small region of the sampling site; it would be expected that the virus would be found in more samples but it was not, perhaps taking a greater number of samples from the area would have found more cases of the virus.

The samples collected for natural weeds were chosen to give as many different types of plant as possible. It was difficult to decide which plants to sample and bias may have been a problem. Quadrats may give a less biased choice of plants, but may not give such a broad range of host plants. Soil samples could also have been tested in this project to search for soil-borne viruses, although they are notoriously difficult to extract viruses from. Future methods may enable efficient testing of soils.

4.4.7.11 Investigating other types of genomic material

Ribonucleic acid was used in this work because most viruses that infect plants have RNA genomes (Roossinck *et al*., 2010). In addition, DNase was used to remove contaminants, as the method is known to be contamination prone. Host DNA was also removed by the DNase meaning the proportion of any viruses compared to other components within the sample was greater. This was important because the small size of viral genomes compared to wheat mean that it would have been less likely that they would have been detected as sensitivity would be reduced otherwise (Barzon *et al*., 2013). There are examples of viruses that infect wheat that have DNA genomes, such as *Maize streak monogeminivirus* (Web reference – Pvo2). However such viruses would have RNA intermediate steps so may have been included in the samples but, if the level of RNA present was low at the time of preparation such viruses may have been missed. Future work using DNA or total nucleic acid from the samples, achieved by not using a DNase step during sample preparation, would be of interest.

4.4.7.12 Species identification

The contents of pit and pan traps were not able to be identified on return to the laboratory due to the state of decay. It should be noted however, that the preliminary work to select a solution to fill the insect traps with (see Section 4.2.2)

allowed successful recovery of viruses, as proven by the results. The contents were homogenised, and then further prepared as the method states. Therefore it is not possible to specify which possible viruses came from which specific insects. However, the total nucleic acid extract that was not exposed to DNase could be sequenced to find which insects were present.

Weeds were identified using The Encyclopaedia of Arable Weeds, from the HGCA and internet sources, along with the help of experts at Fera. Due to the timing of the sample collection most samples were flowering which made identification easier, as plants, especially grasses, are notoriously difficult to identify if not flowering. Identification of the mown area weeds was more difficult because of the mown state of the area, but in some cases where whole plants such as clover were present, identification was possible. The saved total nucleic acid samples without DNase could be re-sequenced to aid identification.

4.4.7.13 Washing samples before extraction

It would be beneficial to wash plant samples before extraction to remove any insects that were on them; this would remove uncertainty about whether a possible virus was present in the plant sample or an insect on the plant. However, the evidence for viruses that infect insects and plants is limited, which makes this seem unlikely (Singh *et al*., 2010).

4.4.7.14 Choice of sampling site

It would be interesting to repeat this survey at other locations around the UK. Organic wheat fields may contain more viruses because insects that are vectors of some viruses of wheat are likely to be present at higher levels due to the lack of pesticides.There is evidence that a high density of aphids causing crowding, leads to aphids developing into winged morphs, and then moving from the location, thereby spreading any viruses that they may be carrying to other plants (Wadley, 1923).

Therefore, an organic site was chosen to maximise the likelihood of detecting viruses. However, organic farming may mean that there are more natural predators to contol virus vectors. For example toxins produced by spiders are effective in aphid control (Michell Powell, Fera, personal communication). Ladybirds (*Coccinella septempunctata* L.) and Green Lacewings (*Chrysoperla carnea*) are also natural enemies of aphids which are likely to be present at higher levels in organic farms (Kaplan and Eubanks, 2002). Future studies to compare to a non-organic site would be of interest. This organic site was chosen specifically because it was the only site to respond to requests to carry out the work. The financial and time constraints of the project meant that only a certain number of samples could be processed and it was decided to survey this site as thoroughly as possible rather than taking fewer samples from a number of sites.

Sampling at different times during the growing season and after periods of different weather conditions may cause different levels of viruses to be be present. For example, higher temperatures and winds may cause increased distribution of insects and any viruses they may be able to transmit to wheat. The samples in this project were collected at the end of the wheat growing season. This was in accordance with the requests of the site owners. Taking samples at this time also meant that the wheat had had almost the whole growing season to become infected and for viruses to become systemic, thereby increasing the likelihood of detecting any viruses present. However, it is possible that plant defence systems such as RNA silencing could have been functional therefore reducing the likelihood of virus detection. It is possible that insects which introduced viruses during the growing season would not have been present in the area at the time of sampling. However, if they had successfully infected a wheat plant or weed with a virus it is suggested that it would have been detected in such plants. The results of this survey apply to the

specific site and specific time at which samples were collected, thereby providing a snapshot of the prevalence of viruses.

4.5 Conclusion

A number of known and potentially unknown viruses were detected in this study. This confirms that the method used was successful. Suggested improvements have been noted, which could enable development of an even more robust tool. This method provides a very significant tool in the detection of viruses and will allow a greater understanding of viruses of wheat and other plants in the future. In addition, this method could be applied to other types of study involving other test subjects. Virology may have been limited by tools for detection in the past but this technology provides great opportunities for research. In the past the cost of this technology has meant that its use was limited; however, the cost is predicted to fall dramatically making this a more accessible option for a future work. An area for improvement in the future is the bioinformatics associated with this work because vast amounts of data are generated which need to be analysed to produce information about which viruses are potentially present and how likely they are to be genuine viruses. Further studies including Koch's postulates will always be required to confirm that a virus or viruses are present, and to explore the impact on the host, therefore next generation sequencing can complement other methods. While the vast amount of data is currently daunting and can leave unanswered questions and there can be 'red herrings', it is valuable to have such information to highlight possible results and direct further studies of samples in which currently known viruses have not been found. The main advantage of this tool is that it can search without bias for viruses (with the caveat that only viruses with similarity to those on the reference database will be found), which is an advantage of more traditional methods such as qRT-PCR. It can be used to detect viruses which could be some of those which pose significant

threats to wheat yield now and in the future, helping us to prepare and strive to meet the demands for wheat. Studies in to the impact of novel viruses on yield and their transmission methods, such as those in Chapter 6, will be required to manage such viruses.

The preliminary work to investigate insect trap storage regimes found a suitable solution, proven by the detection of RNA viruses in samples. This is the first known study in to storage of RNA in insects caught in traps.

Several known viruses were found in this study, which was not unexpected. The detection of such viruses provides information about their prevalence. The most significant result of this study is that regarding a potentially novel virus in wheat samples. Twelve wheat samples, which equates to 25% of the total wheat samples tested, are tentatively involved in the potentially novel genome. This suggests that if one novel virus was present it was relatively highly prevalent in the field. This same virus could be present in other wheat fields in the UK, and could therefore partially explain the plateau in the yield of wheat. Further study would develop this hypothesis and investigate the effects the virus has on specific varieties of wheat.

Chapter 5 - Sequencing the complete genome of Cynosurus mottle virus and using it to develop a real time reverse transcriptase polymerase chain reaction assay

5.1 Introduction

Cynosurus mottle virus (CnMoV) is a virus that has been reported in the UK (Catherall *et al*., 1977), which can infect wheat and cause symptoms such as chlorotic mottling and ultimately yield loss (see Section 1.11.1.7). Much of the research into the virus was done during the 1970s and 1980s (Catherall, 1985; Huth and Paul, 1977, Mohamed and Mossop, 1981) when the diagnostic methods of the time allowed only limited information to be collected. For example, the complete genome was not described. Here, the genome of CnMoV has been sequenced using next generation sequencing technologies that are now available. The purpose was to increase knowledge of the virus but importantly so that it could be included in the annual survey of winter wheat, which requires sequence data to develop qRT-PCR assays (see Section 3.2). In previous years antiserum was produced against CnMoV by Mohamed (1978) by injecting purified virus into a rabbit and removing the serum, and an ELISA test was subsequently developed for CnMoV. While this method is suitable for use, a qRT-PCR assay would be beneficial due to its advantages over ELISA, such as sensitivity (Mekuria *et al*., 2003). In addition, there were a large number of samples in the survey in Chapter 3, and the robotics available to support qRT-PCR made the method preferential to ELISA testing for this project. It was also more efficient to use the same nucleic acid extracts as for the other qRT-PCR tests, rather than using new samples for ELISA testing. The genome can also be included in GenBank and used as a reference for future pyrosequencing and bioinformatics work, so that CnMoV can be identified if it is present.

It was proposed that the genome of CnMoV was a 4.3 kb single stranded RNA genome, with base compositions 24.2% G, 24.3% A, 26.2% C and 25.3% U (Mohamed,

1978b). King *et al*. (2012) placed CnMoV as a tentative member of the *Sobemovirus* genus for which the type species is *Southern bean mosaic virus.* All of the members of the *Sobemovirus* genera have similarities in their particle morphology, capsid stabilization, sedimentation coefficients, sizes of protein subunits and genomic RNA. As a *Sobemovirus*, King *et al*. (2012) predicted that the genome would be 4-4.5 kb in length, consisting of one linear segment, polycistronic with four open reading frames (ORFs), ssRNA (+) and deficient of an envelope. At the 5' terminus there would be a VPg and the 3' terminus would not be polyadenylated, with no tRNA like structures. The currently accepted organisation of the *Sobemoviruses* is shown in Figure 5.1.

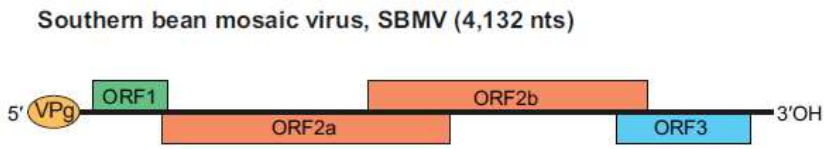

Figure 5.1. The genome organisation of *Southern bean mosaic virus* (King *et al*., 2012).

5.2 Materials and methods

5.2.1 Genome sequencing

5.2.1.1 Virus material and sequencing

Freeze dried CnMoV infected wheat was obtained from DSMZ, Braunschweig, Germany. Sequencing was carried out as detailed in Adams *et al*. (2009). Briefly, total RNA was extracted from the infected plant material and from healthy wheat. Double stranded cDNA was produced using tagged random and oligo dT primers. Polymerase chain reaction amplification was performed using Tag primers. The cDNA from healthy wheat was amplified using a nucleotide mix containing biotin-16-dUTP. The cDNA from infected wheat was amplified with unlabelled nucleotides. A subtractive hybridisation was performed, with the biotinylated uninfected wheat cDNA

becoming bound to streptavidin beads (Invitrogen). The resulting enriched infected sample was amplified again with tagged primers and the ends of the products were made blunt before sequencing. Sequencing was performed using a GS-FLX Genome Sequencer, Roche, according to manufacturer's protocols.

5.2.1.2 Completion of the complete genome

The 5' and 3' ends of the genome were completed by rapid amplification of cDNA ends (RACE), using the SMART RACE kit (Clontech) according to manufacturer's instructions. In order to resolve uncertainty of a region within the genome a portion of it was re-sequenced by Sanger sequencing, (to resolve ambiguity in the consensus sequence). Polymerase chain reaction using primers that flanked the region were produced, and PCR performed followed by cloning the PCR products using the pGEM-T easy vector system (Promega) following manufacturer's instructions. Amplification of clone inserts from transformant colonies was done by PCR using primers M13For and M13Rev, followed by Sanger sequencing.

5.2.1.3 Bioinformatics

Reads produced by the GS-FLX Genome sequencer were assembled in to contigs using the software Newbler v 2.6 (Roche). The resulting contigs and unassembled reads were used in Blast N and X searches to look for homology to plant viruses. The contigs with homology to viruses were assembled to form the genome of CnMoV, using Tablet (SCRI) and the genomes of other *Sobemoviruses* as scaffold. The Sanger sequencing of the problematic region within the sequence was used to resolve base queries in the genome. Sequence data generated by RACE was added to the ends of the genome at the appropriate sites. Vector NTI (Invitrogen) was used to separate the genome in to functional proteins, which were confirmed by Blast P searches.

5.2.2 Developing a qRT-PCR assay for CnMoV

5.2.2.1 Assay design

Primer Express v 2.0 (Applied Biosystems), (specifically the Taqman Probe and Primer design tool), was used to design a qRT-PCR assay to the coat protein (ORF 3) of CnMoV (see Figures 5.2 and 5.3). This specific region was selected because of the high number of other plant viruses whose coat protein sequences are available on the GenBank database, therefore providing numerous sequences for comparison to avoid cross reactions. Suggested primers and probe sets were assessed according to standard assay design criteria such as nucleotide length (approximately 17-30 bp), GC content (approximately 50%), melting temperature (approximately 60°C for primers and 70°C for the probe), terminating nucleotides (not exceeding 3 G or C bases at the 3' terminus), strings of identical nucleotides (not exceeding 4) and length of the amplicon (100). Comparisons to the nucleotide and protein databases of GenBank were performed to ensure specificity of the assay.

5.2.2.2 Testing the qRT-PCR assay

Two isolates of CnMoV that had been extracted from freeze dried material (DSMZ, Germany) by CTAB and RNeasy clean up (Qiagen) according to manufacturer's instructions (see Section 2.1 and Appendix 13) were tested in duplicate wells with the designed assay. Mastermix and cycling conditions were as detailed in Appendix 18 and Section 2.3.

Isolates of other viruses that are known to infect wheat or other Gramineae, were used to test the assay for specificity. Two duplicate wells were spiked with each of the following (origin of the sample is detailed); *Barley mild mosaic virus* (BaMMV), *Barley yellow mosaic virus* (BaYMV), *Cocksfoot mottle virus* (CfMV), *Oat chlorotic stunt virus* (OCSV), *Ryegrass mosaic virus* (RgMV) and *Soil-borne cereal mosaic virus* (SBCMV) (freeze dried plant material from the virology department of Fera), *Barley*

yellow dwarf virus-MAV (BYDV-MAV), *Barley yellow dwarf virus-PAV* (BYDV-PAV) and *Barley yellow dwarf virus-RPV* (BYDV-RPV) (freeze dried plant material from Bioreba) and Cocksfoot streak virus (CSV) and *Wheat spindle streak mosaic virus* (WSSMV) (freeze dried plant material from DSMZ). The isolates were extracted from plant material by CTAB and RNeasy clean up in the same way as CnMoV.

5.2.2.3 Dilution series of positive material and comparison to the existing ELISA test

Dilution series were made from identical infected and healthy material for both DAS ELISA and qRT-PCR methods, using the appropriate grinding buffers. For each method three replicates of the dilution series were tested in parallel. The ELISA was carried out according to the manufacturer's instructions (DSMZ). In the case of qRT-PCR, nucleic acids were extracted from samples using a Kingfisher 96 (ThermoScientific), for downstream testing with the qRT-PCR assay (see Section 2.3, 2.4 and 2.7). Concentrations used were: 1, 1/50, 1/100, $1/10^3$, $1/10^6$, $1/10^7$, $1/10^8$, $1/10^9$, $1/10^{10}$, $1/10^{11}$, $1/10^{12}$, $1/10^{13}$, $1/10^{14}$, $1/10^{15}$, $1/10^{16}$.

5.3 Results

5.3.1 Genome sequencing

5.3.1.1 Sequence data generated and bioinformatics

The pyrosequencer produced 30,102 reads for the CnMoV sample. Newbler assembled these sequences in to 1,152 contigs with 6,243 unassembled reads remaining. Blast N and X searches showed that nine of the contigs had homology to viruses. The contigs were organised to form the majority of the genome of CnMoV. The ends of the sequence were completed using RACE. The re-sequencing of a region within the genome for which there was uncertainty (based on pyrosequencer data), using Sanger sequencing, resolved the issue.

5.3.1.2 The genome of CnMoV

The complete genome of CnMoV is shown in Figure 5.2. It is 4,517 nucleotides long, excluding what appears to be a polyA tail. The base ratios are: A 22.6%; C 26.7%; G 27.9% and U 22.8%.

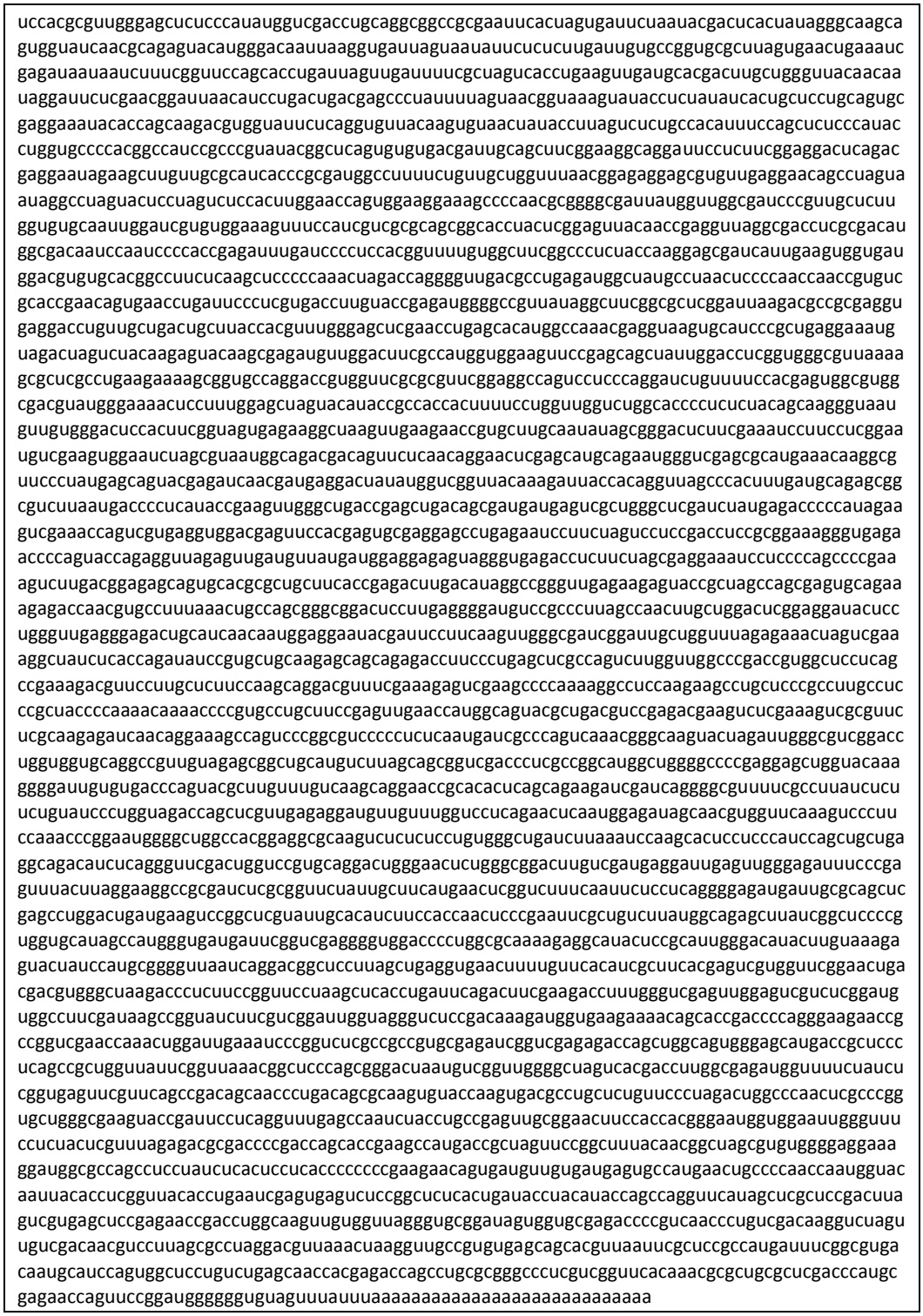

Figure 5.2. The complete genome of CnMoV.

5.3.1.3 Organisation of the genome of CnMoV

Figure 5.3 shows the organisation and translation strategy of CnMoV.

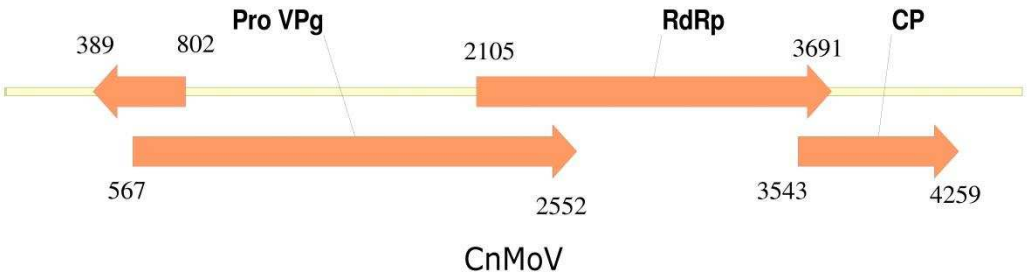

Figure 5.3. The organisation in terms of protein coding regions, of the genome of CnMoV. The arrow on the left represents ORF 1 and Pro VPg, RdRp and CP represent the VPg, RNA dependent RNA polymerase and coat protein respectively.

The proteins of CnMoV are shown in Figures 5.4, 5.5, 5.6 and 5.7. Of note is the GDD domain located in the RNA dependent RNA polymerase, which is characteristic of plant viruses (Koonin, 1991).

MPSIKLQSSDGEIFEVDVEIAKQSVTIKTMLEDLGMDDEGDDDPVPLPNVNAAILKKVIQWCTHHKDDPP
PPEDDENKEKRTDDIPVWDQEFLKVDQGTLFELILAANYLDIKGLLDVTCKTVANMIKGKTPEEIRKTFNIKN
DFTEEEEAQVRKENQWCEEK

Figure 5.4. The translated protein sequence of ORF 1.

MAFSVAGLTERSVLRNSLVIGLVLLVSTWNQWKESPNAGRLWLAIPLLLVCNWIVWKVSIVAQRHLLGVT
TEVRRPRDMATIQSPPRFDPLHGFVASALYQGAIIEVVMDVCTAFSSSPKLDQGLTPEMAMPNSPTNRVA
PNSEPDSLVTLYRDGAVIGFGARIKTPRGEDLLLTAYHVWELEPEHMAKRGKCIPLRKCRLVYKSTSEMLDF
AMVEVPSSYWTSVGVKSARLKKSGARTVVRAFGGQSSQDLFSTSGVATYGKTPLELVHTATTFPGWSGTP
LYSKGNVVGLHFGSEKAKLKNRACNIAGLFEILPRNVEVESSVMADDSSQQELEHAEWVERMKQGVPYEQ
YEINDEDYMVGYKDYHRLAHFDAERRLNDPSYRSWADRADSDDESLGSIYETPIEVETSREVDEFHECEEPE
NPSSPPTSAERVRTPVPEVRVDVMMEESRVRPLLARKSSPAPKVLTESSARAASPRLDIGRVEKSTASQRVQ
KETNVPLNCQRADSLRGCPPLANLLDSEDTPGLRETASTMEEYDSFKLGDRIAGLEKLVERLSHQISVLQEQ
QRPSLSSPVLVGPTVAPQPKDVPCSSKQDVSKESKPQKASKKPAPALPPATPKQNPVPASELNHGSTLTSET
KSRKSRSRKRSTGKPVPASPSQ

Figure 5.5. The Pro VPg of CnMoV.

MSALSQLAGLGGYSWVEGDCINNGGIRFLQVGRSDCWFRETSRKAISPDIRAARAAETFPELASLGWPDR
GSSAERRSLLFQAGRFERVEAPKGLQEACSRLASRYPKTKPRACFRVEPWQYADVRDEVSKVAFSQEINRK
ASPGVPLSMIAQSNGQVLDWASDLVVQAVVERLHVLAAVDPRRHGWGPEELVQRGLCDPVRLFVKQEP
HTQQKIDQGRFRLISSVSLVDQLVERMLFGPQNSMEIATWFKVPSKPGMGLATEAQVSLLWADLKSKHSS
HPAAEADISGFDWSVQDWELWADLSMRIELGDFPSLLRKAAISRFYCFMNSVFQFSSGEMIAQLEPGLMK
SGSYCTSSTNSRIRCLMAELIGSPWCIAM**GDD**SVEGWTPGAKEAYSALGHTCKEYYPCGVNQDGSLAEVN
FCSHRFTSRGSELTTWAKTLFRFLSSPDSDFEDLWVELESSRMWPSISRYLRRIGRVSDKDGEENSTDPREE
PPVEPNWIEIPVSPPCEIGRETSWQWEHDRSLSRWLFG

Figure 5.6. The RNA dependent RNA Polymerase of CnMoV. Of note is the GDD domain, which is in bold font and underlined.

MVKKTAPTPGKNRRSNQTGLKSRSRRRARSVERPAGSGSMTAPSAAGYSVKRLPAGLMSVGASHDLGE
MVFYLGEFVQPTATLTAQVYQVTPALFPRLAQLARCWAKYRFLRFEPIYLPSCGTSTTGMVELGFLYSFRDA
TPTSTEAMTASSGFTTASVWGGKDGASLLSHSSPPPKNSDVVMSAMNCPNQWYNYTSVTPESSESPALT
DTYIPARFIARSDLVVSSENRPGKLWLGCG

Figure 5.7. The coat protein of CnMoV.

5.3.2 Developing a qRT-PCR assay for CnMoV

5.3.2.1 The assay for CnMoV

The qRT-PCR assay for CnMoV is: forward primer: 5'-TTC TAT CTC GGT GAG TTC GTT CAG-3'; reverse primer: 5'-GCA GGC GTC ACT TGG TAC ACT-3' and probe : 5'FAM-CGA CAG CAA CCC TGA CAG CGC-3'TAM Blast searches on the GenBank database did not find homology to any other viruses.

5.3.2.2 Testing the qRT-PCR assay

The results were as follows: CnMoV sample 1 (Ct 5 and 5; ΔRn 2.45 and 2.35) and CnMoV sample 2 (Ct 3 and 3; ΔRn 2.3 and 2.3). All other viruses and negative controls produced negative results (based on no Ct or ΔRn being observed) (data not shown).

5.3.2.3 Dilution series results

5.3.2.3.1 qRT-PCR

Figure 5.8 shows the results of a dilution series of infected wheat tested by qRT-PCR. The average cycle threshold for the three replicates of the test are shown. The qRT-PCR assay was able to detect CnMoV down to a concentration of 10^6.

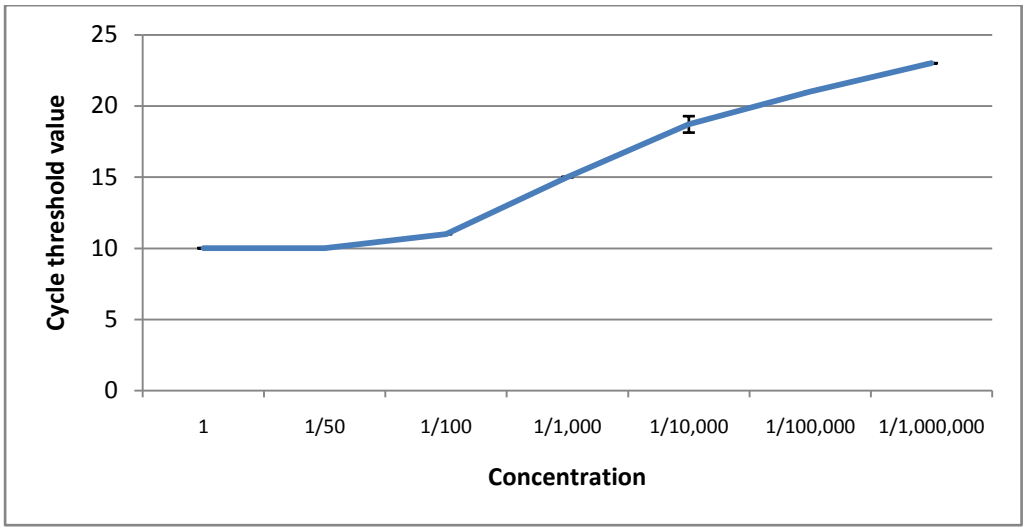

Figure 5.8. The average results of the serial dilution for the qRT-PCR assay for CnMoV, error bars greater than zero for each concentration are plotted.

5.3.2.3.2 ELISA

The results of a dilution series of wheat infected with CnMoV tested by ELISA are shown in Figure 5.9. The ELISA test was able to detect CnMoV down to a concentration of $1/10^5$.

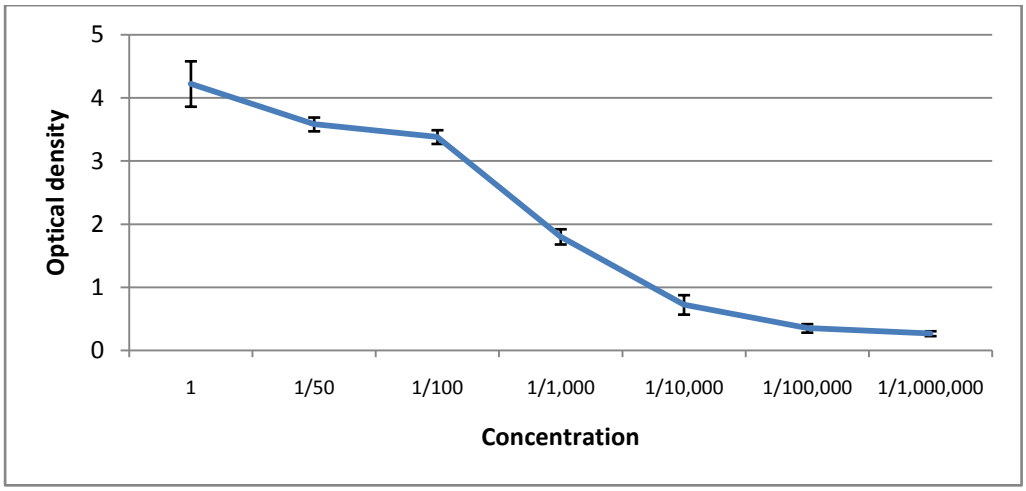

Figure 5.9. The average results of a dilution series of CnMoV tested by ELISA. The cut off point for a positive result was triple the average of the healthy control sample, which was 0.318. Concentrations below $1/10^5$ were all negative and are not shown. Errors bars of standard deviation for each concentration are plotted.

5.4 Discussion

5.4.1 Genome sequencing

5.4.1.1 Should CnMoV be considered a Sobemovirus?

The genome of CnMoV was determined (see Figure 5.2). Cynosurus mottle virus was a tentative member of the *Sobemovirus* genus and sequencing the genome of CnMoV strongly suggests that it should be included as a full member. The length of the genome and ratio of bases are relatively consistent with the predictions made by Mohamed (1978). Blast N searches found that there was homology between the complete genome of CnMoV and other members of the *Sobemovirus* genus, such as CfMV (gb FJ669143.1) (Identities = 1009/1510 (67%), score 385), *Rice yellow mottle virus* (RYMV) (emb AM883057.1) (Identities = 507/721 (70%), score 316) and *Southern bean mosaic virus* (SBMV) (gb AF055888.1; AF055888) (Identities = 416/605 (69%), score 210). According to King *et al*. (2012) species demarcation within the genus is due to three criteria, one of which is that different species have overall sequence identity of approximately 75%. Therefore CnMoV can be considered a species within the *Sobemovirus*, based on sequence homology. The genome organisation of CnMoV is similar to that of the type species for the genus (SBMV) (see Figures 5.1 and 5.3). In contrast to all the supporting evidence that CnMoV is a *Sobemovirus*, the genome of CnMoV does have a polyadenylated tail at the 3' end which does not agree with the predictions made by King *et al*. (2012). However, because the vast majority of evidence supports CnMoV being a *Sobemovirus*, it should be considered so.

Figures 5.4, 5.5, 5.6 and 5.7 show the translated proteins of CnMoV. Of note is the GDD domain located in the RNA dependent RNA polymerase, which is characteristic of plant viruses (Koonin, 1991). Comparisons can be made between the *Sobemoviruses* and the proteins of CnMoV, as are discussed below.

5.4.1.2 First protein at the 5' end

The first protein at the 5' end of *Sobemoviruses* codes for a suppressor of gene silencing, which enables systemic spread of the virus. The translation initiation codon for this protein is often missed by ribosomes, causing a leaky system which allows translation of the next protein, encoded by ORF 2 (King *et al*., 2012)

Blast searches with CnMoV did not find homology for the first protein to any other *Sobemoviruses*, on indeed any viruses. However, comparison of the P1 proteins of other *Sobemoviruses* such as CfMV (gi 82001012; spQ66011.1) and SBMV (gi 139272; spP21406.1) in MEGA 4.1 found only a few random bases were homologous (data not shown). This suggests that the region is not conserved between the *Sobemoviruses*, therefore this does not rule CnMoV out as a member of the *Sobemoviruses*.

5.4.1.3 ORF 2

According to King *et al*. (2012), the first part of ORF 2, (ORF2a) codes for the virus serine protease, VPg, C-terminal proteins P10 and P8 with ATPase and RNA binding properties, respectively. Cleavage of the polyprotein is leaky, and 10% of ribosomes make a -1 frameshift and continue transcribing the second part of ORF2, ORF2b. ORF2b encodes the RdRp of the virus (King *et al*., 2012)

The ORF2 of CnMoV is split in to two parts, labelled Pro VPg and RdRp in Figure 5.3. Blast P searches showed there was homology of the first section to the VPg of *Sobemoviruses* such as RYMV (typical instance- emb CAE81336.1, Identities = 214/663 (32%), score 233). There was also homology to the P10 of *Sesbania mosaic virus* (SeMV) (ref NP 996748.1) (Identities = 36/95 (34%), score 49.3). The second part had homology to the RdRp of numerous *Sobemoviruses*, including CfMV (ref NP 942020.1) (Identities = 321/486 (66%), score 640).

5.4.1.4 ORF 3

The ORF 3, (labelled CP in Figure 5.3) had homology to the coat protein of CfMV in Blast P searches. According to King *et al*. (2012) the coat protein of *Sobemoviruses* is encoded by ORF3. The CP is known to have a role in long distance transport of the *Sobemoviruses*.

Blast P searches of ORF 3 found homology to the coat proteins of numerous *Sobemoviruses*, such as CfMV (gb ACN78882.1) (Identities = 85/209 (41%), Score 156).

5.4.1.5 Discussion of methods

While 454 pyrosequencing provided the majority of the genome sequence, Sanger sequencing was required to determine some bases that were present in a central portion of the genome.There were also issues due to the known problem of the 454 pyrosequencer being unable to distinguish the correct number of bases in a homopolymer. Analysis using Tablet was able to resolve this problem. The overall method was successful and enabled sequencing of the complete genome of CnMoV. However, this does highlight the issue that while pyrosequencing can be useful because in produces massively parallel sequence data, it may need to be complemented by Sanger sequencing as was the case with another study to sequence *Cassava brown streak virus* by Monger *et al.* (2010).

5.4.2 Developing a qRT-PCR assay for CnMoV

5.4.2.1 qRT-PCR assay development

Blast searches and qRT-PCR tests showed that the CnMoV assay detected its target and did not detect other viruses that could potentially be infecting the target plant. As expected lower concentrations of positive sample gave higher Ct results, however this trend does not appear to happen between concentrations 1, 1/50 and 1/100; this may be because if any inhibitors (such as phenolics) were present in the original

extract they would have had a greater inhibitory effect on PCR (and therefore increased the Ct value) when at tested at higher sample concentrations (concentration 1), than when diluted (for example, concentration 1/100) (see Figure 5.8).

The qRT-PCR assay was able to detect CnMoV at a lower level than the existing ELISA tests, hence it was more sentivive (see Figures 5.8 and 5.9). Similar results have been found by other scientists such as Mekuria *et al*. (2003) in the detection of *Prunus necrotic ringspot* in almonds. Additionally the standard deviation bars for each concentration were smaller for the qRT-PCR test than the ELISA, therefore the former produces more consistent results. It would be beneficial to use the qRT-PCR test in diagnostics rather than the ELISA.

5.4.2.2 Discussion of methods

The same starting material was used for the qRT-PCR and ELISA tests, therefore a true comparison of detection ability could be made. Using healthy plant sap in the appropriate buffer for the test, rather than water for the dilution series, meant that this was a fairer test because water may have diluted inhibitors that would have led to artificially improved results. DAS ELISA was used in this study because that was the only type available at the time for CnMoV. However, should other variations of ELISA have been available such as triple antibody sandwich (TAS), or had substrate amplification been carried out as part of the method (not done as this was not included in the manufacturer's instructions) it is possible that the qRT-PCR assay would not have been more sensitive than the ELISA test. For example a TAS ELISA was more sensitive than a DAS ELISA test when testing banana (*Musa* spp.) for *Banana streak virus* (Thottappilly *et al*., 1998). Also, the use of substrate amplification was required rather than *p*-nitrophenyl phosphate, in order for the successful detection of *Beet yellows closterovirus* in *M. persicae* (Stevens *et al*.,

1997). Additionally, the assay available in this study used polyclonal antibodies. Had a monoclonal alternative been available it would have been more specific to CnMoV, but there may not have been a change in sensitivity and infact the polyclonal may be more sensitive because such antibodies are more tolerant to changes in the target epitope (Lipman *et al*., 2005). However, while the purpose of developing a qRT-PCR assay was to aid future diagnostics the main reason was so that the virus could be tested for in the high throughput testing using the same method as other viruses which was qRT-PCR (to work most efficiently in terms of time and money). Therefore, whilst additional sensitivity to the exsiting ELISA was an advantage it was not the main objective of the study.

5.5 Conclusion

The complete genome of CnMoV has been described and annotated. The evidence suggests that CnMoV is an individual species in the genus *Sobemovirus* and should be named as a full member rather than a tentative member, based on sequence data. A qRT-PCR assay, which detects CnMoV and provides reproducible, reliable and accurate results, was developed using the newly sequenced genome. It could be used to consistently detect CnMoV at a lower concentration compared to ELISA. The assay was suitable for use in the annual winter wheat survey (see Chapter 3) and for other future diagnostic applications.

Chapter 6 – Investigating the effect of Cynosurus mottle virus on the yield of wheat and the possibility that it is seed transmitted

6.1 Introduction

Cynosurus mottle virus (CnMoV) can cause symptoms in wheat such as chlorotic mottling 1-3 weeks post inoculation, extensive necrotic streaks and plant death (Catherall, 1985). However, the effect the virus can have on the yield of wheat has not been described for plants that survive CnMoV infection.

It is also important to understand the transmission methods of viruses in wheat, to inform management strategies both within the UK and overseas. Limited information is known about the transmission methods associated with CnMoV. For example, it is transmitted semi-persistently by *O. melanopa* in Britain, but by *R. padi* in New Zealand where the former does not occur (Brunt *et al*., 1996; Mohamed, 1978; Serjeant, 1967). It seems likely that *R. padi* in the UK also transmit the virus, but this has not been tested and reported. It is also transmitted by mechanical inoculation and is readily spread by machinery such as lawnmowers (Brunt *et al*., 1996; Huth and Paul, 1977). Catherall (1985) also states that sap transmission is easily achieved. Seed-borne transmission of viruses is possible amongst the Gramineae and is known to occur in cocksfoot with CSV (Gray and Banerjee, 1999; Torrance *et al*., 1994). Approximately one third of known plant viruses are seed transmitted (Sastry, 2013). Therefore it seemed possible that CnMoV could be. Literature does not discuss seed transmission of CnMoV, so it is unknown if it occurs. Since a small stock of fresh wheat seeds from plants that had been confirmed to have CnMoV infections, from the CnMoV yield trial, was available, experiments were carried out to investigate this possibility.

6.2 Materials and methods

6.2.1 Trial 1

A small scale trial was carried out to develop a method for a further larger scale trial. Six plastic trays (40 cm x 70 cm) and sufficient soil based compost to fill them were autoclaved. Wheat cv. Einstein was sown in each tray at a typical density used by commercial farmers, which is 194kg/hectare (107 seeds/m^2). To further mimic wheat growth in the commercial environment the distance between rows was 11 cm, as this was used by the combine harvester at Fera. Three rows of seed were drilled, each containing 1.81 g, which amounted to 30 seeds. The seeds were left to germinate in the glasshouse at Fera where the temperature was 18°C, under natural lighting conditions.

6.2.1.1 Vernalisation

Vernalisation is a natural period of cold temperatures through which winter wheat survives during the winter months. It is required by winter wheat in order for flowering to occur (Diallo *et al*., 2012). It is most effective between 3 and 10°C for a duration of approximately 35 days (Streck *et al*., 2003). Therefore 10 cm tall plants were incubated at 4°C for 35 days. The plants were then returned to the glasshouse where they were covered with fleecing for three weeks to prevent damage from sunlight.

6.2.1.2 Confirmation of healthy wheat plants

Three random samples from each tray were combined to give one sample, which was tested for CnMoV and a range of other viruses that can infect wheat for which there were qRT-PCR assays available (see Tables 3.1 and 3.3). The purpose was to establish that wheat was virus free (of the viruses tested for) before inoculating with CnMoV, so that any significant results could be attributed to CnMoV.

A DAS ELISA for CnMoV (DSMZ) was carried out using the same wheat samples, according to manufacturer's instructions (see Section 2.4). The samples were combined to make one sample per tray.

CTAB extractions (see Sections 2.1 and Appendix 13) were carried out and qRT-PCR testing for *Barley mild mosaic virus* (BaMMV), *Barley yellow dwarf virus*-MAV (BYDV-MAV), *Barley yellow dwarf virus*-PAV (BYDV-PAV), *Barley yellow dwarf virus*-RPV (BYDV-RPV), *Barley yellow mosaic virus* (BaYMV), *Cocksfoot mottle virus (*CfMV), *Cocksfoot streak virus* (CSV), *Oat chlorotic stunt virus* (OCSV), *Ryegrass mosaic virus* (RgMV), *Soil-borne cereal mosaic virus* (SBCMV) and *Wheat spindle streak mosaic virus* (WSSMV) (see Tables 3.1 and 3.3) was carried out, using Mastermix A and standard qRT-PCR cycling conditions (see Appendix 18 and Section 2.3).

6.2.1.3 Prevention of insect interactions

Insects should not have been able to enter the glasshouse due to its inpenetrable design, however should any enter as the door was opened further control measures were put in place. Intercept 60 WP (active ingredient - imidacloprid) (Bayer) was applied following manufacturer's instructions. The chemical is ingested by insects and according to the manufacturer, 'very soon after they become immobile and cease feeding', therefore limiting direct insect damage on plants, which would weaken them and possibly have an impact on the results, and could contribute to the spread of viruses (including CnMoV which is transmitted by aphids and *O. melanopa* (Mohamed, 1978)) from inoculated to healthy control plants.

6.2.1.4 Mechanical inoculation of CnMoV

One week after the intercept had been applied three trays (1, 2 and 3) were inoculated with CnMoV. Freeze dried CnMoV wheat (DSMZ) was used to mechanically inoculate the plants (see Section 2.5). Trays 4, 5 and 6 were the healthy

controls and were spatially separated from the inoculated trays; the plants were inoculated with buffer and celite only, to mimic the inoculation procedure.

6.2.1.5 Confirmation of infection status of the plants

After two months the ELISA and qRT-PCR tests discussed previously were repeated.

6.2.1.6 Observations, data and sample collection

Visual inspections were carried out regularly throughout the study, to look for symptoms of viral infection and general plant health. Photographic records were kept. Wheat heads were removed as they ripened and stored at4°C until harvest was complete. This was because the wheat did not all ripen at the same time, so was done to prevent grain loss in early ripening heads. Data about grain number, thousand grain weight and the number of head producing plants were collected as this was done in other studies of this type (Budge *et al*., 2008).

6.2.1.7 Grain processing and statistics

A threshing machine was used to separate the grain from the chaff. Thirty grains from each tray were removed and stored at 4°C in trial 1, for seed transmission testing (see Section 6.2.3). The remainder of the grains were dried in a grain drying oven (LTE Scientific) at 90°C overnight and then weighed. The grains were counted using a Numigral seed counter (Sinar Technology). A Mann-Whitney U test was performed manually for thousand grain weights and total number of grains for each tray.

6.2.2 Trial 2

The method used for trial 2 was almost identical to that for trial 1; however, there were some amendments. Two different varieties of wheat were used in the second trial. These were Gladiator and Scout, which were both on the HGCA winter wheat recommended list at the time of planning, and were therefore considered more relevant to farmers and funders (Web reference – HGCA6). Twenty four trays (30 cm

x 20 cm) were used rather than larger trays to allow more replicates in the limited glasshouse space available. Data about the number of surviving plants was also collected. This was assessed by visual analysis with a surviving plant being that which remained green and developed while a plant which had not survived was that which had not developed, was not green and was shrivelled. Genstat version 15 (Web reference – Genstat15) was used to perform two-way ANOVA with replication tests or generalised linear model analyses, depending on the normality of the data.

6.2.3 Seed transmission experiments

Comparisons were made between the seeds and the resulting plants, of the seeds from CnMoV infected plants and healthy wheat plants.

Three batches of five seeds from CnMoV infected wheat plants and three batches of 5 healthy wheat seeds were tested for CnMoV by DAS ELISA (DSMZ) according to manufacturer's instructions (see Section 2.4).

Three trays of thirty seeds from CnMoV infected plants and three trays of thirty fresh healthy wheat seeds (from the same batch as were tested in direct seed testing) were sown. After seven weeks five centimetre long pieces of leaf were taken from three random places in each tray and placed in separate grinding bags. Enzyme linked immunosorbent tests (DSMZ) were repeated using these samples. The test was repeated after a total of ten weeks.

6.3 Results

6.3.1 *Yield experiments*

6.3.1.1 Establishing a virus free set of wheat plants

All samples were negative for CnMoV when tested by ELISA and were negative for the range of other viruses which were tested for using qRT-PCR assay (data not shown).

6.3.1.2 Infection status post mechanical inoculation of CnMoV

Table 6.1 shows that the three trays of wheat plants that were mechanically inoculated with CnMoV were infected with the virus, because they all gave results that were above the threshold (0.702 (triple the healthy control)), and the plants in the three trays that were not inoculated remained free of CnMoV, as the results were below the threshold.

Table 6.1. Results of an ELISA test for CnMoV, post mechanical inoculation of CnMoV to half of the trays of wheat (trays 1-3) (bold font) but not trays 4-6 (italic font).

Sample in well	Optical density at 405nm absorbance (average)
Positive control	0.631
Negative control	0.234
Tray 1	**0.777**
Tray 2	**0.863**
Tray 3	**0.837**
Tray 4	*0.261*
Tray 5	*0.219*
Tray 6	*0.229*

6.3.1.3 Summary of results for trial 1

Table 6.2 shows data about the number of plants which produced heads, the number of grains produced and the thousand grain weights per tray.

Table 6.2. Summary of results from trial 1 (trays 1-3 inoculated with CnMoV (bold font) and trays 4-6 which had not been inoculated (italic font)). Results are per tray.

Tray	Total number of plants producing heads	Average number of heads on head producing plants	Number of grains	Thousand grain weight (g)
1	**34**	**1.79**	**1,115**	**15.77**
2	**38**	**1.30**	**863**	**16.13**
3	**52**	**1.84**	**1,369**	**16.62**
4	*44*	*1.72*	*830*	*15.78*
5	*52*	*1.80*	*928*	*16.33*
6	*56*	*1.56*	*976*	*20.17*

6.3.1.4 Statistical analysis

Mann Whitney U tests did not find significant differences in the total number of grains produced or thousand grain weights per tray when comparing CnMoV inoculated wheat with healthy control wheat (Thousand grain weight-U_1=6 , U_2=3 and grain number-U_1=7 and U_2=2 respectively, U_{crit}= 0 for p>0.05).

6.3.1.5 Observations

Inoculated plants showed symptoms of yellow mottling along leaves approximately one month post inoculation, the healthy plants remained asymptomatic.

6.3.2 Trial 2

6.3.2.1 Summary of results

Table 6.3 shows a summary of the data that were collected at the end of trial 2.

Table 6.3. A summary of data collected at the end of trial 2. Data is the average for all trays of the same type of wheat and infection status providing one value for each.

Type of wheat and infection status	Number of surviving plants	Number of plants producing heads	Average number of heads per head producing plant	Number of grains produced	Thousand grain weight
Gladiator healthy	7.3	3.41	3.12	111.92	28.3
Gladiator CnMoV infected	3.5	1.16	2.62	46.58	19.8
Scout healthy	9.9	2.33	2.05	34.58	17.9
Scout CnMoV infected	3.8	0.42	1.40	5.75	15.3

The approximate reduction in total number of grains for all trays when plants were infected with CnMoV was 58% for cv. Gladiator and 83% for cv. Scout. Cynosurus mottle virus infection caused approximately 30% and 15% reductions in thousand grain weight for cv. Gladiator and cv. Scout respectively.

6.3.2.2 Statistical analysis

Table 6.4 shows the results of the statistical analyses performed on the raw data from trial 2 (shown in Table 6.3).

Table 6.4. Results of statistical analyses of the data from trial 2. Results which are considered significant are those which are below the 5% significance level.

Data type (per tray)	Between cultivar result	Between inoculation treatment result	Interaction between cultivar and inoculation treatment result	Statistics test
Thousand grain weight	0.004	0.013	0.953	Two way ANOVA with replication (ANOVA)
Grain number	<0.001	0.003	0.003	Generalised linear model (GLM)
Number of surviving plants	0.047	<0.001	0.295	GLM
Proportion of surviving plants producing a head	<0.001	0.061	0.703	GLM
Average number of heads per plant	0.016	0.016	0.234	ANOVA

6.3.2.3 Observations

As in trial 1 inoculated plants of both cultivars developed a yellow mottle along leaves after approximately one month, but the healthy control plants did not (see Figures 6.1 and 6.2).

Figure 6.1. Symptoms of a CnMoV infection in wheat cv. Gladiator, two months post inoculation.

Figure 6.2. Healthy wheat cv. Gladiator, showing none of the symptoms that CnMoV inoculated wheat developed, two months post mock inoculation.

6.3.3 Seed transmission experiments

6.3.3.1 Visual observations

The seeds from CnMoV infected plants were visually identical to healthy wheat seeds. Throughout the trial the plants grew from CnMoV infected seeds did not look different to the healthy control plants.

6.3.3.2 Direct seed testing

Table 6.5. The results of DAS ELISA tests of seed from CnMoV infected wheat cv. Einstein (trays 1-3 inoculated with CnMoV (bold font)) and seed from healthy wheat cv. Einstein((trays 4-6) (italic font)).

Sample	Optical density at 405nm absorbance (average of the duplicate wells)
Positive control	2.100
Negative control	0.103
Tray 1	**0.130**
Tray 2	**0.108**
Tray 3	**0.142**
Tray 4	*0.130*
Tray 5	*0.138*
Tray 6	*0.148*

6.3.3.3 Growing infected seeds

None of the plants which grew from seed from CnMoV infected plants were positive in ELISA tests for the virus, seven or ten weeks post sowing the seed (see Table 6.6).

Table 6.6. The results of DAS ELISA tests of plants grown from seed from CnMoV infected wheat cv. Einstein (trays 1-3 inoculated with CnMoV (bold font)) and seed from healthy wheat cv. Einstein ((trays 4-6) (italic font)) after seven weeks.

Sample	Optical density at 405nm absorbance (average of the duplicate wells)
Positive control	0.331
Negative control	0.071
Tray 1	**0.065**
Tray 2	**0.063**
Tray 3	**0.068**
Tray 4	*0.065*
Tray 5	*0.066*
Tray 6	*0.077*

6.4 Discussion

6.4.1 Yield experiments

A set of healthy wheat plants was established in trial 1, because ELISA and qRT-PCR tests for CnMoV and other viruses were negative (data not shown). While these tests could not confirm that no pathogens were present, they were sufficient for this study as these were considered the most likely viruses to be present. In addition, healthy control plants were included in the study to allow comparisons to be made. However, if another virus were present, inparticular a *Potyvirus,* it could have acted syngeristically with CnMoV and resulted in greater yield loss (Tatineni *et al*., 2010). It would therefore have been beneficial to test samples using next generation sequencing rather than qRT-PCR to confirm that no viruses were present, not just known and tested for ones. However, financial and time constraints prevented this. Mechanical inoculation to half of the trays of wheat was successful in trial 1, based on observations of typical symptoms of CnMoV such as chlorotic mottling and the results of the ELISA tests (see Figures 6.1 and 6.2 and Table 6.1). The concentration of the inoculum which was applied to the wheat plants was not calculated beforehand as this was not done in other published studies of this kind (Byamukama *et al*., 2012; Seifers and Martin, 2011). If such information were available it would allow the effect of different viral loads to be studied, however all would likely be higher than that introduced by an insect vector. However, there would not be repetition of virus introduction as would likely occur with insects. It is probable that overall the plants in this study received a higher number of virus particles than would occur in the field, therefore the effects to yield may have been artificially high.

Overall a suitable method for investigating the effect of CnMoV on the yield of wheat was developed in the first trial. Due to the low number of replicates and there being just one treatment, the results of the trial could not be analysed to

produce a convincing conclusion about the effect CnMoV has on the yield of wheat. Therefore, a second trial with a greater number of replicates and two types of wheat (increasing the degrees of freedom from 5 to 47) was carried out. The tests performed during experiment establishment in trial 1, such as confirmation of infection status at various stages, were not repeated because the method was repeated in exactly the same way as had been proven to be successful in the first trial. However, clear symptoms were seen on mechanically inoculated plants but not on the healthy control plants, strongly suggesting that the infection status of the two sets of plants had been confirmed. With hindsight it may have been useful to perform these tests to confirm that the varieties in the second trial behaved in the same way as those in the first trial.

6.4.1.1 Comparison of infected and healthy plants

Trial 1 enabled a method to be developed to investigate the impact of CnMoV on wheat. This informed investigations into the impact on yield of CnMoV on wheat, which until this project had not been investigated. Additionally a model to investigate the impact of a virus on wheat within a glasshouse could not be found in published literature, only outdoor plot based experiments (Miller *et al*., 1992; Perry *et al*., 2000). Data from the trial enabled limited conclusions to be drawn such as CnMoV infections in wheat cv. Einstein decrease the thousand grain weight, but increase the number of grains, although the Mann Whitney U tests did not support significant differences. In addition, CnMoV decreased the number of plants that produce heads and the number of heads on head producing plants (see Table 6.2). Data about the number of surviving plants were not collected in trial 1, so further conclusions about whether CnMoV reduces the number of surviving plants or whether it reduces the proportion of plants that produce a head cannot be drawn.

Tables 6.3 and 6.4 summarise the results and statistical analyses of trial 2. These results indicate that CnMoV does have an impact on the yield of wheat in terms of grain number and thousand grain weight. Wheat cv. Scout showed a higher percentage reduction in terms of grain number but a lower percentage reduction in terms of thousand grain weight per tray compared to cv. Gladiator. It is preferable to have high thousand grain weights, because, for example, if the grain is to be used as seed it will contain larger embryos and reserves for future growth and it will be beneficial for downstream production (Moshatati and Gharineh, 2012). It is also preferable to have higher grain numbers for future sale and use. Therefore as has been found with other examples of viruses, CnMoV can cause yield loss in terms of amount and quality (Budge *et al*., 2008). The reduction in quantity and quality of grain is likely to be because of a decline in plant health and ability to produce energy due to reduced green leaf area, additionally the impact of diversion of the energy that is produced to other sources rather than grain production, such as virus replication or defence mechanisms against the virus. Additionally viruses have been linked with decreased rooting, transpiration and tillering (see Section 1.5). For example CfMV, which is also a *Sobemovirus,* causes stunting and reduced tillering (Serjeant, 1967). Another reason for decreased grain numbers could be linked to there being fewer surviving plants in trays of wheat that had been inoculated with CnMoV than in the healthy control. A'Brook (1972) stated that wheat infected with CnMoV exhibited a severe mottle; however, Catherall *et al*. (1977) observed a lethal mottle. The second observation supports the conclusion that CnMoV reduces plant numbers. The proportion of surviving wheat plants that later developed heads did not significantly differ between CnMoV infected and healthy control plants. However, the average number of heads per plant did decrease when wheat was inoculated with CnMoV. Therefore, the reduction in grain numbers is likely to be due to CnMoV

causing death of plants, and reducing the number of heads produced by any plants that do survive.

6.4.1.2 Comparison of cultivars

Table 6.3 suggests that cv. Gladiator is a higher yielding variety of wheat in terms of thousand grain weight and grain number compared to cv. Scout; however, the HGCA recommended lists suggest that both should yield the same (99t/ha) and that cv. Scout should acheive higher thousand grain weights than cv. Gladiator (45.7 and 44.2 respectively) (Web reference – HGCA6). While it is expected that plants grown in the field will perform differently to those in the glasshouse, likely yielding less grain, the relative yields may remain the same providing one variety is not better adapted to glasshouse conditions. The cultivar Gladiator also produced more surviving plants, a higher proportion of surviving plants which developed a head and a higher average number of heads per tray. The differences in the cultivars are interesting but the most significant for this study is that overall cv. Gladiator was more resistant to the virus than cv. Scout as there was a lower percentage reduction in total number of grains produced (58% and 83% respectively), but the opposite was true for thousand grain weight (30% and 15% respectively). There was a significant interaction between the number of grains per tray and cultivar for Gladiator and Scout (see Table 6.4). It has been reported previously that different cultivars of wheat show different levels of resistance to viruses, therefore this is not unexpected (Budge *et al*., 2008). Genes such as *Sbm1* and *Sbm2* have been implicated with resistance of wheat to SBCMV (Bayles *et al*., 2007). Both Gladiator and Scout, along with other cultivars of wheat could be studied by genetic mapping with the identification of quantitative trait loci to examine the apparent differences in resistance to CnMoV. Any resistance genes found could then be screened for by wheat breeders to develop CnMoV resistant wheat, should it be required. However, an extensive study of wheat in Chapter 3 and

of wheat, weeds and insects in Chapter 4 did not detect the virus, suggesting it is not currently a prevalent virus. The likelihood of CnMoV becoming a severe problem is dependent on the dynamics of its current vector and any other currently unknown insect vectors. Hodson (1929) stated that *O. melanopa* had been a problem in Europe and was increasingly becoming so in England. More recently research has shown that there have repeatedly been sightings and in 2012 the insect was abundant from April until September in England (Web reference – *O. melanopa*). Predictions are that the climate will become more conducive to the survival of, and will increase their spread, therefore increasing the spread of CnMoV (Ordon *et al*., 2009). For example the duration of egg and larval stages decreased with rise in temperature up to 30°C (Guppy and Harcourt, 1978). Breeders have not focussed on CnMoV in the past and it seems that the vector is already quite prevalent suggesting that the virus may not become any more prevalent in wheat. However, reassessment of the situation in the future, perhaps following repetition of the study in Chapter 3 and the main study in Chapter 4 would suggest if this virus was becoming more prevalent therefore developing resistant wheat should be considered.

6.4.1.3 Discussion of methods

The first trial was carried out as a preliminary test for the second, larger trial, which had more replicates and two varieties of wheat, therefore providing more information and data for statistical analysis. This was because a suitable method for this study could not be found in literature, in which there were few experiments into the impact of virus on wheat yield but where there were they were conducted outside on large plots (Miller *et al*., 1992; Perry *et al*., 2000). The method including equipment, experiments and processes such as vernalisation established in trial 1 and used in trial 2 was suitable for use and allowed the hypothesis that CnMoV has an impact on wheat to be investigated. There was not continuity between the

cultivars used in the trials because after using cv. Einstein to trial the method it was decided to use more relevant cultivars to todays farming industry which were both on the most recent HGCA recommended list of winter wheat (2010/11) (Web reference – HGCA6) at the time of planning the experiment. Therefore, the results were relevant to commercial growers. The trial could be repeated in the future using other varieties of wheat to compare results because varieties of wheat differ in susceptibility to viruses (Budge *et al.,* 2008).

The reason for the wheat cv. Einstein being used in the first study was that there was no record in literature of which cultivar of wheat was used in most cases and where there was the cultivars were old ones from New Zealand such as Kopara and Diplomat which it was not possible to obtain (Huth and Paul, 1977; Mohamed and Mossop, 1981). Therefore the winter wheat cultivar available at Fera at the time was used in the preliminary studies. There were no reasons to suspect that this cultivar was not susceptible to virus because it developed visible symptoms, the mechanically inoculated samples contained virus particles found a TEM (see Figure 1.6) and samples were positive in DAS ELISA tests (see Table 6.1). However, it is possible that it had a level of tolerance in terms of impact on grain because the results in Table 6.2 do not suggest significant differences between healthy and infected wheat.

The purpose of the first trial was to develop experimental design and was not focussed on gathering results in the same way as the second trial. There were some issues due to the indoor location of the trials, enforced due to spatial and financial constraints (unfortunately an outdoor plot was not available for this study). In this study, this was shown as a lower number of grains being produced and lower thousand grain weights when compared to expected values (Web reference – HGCA6). In some cases healthy wheat did not produce grain, and it is suggested that

the artificial conditions caused this. However, as the inoculated and healthy control plants were grown under the same conditions, the study did provide a true comparison of the effects of the virus. The low number of grains produced meant that specific weight could not be calculated on a sensible scale, which meant that information about the quality of the grain could not be collected other than thousand grain weight. Again, due to the low numbers of grains produced, the thousand grain weights were based on large projections. While these allow relative comparisons, larger number of grains would have given more confidence in the results as averages could have been taken. Therefore it would be interesting to repeat this experiment in a natural outdoor setting with more plants. This would have allowed more realistic yield losses to be calculated for example in Miller *et al*. (1992) and Perry *et al*. (2000), who conducted larger scale outdoor studies into the impact of BYDV on wheat. If this study were to be repeated outdoors it is unknown if the conclusions reached would be the same because many factors would be changed such as climate, which can have an affect on the impact of viruses within plants and their insect vectors (see Section 1.8). Interactions with other insects would have an impact too, beneficial insects which preyed on *O. melanopa* would reduce the spread of the virus but other insects feeding on the wheat would cause direct damage for example by removing sap thus decreasing its health (Nault, 1997). It is suggested that the overall conclusions would be comparable to those reached in this study, that CnMoV does have an impact of the yield of wheat.

It was important to create conditions as similar as possible to commercially grown wheat, so that the results of the study were applicable. For example, the sowing density was the same because there could be interactions between plants and insect/soil-borne vectors in the field that had an impact on the effects of CnMoV. For example by plants rubbing against each other, or the ease of insects to move

between plants and therefore spreading virus. The vernalisation steps were important to recreate the conditions that outdoor commercial wheat would encounter during the winter period, so that it would flower and produce grain. This meant that this wheat was effectively introduced to the virus in the spring, at growth stages 10-20. Therefore, inoculation at different growth stages would be important to learn how the effects of the virus change. For example, it is known that is a virus infects a wheat crop early the effects can be more severe (Doodson and Saunders, 1970). In a natural environment it is likely that *O. melanopa* would feed on the wheat plant more than once resulting in multiple inoculations over time, therefore experiments to mimic and study this are required. Such studies could incorporate varying levels of *O. melanopa* to study the effect. Also monitoring of insects to predict the likely effects based on insect levels would be useful. This would provide an early warning system akin to what can be drawn from the Rothamsted insect survey (Web refererence - RIS). The lighting conditions in the cold store were low, and this was unavoidable but could have had an impact on the health of the plants. However, both the healthy and inoculated plants were treated in the same way.

6.4.2 Seed transmission experiments

6.4.2.1 Analysis of results

The seeds from the CnMoV infected wheat plants appeared identical to healthy wheat seed. However, this was not unexpected as seed borne viruses are known to be undetectable by eye in many cases as they do not cause visual changes to the seed (Walcott, 2003). None of the resulting plants from either seed type showed the striking chlorotic streaks that are typically caused by CnMoV (see Section 1.11.1.7 and Figures 6.1 and 6.2). This suggested that CnMoV had not been passed from the seed to the plants.

None of the seeds that were produced by plants infected with CnMoV were positive in ELISA tests for the virus (see Table 6.5). Furthermore none of the leaf samples produced from the seeds were positive in ELISA tests for the virus, either after seven or ten weeks (see Table 6.6, ten week data not shown as same conclusion as seven weeks). These results indicate that CnMoV is not seed transmitted. Analysis using Seedcalc (Web reference – Seedcalc) indicates that for the seed and plant material testing which was carried out there is a 95% chance that the true number of infected seeds which would be present in a larger theoretical sample would range from 0-21.8% and 0-33.63% respectively. Therefore, this small scale study can only be used as a pilot. Again using Seedcalc, a future larger scale study would require a total of 70 batches of five seeds to detect 1 infected seed with 95% confidence and 120 batches of 3 samples of leaf material to detect 1 positive sample with 95% confidence.

6.4.2.2 Discussion of methods

A qRT-PCR assay for CnMoV was developed after this study (see Chapter 5), which has been proven to be more sensitive than the ELISA test used in this chapter. The qRT-PCR assay was not used in this study because the samples had already been discarded before the assay was developed. Therefore future studies would benefit from using this alternative method of testing now that it is available. There would be a greater level of confidence in the results, and this level of confidence could also be improved by repeating this study and using more replicates.

The result for the negative (healthy wheat) control in the ELISA test for CnMoV in Table 6.1 does seem high compared to those in Tables 6.5 and 6.6. However, the manufacturer's instructions were followed precisely and including development time for the substrate. There was no reason to suspect contamination of the healthy wheat with CnMoV as good laboratory practices were adhered to. This

may highlight an issue with the test, which used a polyclonal antibody, which may have detected epitopes other than the target virus. However, as the values were lower in Tables 6.5 and 6.6 than 6.1 differences in substrate development time, or quality of the healthy control could have contributed towards the differences. Unfortunately, no other test was available at the time for parallel testing. Therefore the qRT-PCR assay developed after the study would have been preferential.

Seed-borne viruses are presumed to cause full systemic infections as they move through plant cells as they divide (Hull, 2004). Therefore, sampling the leaves was acceptable. The duration of the experiment was ten weeks, and this time period was sufficient, as a period of three weeks for germination and symptom development is acceptable in growing on tests according to Walcott (2003). This study used wheat cv. Einstein, which is a variety of wheat that is known to be susceptible to CnMoV infection (see Table 6.2 and Figure 1.6). Therefore, the lack of a result of transmission was not due to a non-susceptible host. These studies could be repeated using other varieties of wheat to investigate if all varieties show the same results.

Studies by Jones *et al*. (2005) and Lanoiselet *et al*. (2008) to investigate seed transmission of *Wheat streak mosaic virus* concluded that it did occur by testing plants which grew from infected seeds by ELISA, qRT-PCR and visual observations. The studies were of larger scale using five cultivars of wheat in the former study, and planting 20,090 seeds in the latter. Therefore there is more confidence in their conclusion than this study for which further work including greater repetition using more cultivars of wheat is required, not done here as this was a small piece of work within the project. Another reason for the limited number of seeds used was that they were required for grain yield analysis, which involved them being heated to 90°C which would make them unsuitable for growth studies. Direct seed testing is

considered to be useful, but perhaps not as important as testing the resulting plants which grow because a virus could be present within a seed, but may not infect the embryo thus causing disease in the resulting plant. Therefore a positive result in a direct seed test can give an indication of seed transmission, but not a conclusion of whether disease will occur (Sastry, 2013).

There are examples of viruses causing shrivelled discoloured seeds to form in other plants such as peas, but this cannot be used as a reliable diagnostic tool for detection of viruses in wheat seeds because the effects can be subtle and undetectable by eye. For example there may be a decrease in seed size in wheat seed (Latham and Jones, 2001; Lanoiselet *et al*., 2008). It was noted that there were no observable differences in seeds from CnMoV infected plants compared to healthy ones. Therefore other diagnostic methods were used. If seed screening is required, for example at points of entry, it would likely be necessary to send seed to a laboratory where testing by ELISA or qRT-PCR could be done. As explained, visual detection is not reliable and would not be practical for large seed bulks. Approximately one third of plant viruses are known to be transmitted through seed, which does not include those viruses and plants for which seed transmission has not been tested, therefore this figure could be higher (Sastry, 2013). Therefore, seed testing prior to seed movement is important in controlling the spread of viruses, especially when global trade is ever increasing. The rate of seed trasnsmission may be low, for example 0.4% for *Wheat streak mosaic virus* but this is still important (Lanoiselet *et al*., 2008). This is because planting just a small proportion of infected seeds will produce plants from which the virus can be transferred to other healthy plants by other transmission methods such as insect or soil-borne vectors. Therefore large scale studies are required to extend this study which would highlight very low levels of seed transmission of viruses.

6.5 Conclusion

The second yield trial suggests that CnMoV does have an impact on the yield of winter wheat. The thousand grain weight and grain numbers were reduced in trial 2, with the former statement also supported by trial 1. The reason for these differences is that CnMoV causes plant death, as seen in trial 2. In addition the virus reduces the number of heads per plant that are produced, supported by trials 1 and 2. This virus has the potential to cause moderate to high yield loss in UK winter wheat. It is important to investigate the yield effects viruses can have on wheat in order to understand the threat they pose to the amount of wheat which can be produced in the UK. In the past, viruses have not been considered such an important threat as other diseases such as fungal. Studies such as this highlight that viruses can cause large yield losses, optimistically bringing them to the attention of scientists and researchers, which has not necessarily been the case in the past. The large scale survey of wheat in Chapter 3 did not suggest that CnMoV was present at high levels in the UK currently, therefore the yield losses seen in this study are not currently occurring. However, future climate predictions suggest favourable environments for *O. melanopa* perhaps increasing the threat of CnMoV (Ordon *et al*., 2009). Met Office data suggests that spring and autumn temperatures are increasing (see Figure 1.10), which are the critical points for insect vector infection for example spring is when wheat is most vulnerable. Studies on the impact of viruses on wheat in recent years primarily come from Australia and America where there have been reports of high yield losses of up to 100% due to viruses such as *Wheat streak mosaic virus, Triticum mosaic virus* and *High plains virus* (Blunt and Brown, 2003; Byamukama *et al*., 2013; Forster *et al*., 2001; Lanoiselet *et al*., 2008; McNeil *et al*., 1996; Seifers *et al*., 2009; Tatineni *et al*., 2009). These areas have higher temperatures, likely contributing to greater spread of viruses by insects and effects of the virus within the plant for

example due to greater replication of the virus. In the future such climates may occur in the UK and we may experience the problems due to viruses that have occurred in other parts of the world (see Section 1.8).

Secondly, the study into seed transmission of CnMoV in wheat cv. Einstein, suggests that it does not occur. However, due to the nature of the trial, further larger scale studies are required for full conclusions to be drawn. If it was concluded that seed transmission did not occur, plants that are diagnosed with CnMoV could be used for future growth without concern of the virus recurring. Management strategies, both within the UK and globally, can use this fact to develop suitable transport and quarantine measures for wheat that is diagnosed with the virus in the future. The methods used here are suitable for the investigation of seed-borne viruses and could be used in future studies, but with greater numbers of samples. As with yield studies, seed transmission studies and discussions have not been carried out in the UK, rather America and Australia (Dwyer *et al.,* 2007; Jones and Latham, 2001; Lanoiselet *et al*., 2008) but again these could become more of a priority in the future should viruses become more of a problem in the UK.

It is possible that viruses may become more of a problem in the future. It is important to prepare now by investigating which viruses could cause large yield loss and understand the methods by which they spread. In the past this could only be done for known viruses but studies such as that in Chapter 4 using next generation sequencing can highlight currently unknown viruses which could be some of the important viruses facing wheat in the future.

Chapter 7 – Discussion and conclusion

This project had several aims; a summary of how these have been achieved and an overview of results follows.

- **Assess the incidence of known characterised viruses in UK wheat**

  From an extensive survey of wheat (1,356 samples) over a period of four harvests (2009-2012) *Barley yellow dwarf virus-MAV* (6 samples)*, Barley yellow dwarf virus-PAV* (6 samples) and *Soil-borne cereal mosaic virus* (12 samples) were detected. Therefore a selection of twelve viruses currently known to be in the UK were not present at high levels.

- **Investigate the possibility that currently unknown viruses are present in UK wheat**

  It is likely that currently unknown viruses are present in wheat in the UK, because next generation sequencing of 120 samples (consisting of wheat, weeds and insects) from a field in Suffolk detected potentially novel viruses (eight, with four being detected in wheat). One such tentative novel virus was detected in 25% of the wheat samples tested.

- **Sequence Cynosurus mottle virus (CnMoV) and develop a real time reverse transcriptase polymerase chain reaction assay (qRT-PCR)**

  The genome of CnMoV was described following sequencing using next generation technology and Sanger sequencing. The result suggested that the virus is a *Sobemovirus*. A qRT-PCR assay was designed using the genome sequence, and this had a lower limit of detection than an existing enzyme linked immunosorbent assay (ELISA). The assay was included in the extensive survey of wheat, and the conclusion was that CnMoV is not currently present in wheat in the UK.

- **Measure the impact of CnMoV on the yield of wheat**

  The reduction in yield due to CnMoV varies according to the variety of wheat tested; for example the number of grains decreased by 58% for cv. Gladiator and 83% for cv. Scout when compared to the healthy control. Therefore CnMoV can have a significant impact on the yield of wheat.

This project investigated the hypothesis that viruses could be contributing to the plateau in the yield of wheat in the UK, by investigating their impact, prevalence and identification. Such an hypothesis was based on the fact that viruses can have significant impacts on wheat, as was highlighted in the literature review (see Section 1.11) and was further proven in the course of this project in inoculation trials with CnMoV, in which there were significant visual symptoms and losses in yield (30% reduction in the thousand grain weight compared to healthy controls in wheat cv. Gladiator) (see Chapter 6). Despite their potential impact, viruses have not been studied to the same depth as other causes of disease of wheat such as fungi, and the incidence had not been studied on a large scale in the UK previously. Therefore it was possible that there were numerous wheat-virus interactions which were unknown. This is supported by Roossinck *et al*. (2013) who discuss that there are likely to be thousands more plant viruses to add to the 900 we currently know about. In order to find such viruses, it stands to reason that we have to first look for them. This is illustrated by the work of Horvath (1983) who discovered a significant number of previously unknown angiosperm species-virus interactions simply by being the first to test for those interactions. The lack of screening for viruses in wheat is due to several reasons, for example viruses do not consistently produce such obvious symptoms as other diseases of wheat, and so they have been ignored and considered less important. Additionally, the diagnostic tools required to perform reliable large scale

screening for viruses have not been historically available. As a result, the control of viruses has been deficient in the past. While more traditional methods such as analysis of visual symptoms are still useful today to confirm and investigate viruses of wheat, they alone cannot lead to firm conclusions because all known viruses of wheat have as yet caused only a limited range of symptoms, making specific diagnosis difficult (see Section 1.11). Transmission electron microscopy (TEM) is a useful diagnostic tool, but it is time consuming, biased towards the more obvious virus particles, subjective and based on existing knowledge of particle morphology making detecting novel viruses difficult. More modern diagnostic tools such as qRT-PCR and next generation sequencing offer significant opportunities to screen for viruses of wheat in large numbers of samples efficiently and accurately, including novel viruses. The applicability of the methods to large scale testing in relatively short periods of time is one of the main advantages these methods offer over more traditional ones such as TEM or inoculation studies. It offers something extra compared to the other techniques listed because it allows us to more realistically work towards finding these potential thousands of new viruses because it is not target led, which would limit searches. In the literature review in Section 1.11 the first virus discussed was a striate mosaic virus, which was based on the diagnostic tools of the 1950's. It was not possible to make a specific diagnosis and to record specifics of the virus so that it would become a unique virus not to be confused with others, as was likely the case with this virus. More modern techniques should prevent this confusion.

Real time reverse transcriptase polymerase chain reaction tests of UK winter wheat showed that the prevalence of a selection of viruses considered the most likely to be present and causing an impact on wheat (based on their symptoms, host range and historic geographical spread) were not prevalent at high levels. Due to the

large number of samples tested over a long period of time the results of this survey are likely to accurately represent the current situation in the UK. The reason that only a few viruses were detected could be due in part to insecticides which are routinely used in modern farming, thereby decreasing insect vector numbers. Additionally, as discussed in Chapter 3 the weather conditions during critical part of the growing season likely contributed to the result. For example the colder than long term average autumn of 2010 likely caused fewer insect vectors to infect young vulnerable wheat with viruses. While such viruses as those detected in the survey are likely to have a small role in causing the plateau in yield, if viruses are a cause of the plateau it seemed more likely that an as yet unknown virus or viruses were having a more significant impact. Next generation sequencing was used to investigate this possibility, exploiting the major advantage of this technique, that it does not require prior knowledge of targets unlike qRT-PCR. This technique has also been used by Kreuze *et al*. (2009) to detect two novel *Badnaviruses* and one *Mastrevirus* in asymptomatic sweet potato and by Roossinck *et al*. (2010) to detect potentially numerous novel viruses in prairie grass. The samples examined in this project were from an organic centre in Suffolk. As expected, some known viruses were detected, but the most significant result was a potentially novel virus which was found in 25% of the wheat samples. Further work is required to confirm whether this virus is genuine, investigate its impact on wheat and that it actually causes a decrease in yield. If a currently unknown virus was at high prevalence in UK wheat it seems likely that it would be cryptic, otherwise it would have been detected already. Therefore the lack of obvious symptoms in the wheat studied does not rule out the possibility that this virus is a significant cause of yield loss. As discussed a cryptic virus was detected by Kreuze *et al*. (2009) which was confirmed to cause symptoms and yield loss. This is because cryptic viruses have effects on their plant host without producing

visible symptoms, for example by reducing replication of host plant cells and energy available for development (Roossinck *et al*., 2010). Additionally, work to understand the prevalence of the virus is necessary; this novel virus could potentially be a widespread virus, considering its prevalence in the one field tested. It is also possible that a higher number of wheat samples were infected with this virus, but these were missed due to inadequate sampling, ultimately due to financial and time constraints which limited the amount of sequencing which could be done per sample and the number of samples sequenced. In addition to this potentially novel virus there were numerous other examples of potentially novel viruses such as a *Potyvirus* in wheat. *Potyviruses* are known to be transmitted by aphids in a non-persistent, non-circulative manner with a helper component and specific amino acid triplets in the coat protein (DAG for some *Potyviridae*) required (King *et al*., 2012). Such a sequence was not present in the read associated with this result, but this is likely because the homology was to the cytoplasmic inclusion protein and not the coat protein. Further sequencing could lead to the coat protein being detected, therefore enabling searches for the motif. No *Potyvirus* like sequences were detected in insects, therefore either the aphid vector was not present, or perhaps a different method of transmission is involved. It is important to highlight that a large number of potentially novel viruses have been found in a relatively small scale study, which suggests that if this experiment was repeated in different areas of the UK it is possible that many more wheat-virus interactions would be detected. This is supported by Roossinck *et al*. (2010) who concluded that the majority of the data from next generation sequencing of prairie grass, which had no homology to anything on GenBank, were novel viruses. While these conclusions are likely to be exaggerated, because the unknown data could partly be attributed to other novel entities such as bacteria, they do support the theory that there are likely to be numerous currently

unidentified viruses. The experimental approach developed in this project could also be used to test other plant species, providing a powerful tool. Financial and time constraints permitted only one location to be sampled and only a certain number of samples. Repetition in the future at more sites using more samples would provide more information about the prevalence of currently known and unknown viruses in the UK. The potential viruses which were detected in the study would require Koch's postulates to confirm causation and further studies to ascertain if the viruses caused yield loss to wheat and whether they caused visual symptoms or were cryptic. Studies such as those in Chapter 6 would provide such information, and inform control strategies should such viruses be seed-borne. Other possible vectors should be investigated too.

Therefore the current situation is that known viruses are unlikely to be contributing significantly to the plateau in the yield of wheat. It is possible that future studies will conclude that novel viruses have been present for some time, perhaps introduced at in the late 1990s when the yield plateau was becoming established (see Figure 1.1). It is likely that the prevalence and impact of known and as yet to be discovered viruses in wheat in the UK will increase in the future. This is for several reasons, including globalisation of trade and travel which are not new but have increased recently, and will continue to do so, removing natural barriers of virus spread and allowing them to travel long distances to the UK (West *et al*., 2012). One example of this long distance spread is the occurrence of related isolates of *Ryegrass mosaic virus* (RgMV) in Canada, South Africa and Wales (Salm *et al*., 1994). Known transmission methods for this virus are by the insect *A. hysterix* and mechanical inoculation (Mulligan, 1960; Web reference –Pvo). The vector *A. hystrix* is widely distributed in the northern hemisphere and has been reported specifically in Canada, South Africa and the United Kingdom (A'Brook, 1975; Frost, 1992; Hill, 2008; Salm *et*

al., 1994). The vector could have been moved in plant material, especially as the insect has been found on a large range of plants and it is very small (80–250 µm) (Wang *et al*., 2011). Therefore the distribution of the vector is likely in part responsible for the spread of the virus. According to Dwyer *et al*. (2007) the transfer of *Wheat streak mosaic virus* (WSMV) between Australia and the USA can be traced to a port at which infected wheat seed entered, highlighting the importance of understanding the transmission methods of viruses, predicting their spread and putting in place control measures. In this study the potential for seed transmission of CnMoV was investigated as it was unknown if this occurred. This was important because CnMoV causes considerable yield loss in wheat, and because approximately one third of plant viruses are seed transmitted (Sastry, 2013). The study suggests that seed transmission does not occur for this virus in wheat; however this was a small scale preliminary investigation and further work is required using greater numbers of samples from a range of cultivars of wheat before full conclusions can be drawn on which control measures can be based.

Another possible reason that the threat viruses pose to UK wheat yield could increase is that climate change is expected to exacerbate the threat that crop diseases such as viruses pose to food security (Stukenbrock and McDonald, 2008). This is due to direct impacts of the virus on wheat and also the affect on vectors of viruses. By 2050, the UK in general (local climates will differ and there will be seasonal variations) is predicted to experience higher temperatures (an increase of approximately 2°C), unpredictable rainfall, including periods of drought (which may be severe) and floods (Gornoll *et al*., 2010; Web reference – Met4). An increase in temperature is likely to increase replication and spread of viruses through a plant, and also increase the severity of symptoms; however there is also evidence that at higher temperatures symptoms are reduced or disappear, as occurs when *Banana*

streak virus infects banana plants (*Musa* spp.) at 28-35°C rather than 22°C, when *Barley yellow mosaic virus* infects barley above 20°C or in *Cucumber mosaic virus* infections of muskmelon (*Cucumis melo*) above 37°C. This has been attributed to increased host defence responses including production of siRNA, and decreased viral replication (Chellappan *et al*., 2005; Dahal *et al*., 1998; Hill and Evans, 1980; Hull, 2004; Huth, 1988; Roossinck, 1991).

Increased temperatures are likely to increase the importance of viruses transmitted by insects in the UK such as mites (WSMV), aphids (*Barley yellow dwarf virus* (BYDV) and leafhoppers (*Wheat dwarf virus* (WDV)) (Ordon *et al*., 2009). In the UK movement of insects from their specific normal locations occurs seasonally due to temperature; therefore insects and the viruses they transmit may become more widely spread throughout the UK, including the colder northern regions (Cannon, 1998). Some vectors of viruses worldwide may have been unable to survive in the cooler climate in the UK in the past, and would have died on entry. However this may not be the case in the future and they could introduce novel viruses to wheat. For example, Agassiz (1996) found that of the 288 Lepidopteran species introduced to the British Isles, 10% became established. The introduction of new vectors and hosts to an area provides an opportunity for new viruses to be spread as was the case in Brazil when biotype B of the whitefly (*Bemisia tabaci*) became established and transferred viruses from non-cultivated plants to tomato in which a new virus was detected (Fernandes *et al*., 2008).

Of the known global wheat viruses there are two which pose perhaps the most significant risk to UK wheat, these are WDV and WSMV. They have both shown considerable geographic spread (including within Europe such as in France and Germany) and have significant impact on wheat, with the latter being attributed to 100% yield loss in wheat in Australia (Lindblad and Sigvald, 2004; McNeil *et al*., 1996).

There is no evidence of the vector of WDV, *P. alienus* in the UK nor the vector of WSMV (*A. tosichella* Keifer) (Ostoja-Starzewski and Matthews, 2009). This perhaps explains why the viruses have not yet been reported, and were not found in Chapter 4 of this study. However, the threat of these vectors is ever present and realistically possible. For example a related insect to *A. tosichella* Keifer, *Aceria tulipae* Keifer was introduced when onions from the Netherlands were imported and distributed to a number of farms in England in 2006. Control measures involving destruction of crops and monitoring of insects were deemed successful and the insect did not spread (Ostoja-Starzewski and Matthews, 2009). However, this highlights the potential for introduction of novel insects and viruses. The threat of introduction of WDV and WSMV is increased because many UK wheat breeders bulk their seed up in countries such as Germany and France before bringing it back to the UK, and according to wheat breeder DSV virus testing is not carried out except for SBCMV (Matthew Kerton, DSV United Kingdom Ltd, personal communication). This lack of testing is a serious issue. There are no records of WDV being seed transmitted (but this does not mean that it does not occur, just that it has not been tested for), but there are for WSMV (Lanoiselet *et al*., 2008). The insects which transmit the viruses are also very small, for example *A. toschella* Keifer are approximately 0.3mm long making them difficult to see by eye thus avoiding detection (Navia *et al*., 2013). Should these viruses arrive in the UK it is possible that they could form synergistic relationships or recombine with other viruses that are present. This was suspected to be the case in Turkey, where analysis of the sequences of barley strains of WDV from Turkey found there has probably been recombination between a barley strain and an as yet un-described WDV-like *Mastrevirus* species to produce it (Ramsell *et al*., 2009). Additionally it has been shown that when WSMV infects wheat in double infections with *Triticum mosaic virus* (also not present in the UK to date) there is disease

synergism causing worse symptoms and higher viral loads than a single infection (Tatineni *et al*., 2010). While these two viruses (WDV and WSMV) are currently considered two of the most serious threats, it is possible that there are other viruses which have similar vector, distribution profiles which could also enter the UK, but which are currently unknown and undetected.

The predicted conditions in the UK are also likely to enable such insects to survive later in to winter and emerge earlier in spring in greater numbers. Met Office data of long term autumn temperatures shows that there is a trend of increasingly warmer temperatures (see Figure 1.10). Additionally it has been shown that a 1°C increase in temperature in January and February causes aphids to emerge four weeks earlier than normal (Web reference - BBSRC). Therefore insect vectors are active and able to spread viruses for a greater proportion of the year and importantly when wheat is most susceptible to viruses, in its juvenile stages thus exacerbating symptoms (Doodson and Saunders, 1970; Lindblad and Sigvald, 2004). There is also evidence that increased CO_2 levels increase the fecundity of aphids, which is thought to be due to increased plant volatiles; this would mean there were more vectors which could transmit viruses to wheat (Awmack *et al*., 1996).

Soil-borne viruses transmitted by *P. graminis* thrive and show increased spread in warm moist soils. Such conditions may not be common across the UK as a whole, but there may be local examples and therefore localised outbreaks of soil-borne viruses such as *Soil-borne cereal mosaic virus* (SBCMV) (Ledingham, 1939).

The direct impact of climate change on wheat is discussed in detail in Section 1.8, but briefly higher temperatures may increase wheat yields if the increase is during the vegetative stages, but be detrimental to yield if they are in the vegetative stages. Droughts and floods would both be detrimental to wheat yield and it is also

possible that the impact of other pests and disease will be worse (Dodd *et al*., 2011; Foulkes *et al*., 2007; Whalley *et al*., 2006). Therefore the situation is uncertain.

Other methods by which the number of viruses in the UK could affect wheat could include the introduction of varieties of plants which are novel to the UK, due to them having certain qualities such as drought resistance. While this may be positive in the intended sense, such plants could also introduce novel viruses, through seed transmission, (if introduced as seed) or if introduced as green plants, by them acting as a reservoir and source of inoculum for insect vectors which may feed on them and then spread viruses. Thereby increasing the diversity of viruses which can then be transmitted to wheat (Garrett *et al*., 2006). Again, these viruses could form synergistic relationships with other viruses in the UK producing even more damaging effects on wheat than if the viruses infected singularly.

In the future, if the novel perennial wheat which is being developed currently was established in the UK, the problem of viruses is likely to increase because there would not be removal of infected material at the end of each season which occurs normally when wheat is harvested (Hayes *et al*., 2012). As discussed in Section 1.11.1 there are viruses which affect a number of cereal crops, such as BYDV affecting wheat and barley, therefore the year round presence of perennial wheat, potentially acting as a reservoir of viruses could result in more insect transmission of viruses to other cereals.

Ultimately, all of these factors mean that the prevalence and impacts of viruses in wheat in the UK may be greater in the future (Dahal *et al*., 1998; Sacks *et al*., 2012). To focus on the harvest of 2013, the prediction was that the prevalence and severity of viruses of wheat would be lower in comparison to previous years, such as 2012 (see Section 3.3.2.2.4). This is because of the prolonged periods of cold temperatures and snow during the autumn and winter months and in to April. For

example one of the most crucial times for insect transmitted virus inoculation to wheat is autumn, when for example in September the temperature was 0.7°C below the UK long term average and October was 1.3°C below the UK long term average (Doodson and Saunders, 1970: Web reference – Met Office autumn 2012). Additionally a study by Rothamsted Research states that the temperature in the south of the UK (where the majority of UK wheat is grown) was 1-2°C below normal for January and February, and in the north of the UK it was 1°C below normal, thus aphids were likely to emerge 2-4 weeks and 2 weeks later respectively (Web reference – AHDB; Web reference - HGCA4). Therefore, they were not able to transmit viruses to wheat during its vulnerable younger stages; however, the growth of wheat may also have been retarded by the climate making timings relative and therefore insect introductions to a similar stage of the wheat. Additionally according to the NFU president, Peter Kendall, farmers had only managed to plant 75% of the wheat which was planned due to the cold weather. Therefore, yields were predicted to be lower anyway. It was suggested that farmers may be able to sow seed in the spring to make up for this gap, but this would potentially expose wheat to insects during juvenile and vulnerable stages (Web reference – NFU). Soil-borne viruses, such as SBCMV are also likely to have occurred at a lower prevalence in 2013 because of the frozen soils during the winter which limited their spread (Kanyuka *et al*., 2003). The regional yield for 2013 was 7.4 tonnes per hectare, producing 12.1 million tonnes, which was a decrease of 8.7% on 2012. This downfall was said to be due to decreased planting due to prolonged wet conditions, but improved yields helped offset the gap (Defra Farming statistics report 17th October 2013, Web reference – Defra 2013). This was perhaps due to the decreased impact of viruses as discussed, a warm summer (0.8 °C above the long term average) with high levels of sunshine aiding the development of grain and relatively low disease pressure from diseases

such as Septoria and rusts in spring/summer (Farmers Weekly 12th June 2013; Web reference - Met Office summer 2013). Therefore despite poor predictions, if the climate becomes more favourable within a wheat season and appropriate actions are taken to take advantage of that (planting more wheat in the spring) then yields can be successful. It is unknown what the prevalence of viruses was as the practical aspect of this study including the annual winter wheat survey ceased in February 2013. However, it is predicted that viruses would not have been detected at high prevalence and would therefore not have had a large impact on the yield of wheat. In the short term it is unknown what the climate in the UK will be exactly, therefore lessons from the past will be drawn upon to strive to fulfil yield requirements.

It is clear that there is a threat that viruses could enter the UK from abroad, adding to those already present here. In the future, surveillance for known and unknown viruses is required in order to detect them as early as possible and control their spread and impact. Targeted applications such as qRT-PCR are a valuable tool, as this project has demonstrated. The study which was carried out in Chapter 3 could be repeated regularly to monitor the prevalence of viruses in wheat. While there are no current plans to do this, it could be considered as a subject for future project proposals. While such a method is useful, other tools such as next generation sequencing may become the method of choice because it enables a deep investigation into samples, with the caveat being that only targets with some similarity to a known entity whose sequence is on a database such as GenBank can be detected. However, as demonstrated in this project (see Chapter 4) the level of similarity can be low, and as the number of entries to GenBank increases so too does the likelihood of detecting a target. The financial cost previously prevented widespread use of this technology, but this is unlikely to be the case in the future because the cost has decreased dramatically and is likely to continue to do so (cost

for a full plate of sequencing at Fera-£8,000 in 2009 and £6,000 in 2013). An issue with the next generation sequencing method is that vast amounts of data are produced which require skilled bioinformaticians to conduct time consuming analyses. However this issue is currently being debated across the globe and solutions sought to easily handle the data in acceptable time scales (Siqueira *et al*., 2012; Prabha *et al*., 2012). It has been suggested that more questions are raised than answered when using pyrosequencing, and that it usually produces results which require confirmation by other laboratory methods. However despite the daunting prospect of analysing the potentially novel or unexpected viruses which are detected, it is important to do so or scientists may be missing important causes of disease by using targeted diagnostic tools for only those viruses we already know about. Radford *et al*. (2012) state that 'an exciting era of viral exploration has begun' with reference to next generation sequencing. The method of next generation sequencing has also been used in a range of other areas, for example Tran *et al*. (2013) have confirmed its usefulness in analysis of the affects of drugs on genes related to human cancer. Shanks *et al*. (2013) were able to detect novel mutations causing early onset of retinal degeneration in humans and Salvioli and Bonfante (2013) studied the range of soil mycorrhiza. In terms of novel virus discovery next generation sequencing is a powerful method which allows high throughput of samples, with no bias (except the caveat discussed) and is preferential to using other methods such as TEM or visual symptoms. Kreuze and Cueller (2011) have used small interfering RNA (siRNA) in connection with next generation sequencing to identify novel viruses, which has an advantage in that amplification or enrichment are not required because the anti-viral defence mechanism of the plant serves that purpose. A symptomatic sample of Yam beans (*Pachyrhizus* spp.) was tested and it was possible to assemble the siRNA in to the genome of a novel virus which they named, *Yam bean mosaic virus*. This method

is also powerful, and could be used as an alternative, but it requires extensive bioinformatics work to assemble the siRNA data and relies on the host plant having begun the process of siRNA production. In this project a successful method was developed for use with next generation sequencing, including the optimum storage solution for insect traps which enabled good recovery of PCR amplifiable RNA for which a method was previously unpublished (see Section 4.2.2). This method could be repeated for wheat in other locations in the UK, or using other plant hosts. In addition to monitoring viruses of wheat in the UK it is also important to attempt to prevent their entry, therefore stringent measures at points of entry to the UK could exploit technology such as next generation sequencing to test imports. However, the results would currently not be available in acceptable time scales and samples would have to be sent to a laboratory with sophisticated equipment and skilled staff. In the future the method could perhaps be used once it has been developed further, but it is likely the sample would still have to be sent to a laboratory. The method could also be used if other tools have been unable to conclude the cause of symptoms, or if a screen for all viruses including the currently unknown is required. A person testing samples at the point of entry or on farm who may not have scientific experience or sophisticated diagnostic tools requires a practical, robust, readily available tool for example lateral flow devices which are available for some viruses such as *Pepino mosaic virus,* but none are currently known for wheat viruses. Perhaps this is because viruses of wheat have not been given a high level of importance, and that it is thought that once a wheat crop in infected nothing can be done and that the harvest will remove the virus. However that it not the case for soil-borne viruses and in the case of insect transmitted viruses risks insect vectors moving from the wheat to reservoirs but returning to the crop the next year (de Boer and Lopez, 2012; Salomone *et al*., 2002). Should any viruses enter the UK, control measures discussed

in Section 1.7 would become important, with the specifics dependent on the methods of transmission and type of wheat. For example, natural differences in resistance to viruses could be exploited, as were highlighted in the yield study with CnMoV and wheat cv. Gladiator and cv. Scout (see Chapter 6). A currently restricted option for the future could be genetic modification, to transfer resistance genes for viruses to varieties of wheat which may have other benefits such as drought resistance. An additional concern is that while restrictions on chemical pesticides have benefits such as environmental, they may cause a lack of control of insects which may be vectors of viruses (which may not be compensated for by the effects of the joint survival of natural predators), thus exacerbating the effects of viruses (Philips *et al*., 2011). Other methods to bridge the gap in yield may be required, such as growing wheat on a greater area of land. However that would cause release of CO_2, nitrogen loss from such areas and have other impacts such as reducing the habitats of wildlife (Carlton *et al*., 2012; Gregory, 2008).

It is not thought that viruses alone are causing the plateau in the yield of wheat, and even if they were present at higher levels than those found in Chapter 3 and 4, that would not be so. It is likely that there are numerous factors other than viruses which have contributed to the plateau in the yield of wheat, and infact many of these factors may interact with each other and with viruses. While Coakley *et al*. (1999) concluded that studies into climate change and plant diseases and pests were lacking due to experiments studying only one or two factors, performing such experiments in laboratories which are unlike natural conditions and test periods being too short, Garrett *et al*. (2006) later stated that there had been considerable advances including the 'explosive' use of genomics. The ability of both a pathogen and the plant host to evolve separately to survive in the future conditions will dictate how plants such as wheat fare; this is relatively unknown. According to Brisson *et al*.

(2010) over the past two decades weather patterns in France such as decreased rainfall and a decrease in the number of sunshine hours (the latter limiting grain filling) have decreased wheat yields. In the Great Plains of the USA, Graybosch and Peterson (2012) concluded that periods of drought were a major cause of yield loss in wheat. A twenty five year trial in China also concluded that rainfall, in connection with nitrogen levels, limited the yield of wheat (Guo *et al*., 2012). There are suggestions that soil compaction, soil pH and poor drainage of soils have also contributed to yield loss in wheat. Conditions such as these which reduce the fitness of wheat and make it more susceptible to pests and diseases (Garrett *et al*., 2006); increases in ozone can also reduce the resistance of plants to diseases (Gregory *et al*., 2009). However, some climate change predictions such as increased CO_2 levels are expected to have a positive effect on wheat yields, associated with changes in plant architecture such as increased surface area and by the CO_2 fertilisation effect. However, this may not be great enough to meet future wheat yield requirements, and if associated with increased humidity this could cause increases in foliar disease such as rusts (Jaggard *et al*., 2010; Manning and von Tiedemann, 1995; Pritchard *et al*., 1999). Farming practices such as intensification and diversification may be responsible for increasing diseases of crops (Anderson *et al*., 2004). While new varieties of wheat may provide opportunities for increased yield these may not have been chosen, or at least been suited, to the farms they were used on thereby limiting yields (Knight *et al*., 2012). According to Knight *et al*. (2012) the level of nitrogen and sulphur applied to crops has been deficient, because there is 'a slight increase in the optimum N fertiliser dose for new, higher-yielding varieties'. Such requirements would not be prevented due to the rules of nitrate vulnerable zones (RB209) set by the government (Defra) because the guidelines state that if there are higher potential

yields possible with certain varieties, more than the normal amount of nitrogen may be used (Knight *et al*., 2012).

Fischer and Edmeades (2010) state that the majority of cereal yields have shown a decrease and while the yield of oil seed rape (*Brassica napus*) has been increasing since 2004 it had previously been sporadic. Several reasons have been proposed for this, including some in common with wheat, such as nitrogen and sulphur deficiency (Knight *et al*., 2012). While these could be corrected to an extent by investment in fertilisers (which if the return price for produce is good will encourage farmers to do so), it does suggest that nitrogen and sulphur are very important and that good agronomy is vital for successful crop yields. There is also evidence that increased levels of CO_2 cause increased uptake of nitrogen from soils as they grow faster, thus depleting resources more rapidly (Riesenfeld *et al*., 2004). Other diseases such as *Fusarium graminearum* and *Septoria* spp. can infect wheat, with the latter having caused a decrease in yield improvement of 0.01 tonnes per hectare between 1996 and 2002, which was in part due to the development of resistance to fungicides. However investment in fertilisers caused an increase in yield improvement of 0.01 tonnes per hectare between 2002 and 2011. *Fusarium* spp. are predicted to cause more severe impacts in the UK (especially the south) in part due to wetter but warmer conditions in the spring (Knight *et al*., 2012; Madgwick *et al*., 2011; West *et al*., 2012). There is also evidence that changes in farming practices such as minimum tillage rather than ploughing increases infections of crops (oats) by *Fusarium langsethiae* because residues of infected plant material remain near the soil surface, thus able to infect the next crops (Imathiu *et al*., 2013). Rusts have also had an impact on wheat and it is predicted that they may fluctuate in the future because their individual temperature requirements are different (2-15°C for stripe rust, 10-30°C for leaf rust and 15-35°C for stem rust (Roelfs *et al*., 1992).

As discussed in detail in Section 1.8 there is an alternative view of the role of viruses, which suggests that they can help plants to adapt to climate change (Malmstrom *et al*., 2011; Xu *et al*., 2008). This could become a benefit for naturally infected plants in the future, but it seems unlikely that humans would intentionally exploit this by performing a form of vaccination due to the risks if viruses formed synergistic relationships with other viruses and control was lost.

In conclusion viruses can have a significant impact on the yield of wheat. As demonstrated in the literature review in Section 1.11 and yield study in Chapter 6. However, currently the prevalence of known viruses is relatively low, which suggests that they are not a major cause of the plateau in the yield of wheat; however, they are likely to be contributing to it especially when weather conditions are favourable for viruses and their vectors. It seems more likely that if viruses are a cause then a currently unknown virus or multiple viruses, such as those detected using next generation sequencing in Chapter 4 could be responsible. The diagnostic tools used in this study, particularly next generation sequencing will become increasingly valuable in identification as climatic conditions and globalisation of trade and travel threaten to increase the prevalence and impacts of viruses further. While viruses are currently not a major issue in the UK, it is important not to let them be forgotten and ignored as they were in the past, but to monitor the situation with the newly available diagnostic tools.

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VectorNTI (Invitrogen) - http://www.invitrogen.com/site/us/en/home/Products-and-Services/Applications/Cloning/vector-nti-software.html, Accessed 27/1/13.

Suppliers

Applied Biosystems (Warrington, United Kingdom)
ATCC (Middlesex, United Kingdom)
Bayer (Cambridge, United Kingdom)
Biorad (Hertfordshire, United Kingdom)
Bioreba (Peterborough, United Kingdom)
Clontech (Saint-Germain-en-Laye, France)
DSMZ (Braunshweig, Germany)
Eurofins (Wolverhamptom, United Kingdom)
Fermentas (now Thermoscientific)
Fisher Scientific (Loughborough, United Kingdom)

Hamilton (Birmingham, United Kingdom)

Invitrogen (Paisley, United Kingdom)

LTE Scientific (Oldham, United Kingdom)

Promega (Southampton, United Kingdom)

Qiagen (Manchester, United Kingdom)

Roche (Welwyn Garden City, United Kingdom)

Sigma Aldrich (Dorset, United Kingdom)

Sinar Technology (Surrey, United Kingdom)

Thermoscientific (Cheshire, United Kingdom)

```
         *IPMVRTAAENSHARTGLLENLVAMIKRNFNAPELSGIIDIENTA*Wheat 24
         *IPMVRTAAE    +TGLLENLVAMIKRNFNAPELSGIIDIENTA*Consensus
         *IPMVRTAAEMPR-QTGLLENLVAMIKRNFNAPELSGIIDIENTA*TMV
```

Appendix 1. Comparison of the amino acids of one read from Wheat 24 and the replicase of *Tobacco mosaic virus* (gb AAS75432.1) (TMV), the consensus sequence is shown.

```
*TKSSDTYTYEFDVPKFDKSQDMMCFTLILEVMTIMGVSDDFVNYWRRASYEGTIFNSCFS*Contig
*TKSS E D+ KFDKSQ++ +VM GVS++ + W + E + ++*Consensus
*TKSS----LEIDIKKFDKSQELSVLQFECKVMRYFGVSEELIYLWFHSHVESIVKDTRNG*OLYaV

*MVFTLFYGNRSGSGGTLAVNCICLLFAFFTEFSNLNIVAALIKGDDSVLI*Contig
*+ F L RSG GGT N + L+ F ++ AL GDDS+L+*Consensus
*LKFKLQVQRRSGDGGTFFGNTMFLIAVMARNFDLNSLDLALFSGDDSLLV*OLYaV
```

Appendix 2. Comparison of the amino acid residues of the mixed contig to the RdRp of *Olive leaf yellowing-associated virus* (emb CAD29306.1) (OLYaV), consensus sequence is shown. Note the GDD sequence which is a known conserved motif in the RdRp of plant viruses.

```
*AHTELSSRKEREVCAATRYSFYLAFGYTPDEQVAIESYFANHVIEPTFSESGTPARASEC* Pit 1
*A ++LS+RKER++   +RYSFYLAFGYTPDEQVA+E+YF   VI+P FS+SG+PARASEC*Consensus
*ALSKLSTRKERDISTQSRYSFYLAFGYTPDEQVAMENYFDKLVIDPNFSDSGSPARASEC*CfMMV

*LLLQLLPQQPTVVITTAPSTDSRRR*Pit 1
*LLLQ        +I   P+T S +R* Consensus
*LLLQLIPKQPTTHSHKR* CfMMV
```

Appendix 3. Comparison of amino acids from a read from Pit 1 to the p106 of *Cocksfoot mild mosaic virus* (gb ABW74550.1) (CfMMV), the consensus sequence is shown.

```
*EFDKIWPHMVLKYITYYPCSLNWRGMPTIPIVYVSKHFWYELYRTGFLNKLYHCGSWTDI*Contig 00009
*EF KIWPHMVLKYITYYPCSLNWRGMPTIPIVYVSKHFWYELYRTGFLNKLYHCGSWTDI*Consensus
*EFHKIWPHMVLKYITYYPCSLNWRGMPTIPIVYVSKHFWYELYRTGFLNKLYHCGSWTDI*ACBV

*LLLLSGDVETNPGPVETYKDLCRRKNIRKRKSRAREEIKMQQHIDKIIRQENEEYKIINV*Contig 00009
*LLLLSGDVETNPGP+ETYKDLCRRKNIRKRKSR REEIKMQQHIDKIIRQENEEYKIINV*Consensus
*LLLLSGDVETNPGPIETYKDLCRRKNIRKRKSRTREEIKMQQHIDKIIRQENEEYKIINV*ACBV

*NMQGIFSFN*Contig 00009
*NMQGIFSFN*Consensus
*NMQGIFSFN*ACBV
```

Appendix 4. Comparison of the amino acid residues of Contig 00009 to the replicase of *Acute bee paralysis virus (*gb AAN63804.2|AF486073 1) (ACBV), the consensus sequence is shown.

```
*TTDKGYDASLMYYSNVGTNQIVARAGNDDFTFGWLIGTPQTQGITRTETK* Contig 00010
*TTDKGYDASLMYYSNVGTNQIVARAGNDDFTFGWLIGTPQTQGITRTETK* Consensus
*TTDKGYDASLMYYSNVGTNQIVARAGNDDFTFGWLIGTPQTQGITRTETK* ACBV
```

Appendix 5. Comparison of the amino acid residues of Contig 00010 to the capsid protein of *Acute bee paralysis virus* (gb AAO74622.1) (ACBV), the consensus sequence is shown.

```
*GCDNPTLFEILPKLRPFNHGLSLPS*Mown 15
*GCDNPTLFEILPKLRPFNHGLSLPS*Consensus
*GCDNPTLFEILPKLRPFNHGLSLPS*WCMV
```

Appendix 6. Comparison of the amino acids of a read from Mown 15 to the triple gene block 3 protein of *White clover mosaic virus* (ref NP 620718.1) (WCMV), the consensus sequence is shown.

```
*VRNPTVSMSKDSHSITPFYTASSMRKWIRAVGECGLSLTGGMPIKQEYYKCLIRNGQGKGKIHT*Contig
*VRNP VSMSKDSHSITPFYT ++M+KWIRAVGECGLSLTGG+PIKQ YYKC IRNG  KGKIHT*Consensus
*VRNPAVSMSKDSHSITPFYTPNTMKKWIRAVGECGLSLTGGIPIKQSYYKCFIRNGADKGKIHT*CfMMV
```

Appendix 7. Comparison of the amino acids of a contig made of seven reads from Weed 45 to the p106 of *Cocksfoot mild mosaic virus* (ref YP 002117834.1) (CfMMV), the consensus sequence is shown.

```
*ASVTSTGPVIIPPSHNTTYHHEKYQNIEVQK*Contig
*ASVTSTGPVIIPPSHNTTYHHEKYQNIEVQK*Consensus
*ASVTSTGPVIIPPSHNTTYHHEKYQNIEVQK*CfMMV
```

Appendix 8. Comparison of the amino acids of a second contig made of twelve reads from Weed 45 to the p6.8 of *Cocksfoot mild mosaic virus* (gb ABW74552.1) (CfMMV), the consensus sequence is shown.

```
*AALMPADGLIRGPSDTEILAHQTAKQVALHRDAKRRGTNVVNSVEITNGRSDPIAPLITYPQ*Contig
*AALMPADGLIRGPSDTEILAHQTAKQVALHRDAKRRGTNVVNSVEITNGRSDPIAPLITYPQ*Consensus
*AALMPADGLIRGPSDTEILAHQTAKQVALHRDAKRRGTNVVNSVEITNGRSDPIAPLITYPQ*WCMV
```

Appendix 9. Comparison of the amino acids residues of a contig made of 62 reads from Weed 28 to capsid protein of *White clover mosaic virus* (gb ABG88080.1) (WCMV), consensus sequence is shown.

```
*RDCFMEDERLEIDTLEDEVSQDANNNKPTGLQNIEEAVKNNPDLPWAPWLIILQAHNADC*Contig 2
*RDCFMEDERLE DTLEDEVSQ+ANNNKPT LQNIEEAVKNNPDLPWAPWL+ILQAHNADC*Consensus
*RDCFMEDERLETDTLEDEVSQNANNNKPTSLQNIEEAVKNNPDLPWAPWLLILQAHNADC*WCMV

*TEKQYDPENNLILPIQEINTLPKHQHPDIPRDLLTLLTKLHREPTTVPLDNHRARAYGSD*Contig 2
*T+KQYDPENNLILPIQEINTLPKHQHPDIPTDLLTLLTKLHREPTTVPLDNHRARAYGSD*Consensus
*TQKQYDPENNLILPIQEINTLPKHQHPDIPTDLLTLLTKLHREPTTVPLDNHRARAYGSD*WCMV

*VKNLRIGAL*Contig2
*VKNLRIGAL*Consensus
*VKNLRIGAL*CfMMV
```

Appendix 10. Comparison of the amino acids residues of a contig made of 62 reads from Weed 28 to the RNA replication protein of *White clover mosaic virus* (ref NP 620715.1) (WCMV), the consensus sequence is shown.

```
*SQNKASEAEPYGKILEFDFLKRHFKADELIPSLFHAPLHKRSIEEQVYWIREGGNSLELLEANIENALYEAHHH *Weed 50
*++NKASEA+PYGKILEFD FLKR HFKADELIPSLFHAPLHKRSIEEQVYWIREGGNSLELLEANIENALYEAHHH *Consensus
*AKNKASEAKPYGKILEFDFLKRHFKADELIPSLFHAPLHKRSIEEQVYWIREGGNSLELLEANIENALYEAHHH *PYFV
```

Appendix 11. Comparison of the amino acids from one read from Weed 50 to the RdRp of *Parsnip yellow fleck virus*(ref NP 734447.1) (PYFV), consensus sequence shown. Note the FLKR domain.

```
*RCRFAGIPSGIFCTQFWGSFYNSVMVVSVLKALGISMTNDYFIKVLGDDVLFGILKLVPV*Weed 92
*R RFAG+PSGIFCTQFW SFYN +MVV+ L+ALG +T+ YF+KVLGDDV+FGILK +P+*Consensus
*RRRFAGMPSGIFCTQFWDSFYNCIMVVTTLEALGFRITDRYFLKVLGDDVIFGILKHIPI*RSCV

*CQWADFLEAFSAEAKRRFNSNLNSRKSGVTTGIHGAQVLSYKNWNGYPKRDAELLLAQLL*Weed 92
* +WADFL+ FS EA+RRFNS LNS+K G ++GIHGAQVLSY NWNGYPKRD+  LLAQLL*Consensus
*SKWADFLQDFSTEARRRFNSKLNSKKCGASSGIHGAQVLSYINWNGYPKRDSNQLLAQLL*RSCV

*HPKSLRDT*Weed 92
*HPKSLRDT*Consensus
*HPKSLRDT*RSCV
```

Appendix 12. Comparison of the amino acid residues of read from Weed 92 to the RNA-dependent RNA polymerase of *Raphanus sativus cryptic virus* 1 (gb AAX51289.2) (RSCV), the consensus sequence is shown. Note the GDD motif which is characteristic of the RdRp of plant viruses.

Appendix 13. CTAB buffer – used in CTAB extractions throughout the project

2% Cetyl trimethylammonium bromide; 100 mM pH 6.4 trisaminomethane, pH 8.0; 20 mM ethylenediaminetetraacetic acid (EDTA); 1.4 M sodium chloride; 1.0 % sodium sulphite; 2.0 % poly vinyl pyrrolidone-40.

Appendix 14. pH 6.4 GITC 1 –used in Kingfisher extractions in* Chapter *3

5.25 M guanidiniumthiocyanate; 50 mM pH 6.4 trisaminomethane-hydrochloride buffer (1M trisaminomethane-hydrochloride; 1M trisaminomethane). Set to pH 6.4

Appendix 15. pH 6.4 GITC 2 – used in Kingfisher extractions in* Chapter *3

5.25 M guanidiniumthiocyanate; 20 mM EDTA; 1.3% (wt/vol) triton X-100; 50 mM pH 6.4 trisaminomethane-hydrochloride buffer (see Section 2.3). Set to pH 6.4.

Appendix 16. TnaPP (8.38%) – an additive to make Kingfisher grinding buffer

16 mM tetrasodium pyrophosphatedehydrate; 50 ml molecular grade water.

Appendix 17. PBS pH 7.4 – a solution trialled for storage of insects

0.14 M sodium chloride; 1.47 mM potassium di-hydrogen orthophosphate 8.097 mM di-sodium hydrogen orthophosphate dodecahydrate, 2.68 mM potassium chloride; 1 L distilled water.

Appendix 18. qRT-PCR mastermix A

For each reaction 11.3 µl DEPC treated nuclease free water, 2.5 µl Buffer A (Applied Biosystems), 5.5 µl $MgCl_2$ (25 nM) (Applied Biosystems), 2 µl

dNTPs(deoxyribonucleotide triphosphates) (6.25 mM) (Fisher Scientific), 1 µl forward primer (7.5 pmol), 1 µl reverse primer (7.5 pmol), 0.5 µl probe (5.0 pmol), 0.125 µl AmpliTaq Gold (5U/µl) (Applied Biosystems) and 0.05 µl RevertAid (200U/µl)(Fermentas) and 1 µl sample was prepared to give a final volume of 25 µl. DEPC treated nuclease free water replaced samples in the negative controls.

Appendix 19. qRT-PCR mastermix B

This was identical to qRT-PCR mastermix A, except 2.3 µl DEPC treated nuclease free water was used, rather than 11.3 µl and 10 µl sample was used rather than 1 µl.

Appendix 20. Mechanical inoculation buffer

9.5 : 0.5 stock A : stock B (stock A: 9.46 g of di-sodium orthophosphate (Na_2HP0_4) per litre of molecular grade water, stock B: 9.07 g of potassium di-hydrogen orthophosphate (KH_2PO_4)per litre of molecular grade water)).