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# Student Report No. 32

# The identification, prevalence and impacts of viral diseases of UK winter wheat

Flint, L.J[1,2].

[1]The Food and Environment Research Agency, York YO41 1LZ. United Kingdom

[2]The University of Nottingham, School of Biosciences, Sutton Bonington, Leicestershire LE12 5RD. United Kingdom.

**Supervisor: Boonham, N.[1], Dickinson, M.[2], Fox, A.[2] and Turner, J.[2]**

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# 1. 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 (qRT-PCR) testing of 1,356 UK wheat samples from 2009–2012 using eleven assays 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 (required for downstream testing) 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. Additional 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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## 2. Introduction

The aims of this project were 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 (qRT PCR) assay
- Measure the impact of Cynosurus mottle virus on the yield 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. 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). Therefore it is of great concern that the yield of wheat in the UK appears to have reached a plateau which began around the year 2000, see Figure 1.

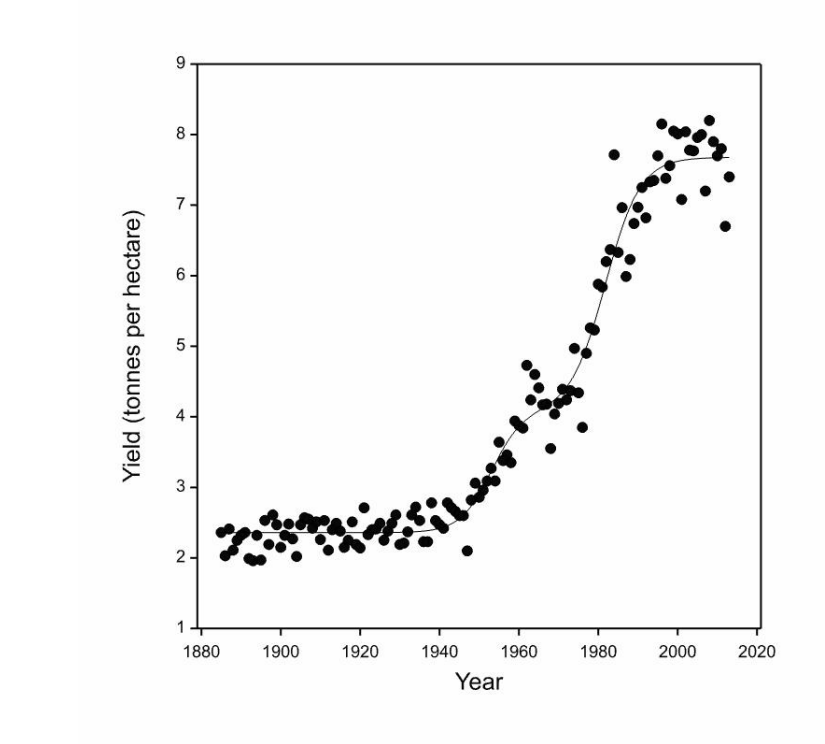


**Figure 1. The yield of wheat achieved in the UK between 1880 and 2013. Modified from Defra statistics (Stephane Pietravalle, Fera, personal communication, data for 1880–2005). Data added for 2006–2013 from Defra farming statistics (Web reference – Defra farming statistics).**

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 *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). Visual symptoms in wheat 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) (see Figures 2 and 3).

**Figure 2. Cynosurus mottle virus causing shortening of internodes which results in stunting in wheat cv. Gladiator (image taken in this project).**

**Figure 3. Cynosurus mottle virus causing a severe chlorotic mottle (central image) and necrosis (background) in wheat cv. Scout (image taken in this project).**

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). Viruses have had significant financial impacts and worldwide total losses 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 focussed on protection from pests and disease, and therefore viruses. It is predicted this would 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.

It is acknowledged that it is unlikely that there is just one cause of the yield plateau and other contributory factors which won't be investigated in this project include wheat bulb fly (*Hylemia coarctatain*) and orange wheat blossom midge (*Sitodiplosis mosellana)* (Web reference – HGCA 3 and HGCA 5) and fungal diseases such as *Septoria tritici* and *Fusarium* spp. (Willocquet *et al*., 2008).

Historically viruses have not been given such a high level of importance as other diseases of wheat, perhaps this is due in part to a lack of simple methods to detect them, therefore they have not been included in disease surveys of UK wheat (Cook *et al*., 1991; Polley and Thomas, 1991). Such techniques are now available and are exploited in this project. For example, next generation sequencing technology which has revolutionised plant virology according to Prabha *et al*., (2013).

It is possible that there are many viruses present in UK wheat which we do not know about, and more could be arriving all the time. While it is accepted that 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), wheat seed is not tested on entering the UK, providing a route for entry. The added danger is that while in the past the climate of the UK would mean insect vectors of virus and the viruses themselves would not become significant problems, the predictions of climate change such as global warming may mean this is not the case in the future (Ordon *et al*., 2009). At farm level, 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 occur in reality, perhaps due to time or financial pressures. 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.

A literature review, which can be found in the full version of the thesis, shows that there are a vast number of viruses that have already been reported in wheat globally, which, if tested for, could potentially be detected in wheat in the UK. 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. These two viruses are those which most farmers/agronomists asked about viruses of wheat know about.

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 *Wheat dwarf virus*, *Soil-borne wheat mosaic virus* and *Wheat streak mosaic virus*. 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. As discussed, the UK may become a more favourable environment for vector survival in the future, thereby allowing their establishment.

# 3. Section 1 - 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. Modern diagnostic techniques are now available that provide opportunities for high throughput 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 qRT-PCR assays for a selection of such viruses.

## 3.2. Materials and methods

### 3.2.1. Selection of viruses to test for

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. Development of qRT-PCR assays

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 Section 4 for CnMoV assay design). Briefly, primer design involved the use of MEGA 3.0 to align the sequences for each virus (downloaded from GenBank). Areas of shared sequence 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 alignments and by BlastN searches on the GenBank website, to choose the set which matched the target best. 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. The assays were validated in physical testing before use in the survey, for qualities such as specificity to target virus.

### 3.2.3. Samples for the survey

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. Winter wheat samples at growth stage 75, from the Defra winter wheat disease survey conducted by Fera, were sub-sampled 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. 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.

#### 3.2.4. Sample preparation and testing

Automated nucleic extraction was carried out using a Kingfisher 96 (ThermoScientific) (see Appendices 1.2, 1.7 and 1.8). An automated liquid handling robot (Star line, Hamilton) was used to prepare qRT-PCR plates (see Appendices 1.3 and 1.12). All samples were tested for wheat phenylalanine ammonia-lyase gene before use in the survey (a wheat internal control gene) (Walsh *et al*., 2005). This was to ensure the extraction of total nucleic acid had been successful and that samples were suitable for testing. All samples were tested in duplicate with all assays developed in this project and two by Mumford *et al*., (2004) for BaYMV and BaMMV.

## 3.3. Results

#### 3.3.1. Developed assays

Ten of the assays designed proved to be specific for their target virus and showed repeatability. However, despite troubleshooting, the assay for *Oat mosaic virus* did not and was therefore not used.

#### 3.3.2. Results of the survey

***2009 -*** None of the samples were positive for any of the assays.

***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).

***2011 -*** None of the samples were positive for any of the assays.

***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 1). 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 2).

**Table 1. 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 2. 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. Details of the samples location, variety of wheat and qRT-PCR result are shown in Table 3.

**Table 3. 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 4 shows the location of wheat samples that were positive for a virus in this study.

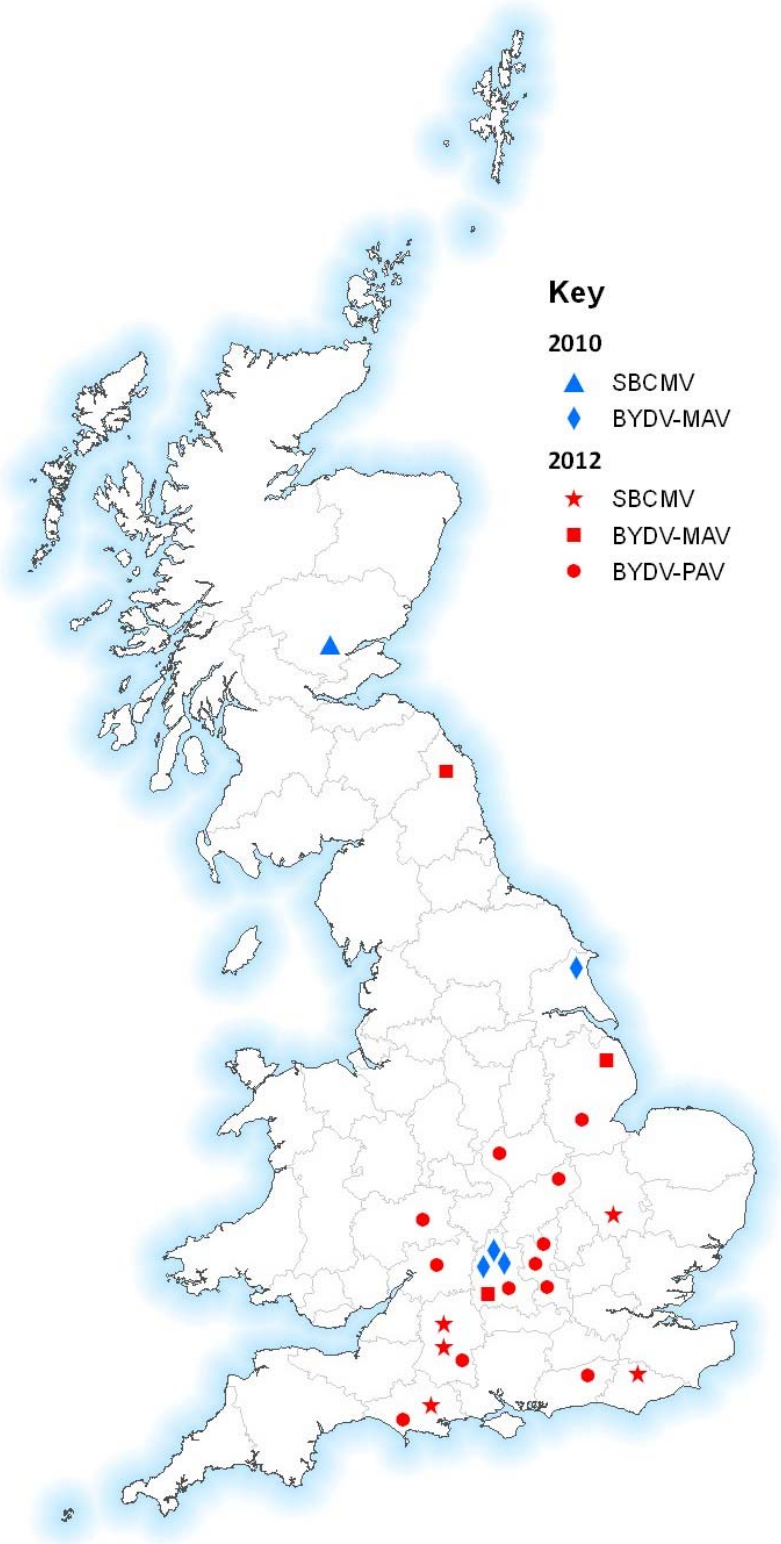


**Figure 4. The prevalence of viruses of wheat in the 2009/2010, 2011 and 2012 surveys. Results are shown at county level, the location of the symbol within the county does not reflect the location of the site because such specific data was unavailable for the majority of samples.**

## 3.4. Discussion

### 3.4.1. Positive results and metadata

In 2010 there were findings 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 (Hoffman and Kolb, 1997; Mastari *et al*., 1998). 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 11th October 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 is actually perhaps irrelevant because the critical time for aphid transmission of viruses to wheat is in the autumn. 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* (SBCMV) 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 (Budge *et al*., 2008; Kanyuka *et al*., 2003; Ordon *et al*., 2009), and therefore supports the conclusion that the virus was present.

The results of wheat testing from the virology department of Fera support the results of this study to an extent (data not shown). The department also detected 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. 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). Early infections are known to cause worse symptoms due to BYDV (Kennedy and Connery, 2001). *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 (Annual 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 (Annual 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, Rothamsted 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 the AHDB Cereals & Oilseeds project RD-2008-3475 (2010) 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. 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; Annual 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. 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 (Annual 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 climatic 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, 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). While this is important in terms of symptoms, it also increases the amount of virus in the plant, thus the chance of detecting a virus by qRT-PCR (Dahal *et al*., 1998; Hull, 2004).

### 3.4.3. Location of viruses

Figure 4 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. 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 (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).

### 3.4.4. Varietal differences

Tables 1, 2 and 3 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 AHDB Recommended List for 2013/2014 (Web reference Fwi; HGCA 7). 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.

### 3.4.5. Breeding strategies

The viruses that were found were BYDV-MAV, BYDV-PAV and SBCMV. It is not surprising that these viruses were found, that have been found in the UK in the past, are the only viruses of wheat that a selection of farmers at the Cereals industry event whom I spoke with were aware of. 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 Section 2.

# 4. Section 2 - 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. 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 1), therefore these are unlikely to be causing the plateau.

While target-designed applications such as polymerase chain reaction (PCR), real-time reverse transcriptase PCR (qRT-PCR), enzyme-linked immunosorbent assay (ELISA) and transmission electron microscopy (TEM) are valuable for diagnosis of certain viruses, they are inherently biased to their target and require prior knowledge of it, such as a sequence to design the assay. 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). The method effectively involves obtaining the sequence of everything in the sample and comparing those sequences to databases of sequences of known organisms such as GenBank, with the results showing if there are any known organisms, and in this cases viruses present.

## 4.2. Materials and methods

### 4.2.1. Samples

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, therefore screening them could reveal viruses that may already be in the wheat crop or could transfer to it.

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 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 3.

#### 4.2.2. Sample preparation and sequencing

Total nucleic acid was extracted from the samples using a CTAB extraction followed by an RNeasy column with on-column DNase digestion (see Appendices 1.1 and 1.6) (Qiagen, following manufacturer's instructions). Quantification was carried out using a spectrophotometer (Nanodrop ND-1000, ThermoScientific) and a fluorometer (Qubit machine and Qubit-iT™ RNA HS kit, Invitrogen) to ensure the samples were of suitable quality for further testing.

The samples were prepared according to the methods recommended by the manufacturer of the pyrosequencing machine (Roche). The sequence data which was produced was analysed using several bioinformatics tools including Newbler v 2.6 (Roche), MEGAN (Huson *et al*., 2007) and blast searches of GenBank on the NCBI website. The aim was to compare the data to the database of sequences and identify if there were any sequences which matched those of any plant viruses, therefore suggesting a virus was present in the original sample.

### 4.3. Results

Table 4 shows some of viruses on GenBank to which sequences from the samples tested had similarity.

**Table 4. Summary of the viruses to which samples tested had similarity.**

| Wheat | Insect traps | Mown area | Weeds |
|---|---|---|---|
| *Tobacco mosaic virus* | *Cocksfoot mild mosaic virus* | *Soybean dwarf virus* | *Cannabis cryptic virus* |
| *Freesia mosaic virus* | *Acyrthosiphon pisum virus* | *Ryegrass mosaic virus* | *Raphanus sativus cryptic virus* 1 |
| *Turnip mosaic virus* | *Acute bee paralysis virus* | *White clover mosaic virus* | *Rhopalosiphum padi virus* |
| ***Epirus cherry virus*** | *Rosy apple aphid virus* | *Acute bee paralysis virus* | *Parsnip yellow fleck virus* |
| ***Citrus leprosis virus C*** | *Rosy apple aphid virus* | *Barley yellow dwarf virus* PAV | *Grapevine virus B* |
| ***Brugmansia mild mottle virus*** | | *Ryegrass mosaic virus* | *Cocksfoot mild mosaic virus* |
| ***Mint vein banding-associated virus*** | | | *Cocksfoot mild mosaic virus* |
| ***GB virus C*** | | | *White clover mosaic virus* |
| ***Grapevine virus B*** | | | *White clover mosaic virus* |
| ***Olive leaf yellowing-associated virus*** | | | *Tobacco mosaic virus* |
| *Grapevine leafroll-associated virus* 1 | | | *Chronic bee paralysis virus* |
| | | | *Pepino mosaic virus* |

## 4.4. Discussion

### 4.4.1. The viruses which were potentially detected

The viruses highlighted in bold which were associated with wheat (see Table 4) were all from related viral genera (groups). This suggests that in fact there could be one novel virus present which is not exactly the same as any of the viruses but was similar enough to be picked out of GenBank. This could have occurred if the novel virus evolved from one of the other viruses and/or if two or more viruses recombined to form a novel virus. This is especially interesting because 25% of all wheat samples potentially had this novel virus. While no obvious viral symptoms were observed in the wheat field, it is possible that this project has detected a novel cryptic virus which is capable of capping the yield of wheat in the UK.

The details of the similarity to sequences and the viruses shown in Table 4 are not shown here but in some cases there is more confidence that a virus was genuinely present because there was a long region of sequence which was very similar if not identical to the virus on GenBank. For example, it is certain that *Acute bee paralysis virus* was found in an insect trap sample.

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 could be 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.

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 (McGrath and Bale, 1990). 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. *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 Sections 1) also suggest this virus does not infect UK wheat.

### 4.4.2. Further work

Further work is required to complete Koch's Postulates and the modern derivatives such as those stated by Fredericks and Relman (1996) for the viruses which were potentially detected. Unfortunately, time constraints meant that this was not possible in this project, although two viruses were investigated but the final conclusion was that a genuine virus was not present in those cases (data not shown). That raises one of the disadvantages of using next generation sequencing technology at the current time, it is a very slow process. For example it took one year just to sequence the samples with bioinformatics to follow. However, as developments are made to the technology and bioinformatics the time required has and is likely to continue to drop dramatically. The high financial cost which currently limits its use is also predicted to do so, and has already. Medicine which has greater funding than plant pathology has been the main area in which the technique has been developed, and this will continue to occur, with plant pathology able

to use the results too (Siqueira *et al*., 2012). As discussed, 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. The financial and time constraints on this project meant that only a certain number of samples could be sequenced. 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 viruses 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 in-between the samples taken here would give a more thorough representation and be more likely to sample any viruses present. An organic site was chosen here because it was likely to have more insects which could act as virus vectors, and therefore more viruses present. However Ladybirds (*Coccinella septempunctata L.*) and Green Lacewings (*Chrysoperla carnea*) are also natural enemies of aphids which are likely to be present at higher levels on organic farms thus reducing numbers (Kaplan and Eubanks, 2002). It would be interesting to sample a conventional farm to compare results. It would also be interesting to sample at different points in the growing season because the number and range of viruses may fluctuate due to time of inoculation or effective removal due to plant defence mechanisms.

# 5. Section 3 - Investigating storage regimes for insect traps for the preservation of insect and viral RNA

## 5.1. 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 which could be used in testing. 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 2; 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).

### 5.1.1. Materials and methods

Storage regimes trialled were: dry (no solution); DEPC treated nuclease free water; 100% acetone; 100% hexane; 100% ethanol; 100% methanol; CTAB (see Appendix 10.6); phosphate buffered saline (PBS) (see Appendix 1.10); 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). Three replicates of each solution were used.

Ten *S. avenae* were put in to each beaker, which was sunk in the ground between wheat plants to mimic the environment the pit traps would be in in Section 2 (see Figure 5). After 7 days the insects were removed. Their RNA was extracted using Chelex (Biorad) extractions. Briefly 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°C for 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. qRT-PCR tests were carried out to detect the level of insect and viral RNA present. This was done using an 18s rRNA assay (Applied Biosystems) and the BYDV-PAV assayed developed in Section 1 (see Appendices 1.3 and 1.11).

The level of the liquid in the beakers was measured after three and seven days. Weather data for the site were also collected.

**Figure 5. An example of test beakers in the field, during the trial of storage regimes for recovery of RNA from insects.**

### 5.1.2. Statistical analysis

A multivariate analysis of variance (MANOVA) was run on the results of the insect and viral tests from 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.

## 5.2. Results

### 5.2.1. Contents of the trap

The level of liquid in the beakers after three and seven days is shown in Figure 6. Weather data during the trial are shown in Figure 7.

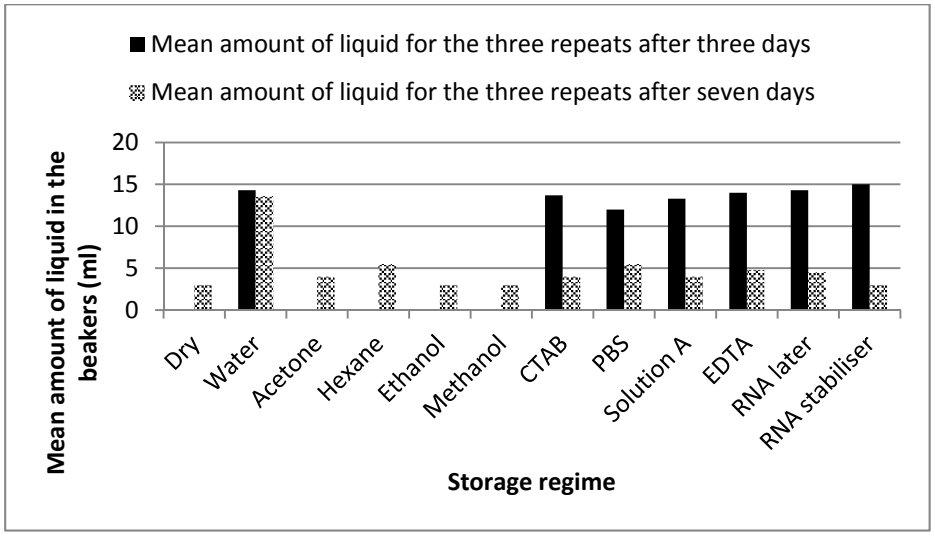


**Figure 6. Mean amount of liquid remaining in the beakers for each storage regime, after three days (black bars) and seven days (hatched bars) in the field.**

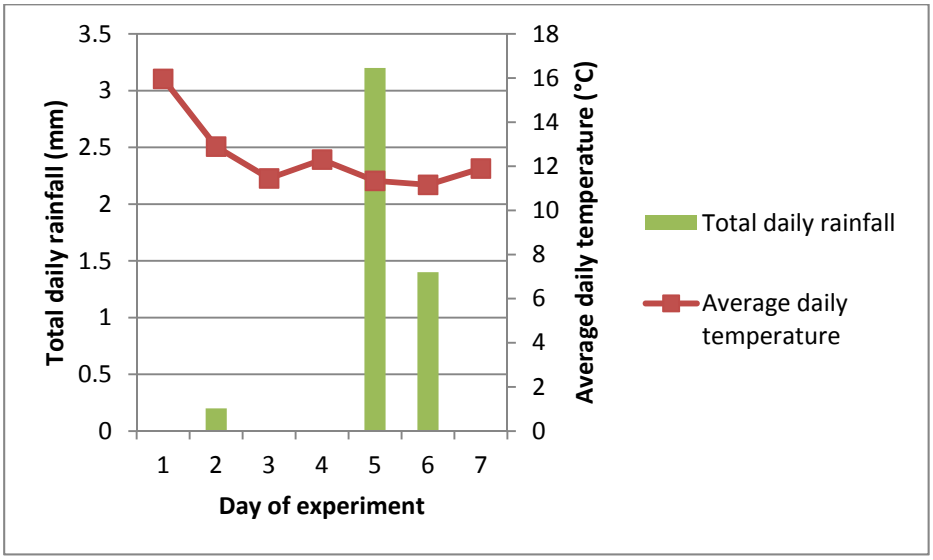


**Figure 7. The total daily rainfall and the average daily temperature during the experimental period.**

### 5.2.2. qRT-PCR tests

None of solutions were consistently the best in enabling insect or viral RNA to be recovered. However overall 0.5 M EDTA was the most successful (data not shown).

### 5.2.3. 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 ∆Rn 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 ∆Rn 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 –$p = 0.01$).

## 5.3. Discussion

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 6 and 7). Figure 6 shows that acetone, hexane, ethanol and methanol were not present in the beakers after three days, due to evaporation. However liquid was present when levels were assessed after seven days, Figure 7 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.

The qRT-PCR tests showed that none of the solutions were consistently the best in testing. However, 0.5 M EDTA was the best overall. The statistical analyses that were carried out suggested that while none of the regimes were significantly better than the others, there were significant overall differences in one test result type (Ct) which leads to the conclusion that 0.5 M EDTA was the best solution to use.

### 5.3.1. 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.

In conclusion, 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 Section 2.

# 6. Section 4 - Sequencing the complete genome of Cynosurus mottle virus and using it to develop a real time reverse transcriptase polymerase chain reaction assay

## 6.1. Introduction

Cynosurus mottle virus (CnMoV) is a virus that has been reported in the UK which can infect wheat and cause symptoms such as chlorotic mottling and ultimately yield loss (Catherall *et al*., 1977). 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 1). 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 Section 1, 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 CnMoV was a member of the *Sobemovirus* genus for which the type species is *Southern bean mosaic virus.* This was because it shared several important traits with the group for example, at molecular level. As a *Sobemovirus*, King *et al*. (2012) predicted several values in connection with the genome of CnMoV, including size and the organisation, see Figure 8.

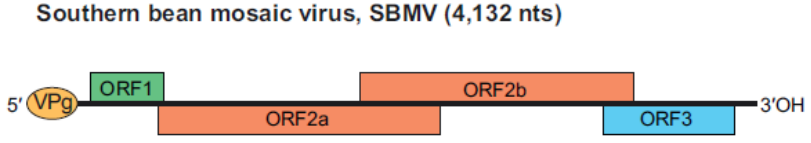


**Figure 8. The genome organisation of *Southern bean mosaic virus* (King *et al*., 2012).**

## 6.2. Materials and methods

### 6.2.1. Sample preparation 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. The sequences common to wheat were then removed, with the aim of leaving on viral sequence. Sequencing was performed using a GS-FLX Genome Sequencer, Roche, according to manufacturer's protocols. A SMART RACE kit (Clontech) was used according to manufacturer's instructions, in order to sequence the ends of the genome which often do not appear in the first sequence from the GS-FLX genome sequencer. Finally Sanger sequencing of cloned regions was used to complete any gaps in the sequence.

### 6.2.2. Genome assembly using bioinformatics

Sequences were grouped according to similarity using the software Newbler v 2.6 (Roche). Blast searches of GenBank were done to find other *Sobemoviruses* to help suggest in which order the grouped segments of sequence should be placed, Tablet (SCRI) was used for this. Finally Vector NTI (Invitrogen) was used to suggest what functions the segments of the genome were responsible for, to produce a genome organisation diagram. This was checked using BlastP searches against other *Sobemoviruses*.

### 6.2.3. Developing a qRT-PCR assay for CnMoV

A qRT-PCR assay was developed to the coat protein (ORF 3) of CnMoV (see Figure 9), see 3.2.2 for details of assay design.

### 6.2.4. 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 (see Appendix 1.4). 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 Appendices 1.2, 1.3 and 1.12). Concentrations used were: 1, 1/50, 1/100, $1/10^3$, $1/10^6$, $1/10^7$, $1/10^8$, $1/10^9$, $1/10^{10}$, $1/10^{11}$, $1/10^{12}$, $1/10^{13}$, $1/10^{14}$, $1/10^{15}$, $1/10^{16}$.

## 6.3. Results

The complete genome of Cynosurus mottle virus was obtained through sequencing (data not shown). Figure 9 shows the organisation and translation strategy of CnMoV.

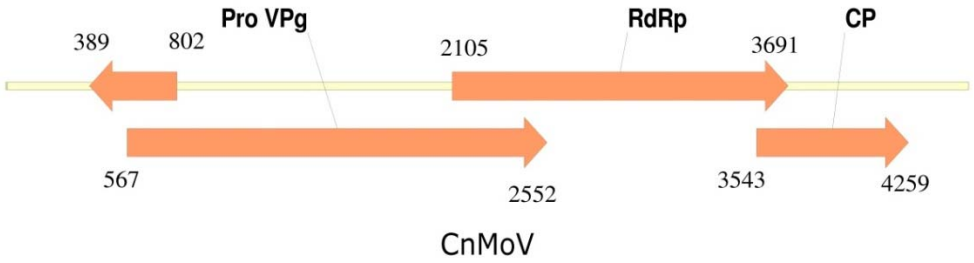


**Figure 9. The organisation in terms of functional protein coding regions, of the genome of CnMoV. The arrow on the left represents ORF 1 and Pro VPg, RdRp and CP represent the VPg, RNA dependent RNA polymerase and coat protein respectively.**

A characteristic region of sequence often found in plant viral genomes was detected which adds confidence to the result.

### 6.3.1. The qRT-PCR assay

A qRT-PCR assay was successfully developed. Blast searches on the GenBank found no similarity to other viruses which would cause cross reactions. In physical tests the assay soley detected the target virus and not other viruses which are likely to be present in wheat.

Figure 10 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 lower the Ct value the better the result. The qRT-PCR assay was able to detect CnMoV down to a concentration of $10^6$.

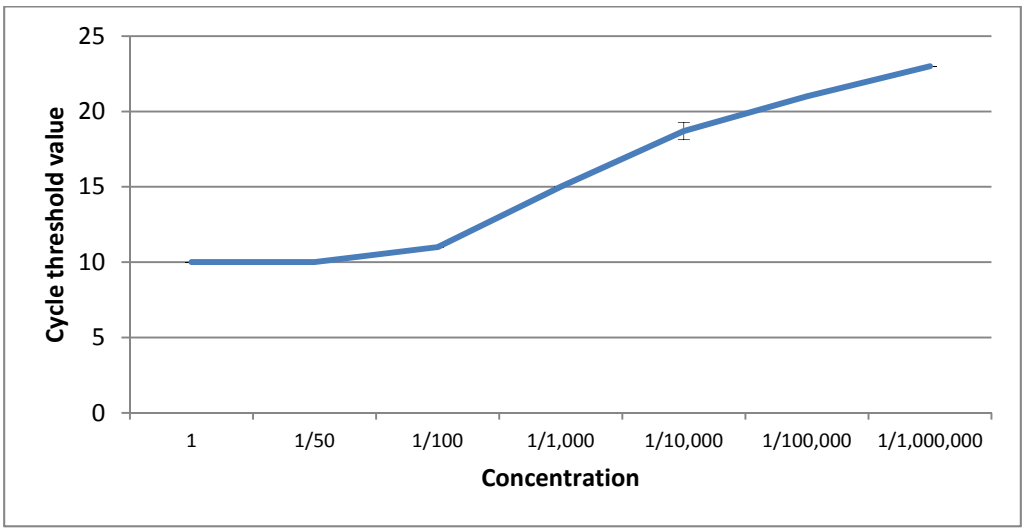


**Figure 10. The average results of the serial dilution for the qRT-PCR assay for CnMoV, error bars greater than zero for each concentration are plotted.**

The results of a dilution series of wheat infected with CnMoV tested by ELISA are shown in Figure 11. The ELISA test was able to detect CnMoV down to a concentration of $1/10^5$.

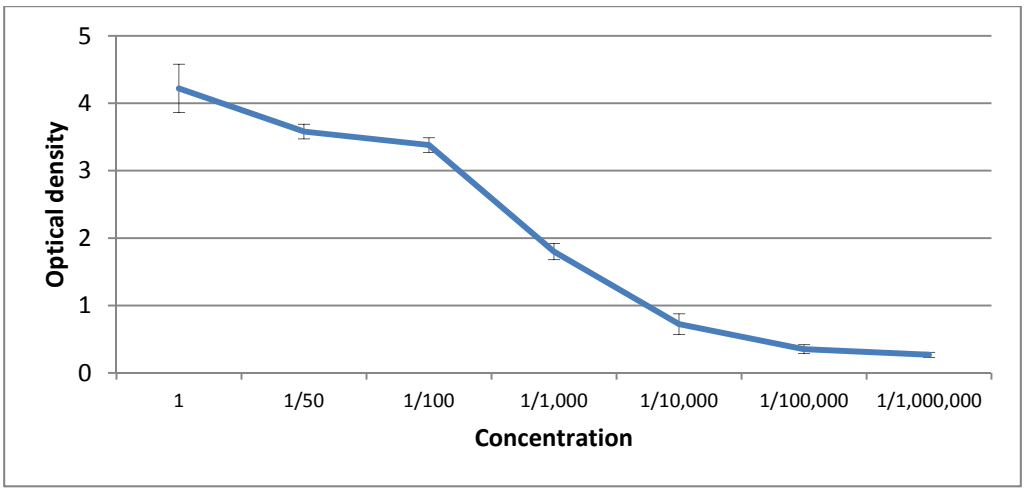


**Figure 11. The average results of a dilution series of CnMoV tested by ELISA. The cut off point for a positive result was triple the average of the healthy control sample, which was 0.318. The higher the optical density value the better the result. Concentrations below $1/10^5$ were all negative and are not shown. Errors bars of standard deviation for each concentration are plotted.**

## 6.4. Discussion

### 6.4.1. The genome

The genome of CnMoV was determined (see Figure 9). 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. This is because the predictions made by Mohamed (1978) about length and base composition were proven correct. Blast N searches found that there were similarities between the complete genome of CnMoV and other members of the *Sobemovirus* genus. According to King *et al*. (2012) overall sequence similarity to be a *Sobemovirus* should be approximately 75%, this was the case with CnMoV. The genome organisation of CnMoV is similar to that of the type species for the genus (SBMV) (see Figures 8 and 9). In contrast to all the supporting evidence that CnMoV is a *Sobemovirus*, the 3' end of the genome is different to predicted by King *et al*. (2012). However, because the vast majority of evidence supports CnMoV being a *Sobemovirus*, it should be considered so.

### 6.4.2. qRT-PCR assay

A successful qRT-PCR assay was developed which can detect CnMoV specifically in a repeatable manner. In the comparison to the existing ELISA test the qRT-PCR assay was able to detect CnMoV at a lower level, hence it was more sensitive (see Figures 10 and 11). 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.

# 7. Section 5 - Investigating the effect of Cynosurus mottle virus on the yield of wheat and the possibility that it is seed transmitted

## 7.1. Introduction

Cynosurus mottle virus 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 (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.

## 7.2. Materials and methods

### 7.2.1. Trial

A trial was carried using the cultivars Gladiator and Scout, which were both on the AHDB winter wheat Recommended List at the time of planning, and were therefore relevant to farmers and funders (Web reference – HGCA 6). Twenty four trays (30 cm x 20 cm) and sufficient soil based compost to fill them were autoclaved. Seeds were sown at recommended density. To 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. The seeds were left to germinate in the glasshouse at Fera where the temperature was 18°C, under natural lighting conditions.

### 7.2.2. 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.

### 7.2.3. 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, developed in Section 1 and 4. 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 Appendix 1.4). The samples were combined to make one sample per tray.

### 7.2.4. Prevention of insect interactions

Insects should not have been able to enter the glasshouse due to its impenetrable 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.

### 7.2.5. Mechanical inoculation of CnMoV

One week after the intercept had been applied half the trays of each variety of wheat were inoculated with CnMoV. Freeze dried CnMoV wheat (DSMZ) was used to mechanically inoculate the plants (see Appendices 1.5 and 1.13). The healthy controls and were spatially separated from the inoculated trays.

### 7.2.6. Confirmation of infection status of the plants

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

### 7.2.7. 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 at 4°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). 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.

### 7.2.8. Grain processing and statistics

A threshing machine was used to separate the grain from the chaff. 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). Genstat version 15 (Web reference – Genstat 15) was used to perform two-way ANOVA with replication tests or generalised linear model analyses, depending on the normality of the data.

### 7.2.9. Seed transmission experiments

Seeds from a first preliminary trial were used in seed transmission experiments. These seeds were separated prior to grain drying so their potential to germinate, or for the virus to be destroyed were not affected, instead they were stored at 4°C. These seeds were separated prior to grain drying so their potential to germinate, or for the virus to be destroyed were not affected, instead they were stored at 4 °C. The variety was Einstein. Visual 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 Appendix 1.4).

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.

## 7.3. Results

### 7.3.1. Trial

Table 5 shows a summary of the data that were collected.

**Table 5. A summary of data collected. 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.

### 7.3.2. Statistical analysis

Table 6 shows the results of the statistical analyses performed on the raw data from the trial (see Table 5).

**Table 6. Results of statistical analyses of the data from the trial. 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 |

### 7.3.3. Observations

Both varieties of wheat that were inoculated with CnMoV developed a yellow mottle along leaves after approximately one month, but the healthy control plants did not (see Figures 12 and 13).

**Figure 12. Symptoms of a CnMoV infection in wheat cv. Gladiator, two months post inoculation.**

**Figure 13. Healthy wheat cv. Gladiator, showing none of the symptoms that CnMoV inoculated wheat developed, two months post mock inoculation.**

### 7.3.4. Seed transmission experiments- 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.

### 7.3.5. Direct seed testing

Table 7 shows the results of ELISA testing of seeds from inoculated and healthy control plants.

**Table 7. The results of DAS ELISA tests of seed from CnMoV infected wheat cv. Einstein (sets 1–3 inoculated with CnMoV (bold font)) and seed from healthy wheat cv. Einstein ((sets 4–6) (italic font)).**

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

### 7.3.6. 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 8).

**Table 8. The results of DAS ELISA tests of plants grown from seed from CnMoV infected wheat cv. Einstein (sets 1–3 inoculated with CnMoV (bold font)) and seed from healthy wheat cv. Einstein ((sets 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* |

## 7.4. Discussion

### 7.4.1. Comparison of infected and healthy plants

The trial was based on preliminary experiments, which allowed the development of a successful method; 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).

Tables 5 and 6 summarise the results and statistical analyses of the main trial. 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 stunting and reduced tillering for example CfMV, which is also a *Sobemovirus* (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.

### 7.4.2. Comparison of cultivars

Table 5 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 AHDB recommended lists suggest that both should yield the same (99t/ha) and that cv. Scout should achieve 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 tolerant 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). 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.

### 7.4.3. CnMoV prevalence

An extensive study of wheat in Section 1 and of wheat, weeds and insects in Section 2 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 Section 1 and Section 2 would suggest if this virus was becoming more prevalent therefore developing resistant wheat should be considered.

### 7.4.4. Seed transmission testing

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 Figures 12). 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 7). 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 8, 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.

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.

# 8. Final discussion

This project had several aims; a summary of how these have been achieved and an overview of results in the context of published work 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 qRT-PCR assay**

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 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 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 Section 5). 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. One reason being a lack of suitable diagnostic tools, however more modern diagnostic tools such as qRT-PCR and next generation sequencing offer significant opportunities to do high throughput screens for viruses of wheat in large numbers of samples efficiently and accurately, including novel viruses. A significant advantage is that there is no target bias from the latter. More modern sequencing techniques also allow specific diagnosis rather than those based on symptoms which can be confusing as there are only so many symptoms wheat can exhibit.

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 Section 1, the weather conditions during critical part of the growing season likely contributed to the result. 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 Roossinck *et al*. (2010) to detect potentially numerous novel viruses in prairie grass. 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.

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. 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.

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). 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 pose to food security (Stukenbrock and McDonald, 2008). This is due to direct impacts of the virus on wheat and also the effect 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 (*Wheat streak mosaic virus* (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. 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 such as increased temperature 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). 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). 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 Section 1 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 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*., 2013). 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. 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. 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 3). 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 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 Section 5). 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 (e.g. 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 Sections 1 and 2, 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 in fact 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 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 oilseed 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).

In conclusion, viruses can have a significant impact on the yield of wheat. 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 some 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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Tablet (SCRI) - http://bioinf.scri.ac.uk/Tablet/
VectorNTI (Invitrogen) - http://www.invitrogen.com/site/us/en/home/Products-and-Services/Applications/Cloning/vector-nti-software.html, Accessed 27/1/13.

## 9.4. 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)

# 10. Appendix

Methods and chemicals used repeatedly in the project are detailed here.

## 10.1. CTAB extraction

Total nucleic acid was extracted by macerating the sample in CTAB grinding buffer (see Appendix 10.6). 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.

## 10.2. Total nucleic acid extraction by Kingfisher96

The machine was loaded as follows: block A- 1 ml sample (sample ground in GITC 1, 1% Antifoam and 2% TnaPP (Sigma Aldrich and see Appendix 1.9) 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 in 2009 and 2010 and GITC 2 in 2011 and 2012 (Appendices 1.7 and 1.8), 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).

## 10.3. 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 1.11 and 1.12 for mastermix constituents.

## 10.4. 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.

### 10.5. 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 1.13). 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.

### 10.6. 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.

### 10.7. pH 6.4 GITC 1 – used in Kingfisher extractions

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

### 10.8. pH 6.4 GITC 2 – used in Kingfisher extractions

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.

### 10.9. TnaPP (8.38%) – an additive to make Kingfisher grinding buffer

16 mM tetrasodium pyrophosphatedehydrate; 50 ml molecular grade water.

### 10.10. 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.

### 10.11. 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.

### 10.12. 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.

### 10.13. 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)).

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