

# Virus Yellows: the consequences of strain variation on future sugar beet varieties

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*In memory of my grandmother, Nancy Matthey, who delighted in  
looking after 'the special yellow sugar beets'  
during the 2020 lockdown.*



## Thesis Abstract

Virus yellows disease is a major threat to the UK sugar beet industry, reducing yields in sugar beet crops by up to 47%. Reductions in available chemical control options, and a shift to reduce the environmental impact of farming practices, means virus yellows-resistant varieties are now seen as the most sustainable and long-term solution. Progress is being made by European sugar beet breeders in the development of such sugar beet varieties, however our understanding of the diversity of strains which may challenge these varieties in the field remains limited. A three-year survey, utilising next generation sequencing of small RNAs and conducted across sugar, fodder, and sea beet plants, identified Beet chlorosis virus (BChV) as more prevalent than Beet mild yellowing virus (BMV) in symptomatic plants – a finding which goes against previous research but supports more recent studies conducted in France. Further analysis of this sequencing data showed genetic variation within the yellowing virus species. The resilience of resistant varieties to infection with differing virus isolates was then determined. Various plant growth facilities were trialled to establish their suitability for phenotyping the performance of sugar beet varieties under virus infection. The results of these trials indicated that varieties developed against BMV also performed well under BChV infection, however sugar yield could not be determined under controlled environment conditions. Novel field trials, challenging susceptible and resistant sugar beet varieties with six differing virus isolates were conducted in 2022 and 2023. The results of these trials indicated that despite differences in pathogenicity between virus isolates, the resistant varieties tested proved to have a robust resistance mechanism.

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## Abbreviations

<b>ABBREVIATION</b>	<b>FULL FORM</b>
°C	Degrees Celsius
μl	Microlitre
AGO	Argonaute (protein)
BBRO	British Beet Research Organisation
BChV	Beet chlorosis virus
BMYV	Beet mild yellowing virus
BtMV	Beet mosaic virus
BWYV	Beet western yellows virus
BYV	Beet yellows virus
CABYV	Cucurbit aphid-borne yellows virus
DAS-ELISA	Double antibody sandwich enzyme-linked immunosorbent assay
DCL	Dicer-like protein
DEPC	Diethyl pyrocarbonate
DNA	Deoxyribonucleic acid
dsRNA	Double-stranded ribonucleic acid
ELISA	Enzyme-linked immunosorbent assay
HD	High definition
ICTV	International Committee on Taxonomy of Viruses
IGV	Integrative Genomics Viewer
Kb	Kilobases
LSD	Least significant difference
mg	milligram
ml	millilitre
NCBI	National Center for Biotechnology Information
nm	nanometre
nt	Nucleotide
PBST	Phosphate buffered saline with 0.05% Tween
PCR	Polymerase chain reaction
PLRV	Potato leafroll virus
qPCR	Quantitative real-time polymerase chain reaction
rcf	Relative centrifugal force
RdRp	Ribonucleic acid-dependent ribonucleic acid polymerase
RISC	Ribonucleic acid-induced silencing complex
RNA	Ribonucleic acid
RNAi	Ribonucleic acid interference
RT	Reverse transcription
RT-qPCR	Reverse transcription quantitative real-time polymerase chain reaction

sRNA	Small RNA
ssRNA	Single-stranded ribonucleic acid
TAS-ELISA	Triple antibody sandwich enzyme-linked immunosorbent assay
TBE	Tris-Borate-EDTA Buffer
TuYV	Turnip yellows virus
UK	United Kingdom
USA	United States of America
V	volts
VPg	Viral genome-linked protein
w/v	Weight per volume
w/w	Weight by weight
YWP	Yellow water pan trap

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# Chapter 1: General Introduction

## 1.1 Plant viruses

Plant viruses cause severe yield losses to agricultural crops worldwide. Whilst some viruses reduce the quality and appearance of produce reducing their commercial value, other viruses can cause entire crops to fail. When such crop failures occur in staple food crops, such as potato or rice, food security is threatened with potentially devastating consequences. The need to feed a rapidly growing human population, particularly under the increasing pressures of climate change, makes protecting crops from these viral diseases increasingly important.

### 1.1.1 Virus classification

As of July 2022 there were 11273 recognised virus species (Zerbini et al., 2023). The variation between these species is large, with virus species parasitising all known living organisms including animals, plants, fungi, and bacteria (Cann, 2012b). Given this huge diversity a classification system is vital for the organisation of research and inference of characteristics. The International Committee on Taxonomy of Viruses (ICTV) is responsible for the classification of all viruses. Established in 1966, and renamed to its current title in 1977, the ICTV determines the criteria for assigning viruses to the recognised taxonomic groups: order, family, subfamily, genus, and species (Lefkowitz et al., 2018a). The high mutation rate of viruses, particularly RNA viruses, can make the assigning these taxa difficult and, occasionally, changes to taxonomic classifications are necessary (Yuan et al., 2022). Such taxonomic changes are made annually after being ratified by ICTV members (Walker et al., 2021). One notable change occurred in March 2021, when a binomial nomenclature format was adopted (Zerbini et al., 2022). As such viral binomial names are used where relevant and helpful in this thesis.

In 1991 the ICTV defined a virus species as:

*“A viral species is a polythetic class of viruses that constitutes a replicating lineage and occupies a particular ecological niche”*

(Francki, Knudson and Brown, 1991)

Virus species are not entirely uniform. As with animals or plant species, a virus species is comprised of a genetic population expressing variation (Hull, 2009a). This variation can make distinguishing what constitutes a different species difficult. Differences in mutation rates between virus families and genera can further compound this problem. To overcome this, species demarcation criteria are outlined for each genus by the ICTV subcommittee study groups (Lefkowitz et al., 2018a). For example, the criteria considered when defining species in the *Polerovirus* genus compared to the *Closterovirus* genus are shown in Table 1.1.

Table 1.1: Factors considered by the ICTV when defining new polerovirus and closterovirus species. Adapted from Fuchs et al., 2020 and Sömera et al., 2021.

Polerovirus	Closterovirus
Host range	Host range
Occurrence of cross-protection	Particle size
Serological specificity with discriminatory antibodies	Serological specificity with discriminatory antibodies
Gene products differing in amino acid sequence by more than 10%	More than a 25% difference in amino acid sequence of the: <ul style="list-style-type: none"> <li>• Coat protein</li> <li>• RNA dependent RNA polymerase</li> <li>• 70-kDa heat shock protein homologue (HSP70h)</li> </ul>
	Coat protein size
	Vector species
	Cytopathological features

The terms ‘strain’ and ‘isolate’ are frequently used by virologists and worthy of defining here. The key difference between these terms is that a virus strain exhibits some unique and recognisable phenotypic characteristics. For example, there are multiple strains of the species *Potyvirus potato virus Y* which exhibit different symptoms in different host plants (Hull, 2009b). Whereas a virus isolate is simply the name given to a specific virus culture that is being studied and is not associated with any taxonomic level (Van Regenmortel, 2007).

### 1.1.2 Virus transmission

Viruses are obligate intracellular parasites which rely on host cells to replicate. Unlike other organisms, viruses do not grow and then divide but instead produce components which assemble (Cann, 2012b). To produce these components all viruses are at least somewhat dependent on accessing the cellular machinery contained within the host cell (Cann, 2012c). Therefore the ability to move between hosts and infect new cells is a fundamental feature of any virus. Plant viruses have many different transmission pathways. Some plant viruses are directly transferred from one host to another via mechanical transmission, where the tissue of an infected plant rubs against an uninfected plant. Others infect or contaminate seeds or are transferred via contaminated soil or water. Examples of the different transmission mechanisms used by plant viruses are given in Table 1.2.

Table 1.2: different transmission mechanisms used by plants viruses and examples of agriculturally important viruses which utilise each mechanism.

Transmission mechanism	Example of agriculturally important virus	Key crop affected	
Mechanical	Potato virus X	<i>Solanum tuberosum</i> L. (Potato)	(Roberts, 1946)
Seed-borne	Barley stripe mosaic virus	<i>Hordeum vulgare</i> L. (Barley)	(Slack, 1975)
Pollen-borne	Tobacco streak virus	<i>Gossypium hirsutum</i> (Cotton)	(Sdoodee and Teakle, 1993)
Fungal vector	Mirafiori lettuce virus	<i>Lactuca sativa</i> (Lettuce)	(Lot et al., 2002)
Nematode vector	Tobacco rattle virus	<i>Solanum tuberosum</i> L. (Potato)	(Ploeg et al., 1992)
Insect vector	Beet yellows virus	<i>Beta vulgaris</i> ssp. <i>Vulgaris</i> (Sugar beet)	(Watson, 1946)
Arachnid vector	Wheat streak mosaic virus	<i>Triticum aestivum</i> L.	(Tatineni and Hein, 2018)

### 1.1.3 Insects as virus vectors

Vector transmission is one of the most common ways plant viruses use to move between hosts (Stevens and Lacomme, 2017). Fungi, bacteria, nematodes, insects, and arachnids are all known to spread viruses (Cann, 2012c). But of all these organisms, aphids are particularly important, utilised by an estimated 50% of insect-vectoring viruses (James and Perry, 2004; Nault, 1997). Aphids make ideal plant virus vectors thanks to their feeding behaviour. All aphids use needle-like stylets to pierce into plant tissues and access the food source of phloem contained within the plant. Through the stylet phloem assimilates are taken up and virus particles may be transmitted in either direction. Species such as *Myzus persicae* (Figure 1.1) make particularly good virus vectors because of their polyphagous nature. By feeding on multiple plant species, aphids such as *M. persicae* enable virus infection of multiple plant species from a single vector species.

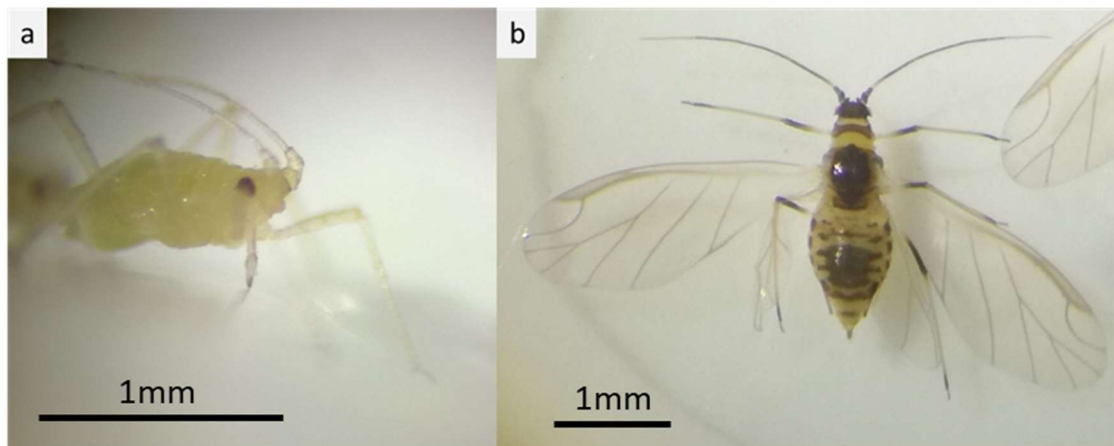


Figure 1.1: images of *Myzus persicae* taken through a dissecting microscope (photos by Suzannah Harder). Scale of each image shown in millimetres by horizontal line. a) apterous (wingless) form of *M. persicae*, b) alate (winged) form of *M. persicae*.

#### 1.1.3.1 Types of aphid transmission

The transmission of viruses by aphids can be categorised as circulative or noncirculative. Non-circulative viruses remain in the mouthparts and foregut of their aphid vector and do not enter the haemocoel (James and Perry, 2004). Non-circulative viruses can be further defined as either non-persistently or semi-persistently transmitted. Non-circulative, non-persistent virus transmission is the most common form of transmission for aphid vectored viruses (Stevens and Lacomme, 2017). The time it takes for an aphid to take up virus particles is termed 'acquisition time'. Non-persistent viruses have a very short acquisition time of less than one minute. However, the virus is not retained within the aphid for very long and can be 'lost' within minutes of the aphid feeding on an uninfected

plant (James and Perry, 2004). Semi-persistent viruses have longer acquisition times, normally requiring at least 15 minutes of aphid feeding, but can then be transmitted by the aphid for days (Stevens and Lacomme, 2017). Notably, both nonpersistent and semipersistent viruses are lost during aphid moulting (James and Perry, 2004), with previously viruliferous aphids returning to a non-viruliferous state.

Circulative viruses, in comparison, travel much further through their aphid hosts crossing multiple membrane barriers (Figure 1.2). After being taken up by an aphid through its stylet, circulative virus particles pass through the food canal, foregut, midgut and hindgut before entering the haemocoel. Once in the haemocoel, the virus travels to the accessory salivary gland and then leaves the aphid through the salivary canal into the plant (James and Perry, 2004). Due to the longer routes circulatively transmitted viruses take within the aphid, and because they are normally phloem limited (restricted to the phloem cells of host plants), the acquisition period is much longer than for noncirculative viruses (taking hours or days). The persistency of these viruses within the aphid, and the fact they are retained even during aphid moulting, has led to them being termed 'persistent' even though not all are maintained throughout the aphid's life.

Some viruses can replicate within their aphid vectors and are called 'propagative'. These viruses belong to the *Reoviridae* and *Rhabdoviridae*, families and are transovarial meaning the virus is passed from the aphid to its progeny. 'Non-propagative' circulative viruses, do not replicate within the aphid vector and do not exhibit transovarian transmission (Hull, 2009c).

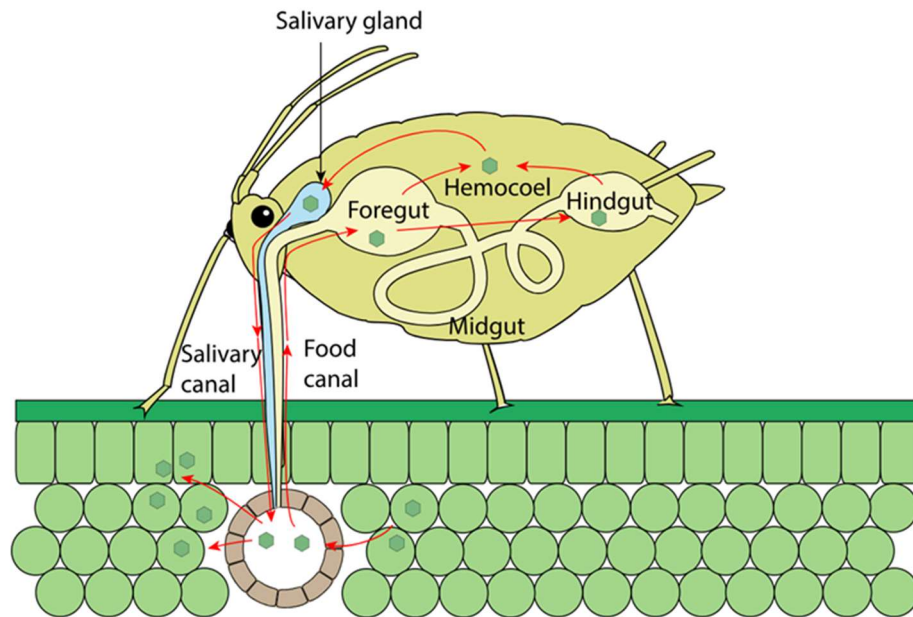


Figure 1.2: cartoon depicting the route of circulative viruses within aphids. Virus particles (shown as dark green hexagons) are taken up by the aphid through the food canal within the stylet, particles then cross multiple membrane barriers through the foregut, midgut and hindgut entering the hemocoel and into the salivary gland. From the salivary gland virus particles then travel through the salivary canal contained within the stylet into the plant. Reproduced from (Hulo et al., 2011).

#### 1.1.4 Spread of viruses

Insect vectors facilitate the spread of viruses across large geographical areas. This is especially true for windborne vectors such as aphids and leafhoppers carrying persistent or semi-persistent viruses. Although often not strong fliers, aphids and leafhoppers can be taken up by wind currents and transported hundreds of miles (Irwin and Thresh, 1988). This can have a major effect on disease epidemiology. One recent example of this is the rice stripe mosaic virus outbreak, which spread from the Jiangsu province of eastern China to Japan and Korea due to a slow-moving cold vortex system transporting the planthopper vector species (Otuka, 2013).

Given the impact of vector distribution on disease occurrence many attempts to control plant viruses target the vector. Detailed monitoring and forecasting systems, enable control strategies to be optimised both in terms of their timing and location of use (Harrington and Hullé, 2017). For example, the Rothamsted Insect Survey aphid forecast predicts the first flight and abundance of different aphid species, including *M. persicae*, based on winter temperatures (Greenslade and Bell, 2023). This information is then used by the sugar beet industry to inform when close monitoring of crops for aphids should begin (Stevens and Bowen, 2023a).

## 1.2 Sugar beet

There are two major crops grown as a source of sugar throughout the world: sugar beet (*Beta vulgaris* ssp. *vulgaris*) and sugar cane (*Saccharum officinarum*). Sugar beet dominates sugar production in temperate countries, whilst sugar cane is cultivated in tropical and sub-tropical regions (Draycott, 2006). The UK is the tenth largest producer of sugar beet globally, growing 7,420,000 tonnes in 2021 (FAOSTAT, 2023). All UK sugar beet is grown under contract to British Sugar and is focused around their four processing factories in East Anglia and the East Midlands.

Sugar beet is a biennial plant, with a vegetative growth stage followed by a reproductive phase (Milford, 2006). During the vegetative stage, a storage root is produced with a sucrose concentration of around 20% (Draycott, 2006). The production of a flowering shoot and seeds is termed 'bolting' and is triggered by a period of cold vernalisation (Francis, 2006). Bolting reduces sugar yield, therefore commercial crops are planted in the spring and harvested in the autumn or winter before the reproductive stage is initiated (Milford, 2006). During harvesting the leaves of sugar beet plants are removed and the roots lifted and transported to a processing factory where the sugar is extracted and purified (British Sugar, 2023a).

### 1.2.1 Factors which affect sugar beet yield

Weeds, pests, diseases and adverse weather conditions can all impact sugar beet yields (Draycott, 2006). The distribution and relative importance of these factors has changed over time, affected by changes to agricultural practices and climate change. For example, historically, UK summers were not warm or humid enough to permit the development of the foliar fungal disease cercospora leaf spot (Dunning and Byford, 1982). However now it is considered a major threat to the UK sugar beet crop with multiple fungicide applications used to control the disease in outbreak years. Other examples include insect pests such as tortoise beetle (*Cassida nebulosa*), which are being observed more frequently in UK sugar beet crops (Dunning and Byford, 1982; Stevens and Bowen, 2023b), and beet moth (*Scrobipalpa ocellatella*) which for the first time caused significant damage to sugar beet crops in 2022 likely due to the exceptionally hot and dry summer (Dunning and Byford, 1982; Stevens et al., 2023).

### 1.2.2 Viruses of sugar beet

Some of the most damaging sugar beet diseases are caused by plant viruses. In western USA the sugar beet industry came close to ruin in the 1920s because of curly top disease (Harveson, Hanson

and Hein, 2009). Caused by three viruses from the *Curtovirus* genus of *Geminiviridae*, and spread by the beet leafhopper (*Circulifer tenellus*), curly top disease can completely destroy infected plants in the arid regions where it is found (Dunning and Byford, 1982). In Europe, Rhizomania (“root maddess”) caused by beet necrotic yellow vein virus (BNYVV) and transmitted by *Polymyxa betae*, was first reported in Italy in 1959 (Harveson et al., 2009). By the 1980s the virus was identified in the UK where it caused yield losses of up to 72% and left some fields unviable for sugar beet production (Henry, 1966). Both curly top disease and rhizomania have been brought under control by the development of partially resistant sugar beet varieties. However resistant varieties have not been developed for all sugar beet viruses.

### 1.2.3 Virus yellows complex

Virus yellows is a disease of sugar beet caused by a complex of aphid-transmitted viruses (Table 1.3). The disease can significantly reduce sugar yields, as evidenced during the 2020 virus yellows outbreak which saw UK yields drop by 25% causing a £67 million loss to the UK sugar industry (Department for Environment, 2023). Historically, however, yield loss during virus yellows epidemic years has been even greater, with the most severe losses to date occurring in 1974 when an estimated 40% of sugar yield was lost to the disease (Werker, Dewar and Harrington, 1998).

Table 1.3: viruses which cause virus yellows disease in sugar beet.

Virus name	Abbreviation
Beet mild yellowing virus	BMYV
Beet yellows virus	BYV
Beet chlorosis virus	BChV
Beet western yellows virus	BWYV

#### 1.2.3.1 History of discovery

Many pathogens and nutrient deficiencies cause yellowing in sugar beet leaves (Figure 1.3). Capsid beetles can bite into main leaf veins, causing yellowing and necrosis above the puncture point. Magnesium deficiency can cause interveinal leaf yellowing. Downy mildew (*Peronospora farinosa*) can cause yellowing to older leaves (Dunning and Byford, 1982). This ubiquity of leaf yellowing as a symptom of disease and deficiency made early identification and characterisation of sugar beet viruses difficult (Russell, 1958).





Figure 1.3: images of yellowing symptoms on sugar beet caused by a) magnesium deficiency, b) downy mildew infection, c) virus yellows infection, d) manganese deficiency, e) herbicide damage due to suspected sprayer contamination. (Photos a,b,d,e provided by Simon Bowen; photo c by Suzannah Harder).

The virus we now know as Beet yellows virus (BYV) was first identified in 1940 by Watson whilst investigating yellowing sugar beet plants in England (Watson, 1940). However, suggestions of a viral cause for these symptoms had been persisting since the 1930s (Quanjer, 1934; Roland, 1936). Naming the virus Beet yellows virus, Watson went on to show it was transmitted by aphid species including *Aphis fabae* and *M. persicae*, which was deemed the principal vector (Watson et al., 1951).

The discovery and classification of Beet mild yellowing virus (BMV) was less straightforward. In 1948 a virus thought to be a strain of BYV was reported in Ireland and named “Irish mild yellows” (Clinch and Loughane, 1948). In 1954 a similar virus was found in sugar beet in England (Hull and Blencowe, 1954). The presence of this second yellowing virus was confirmed by Russell who, when studying the distribution of yellowing virus strains in East Anglia in 1955 to 1957, identified a virus which did not react with BYV antisera (Russell, 1958). Crucially, when viewed under an electron microscope, the infected sap also did not contain the long filamentous particles known to be BYV. Naming the virus “sugar beet mild yellowing virus” Russell recorded it decreased root yield and caused leaf yellowing but to a lesser extent than sugar beet yellows virus (BYV).

Meanwhile in the USA, Duffus also identified a second sugar beet yellowing virus which he initially named “radish yellows” before re-naming it Beet western yellows virus (BWYV) (Duffus, 1960). These joint discoveries have been marked as the start of confusion surrounding the poleroviruses which cause virus yellows (Stevens et al., 2005a).

Duffus and Russell undertook many studies to elucidate the relationship between their respective viruses (Duffus and Russell, 1970, 1972, 1975). Sugar beet mild yellowing virus, or Beet mild yellowing virus (BMYV) as it became known, had a narrower host range to BWYV and was unable to infect *Brassica* sp. (Duffus and Russell, 1970). BWYV isolates were found in Europe which had a broad host range like American BWYV but could not infect sugar beet. Ultimately these non-sugar beet infecting European strains of BWYV were categorised as a separate species; Turnip yellows virus (TuYV), (Graichen and Rabenstein, 1996).

A similar situation of co-discovery occurred in the late 1980s early 1990s with the virus now named Beet chlorosis virus. In 1991 (Duffus and Liu, 1991) identified a virus causing mild yellowing symptoms which were serologically distinct from BYV yet failed to infect the BMYV indicator species *Capsella bursa-pastoris*. In 1994, Stevens, Smith and Hallsworth (1994a) reported what they referred to as a distinct strain of BMYV which failed to react with the BMYV diagnostic antibody BYDV-PAV-IL-1 and did not infect *C. bursa-pastoris*. Owing to the chlorosis symptoms seen in sugar beet infected with this ‘new’ virus the name Beet chlorosis virus (BChV) was proposed and approved in 2002 (Mayo, 2002).

## 1.2.4 Beet poleroviruses

### 1.2.4.1 Taxonomy

The term beet polerovirus is used to define viruses belonging to the Genus *Polerovirus* which infect *Beta vulgaris* and cause yellowing symptoms (Stevens et al., 2005a). The three viruses belonging to this group are *Polerovirus Beet mild yellowing virus*, *Polerovirus Beet chlorosis virus*, and *Polerovirus Beet western yellows virus* (Walker et al., 2021). The genus *Polerovirus* was moved from the family *Luteoviridae* to the family *Solemoviridae* following a proposal to abolish the *Luteoviridae* classification in 2020, placing a greater emphasis on RNA dependent RNA polymerase phylogeny (Scheets, Miller and Somera, 2020; Walker et al., 2021).

The *Solemoviridae* family contains many economically important plant pathogens. Viruses within the family have icosahedral shaped particles which are non-enveloped and between 20 to 34nm in

diameter. They have a non-segmented positive-sense RNA genome with a genome-linked protein (VPg) at the 5' terminus but no polyadenylated tail (Sömera et al., 2021).

#### 1.2.4.2 Genome organisation

Within the *Solemoviridae* family there are four genera, each with slightly differing genomic organisation. Polerovirus genomes are between 5 to 6kb in size and organised into overlapping open reading frames (ORFs), which increase the amount of genetic information that can be encoded (Figure 1.4), (Pagán and Holmes, 2010). The beet poleroviruses have 6 ORFs, compared to the 8 ORFs identified in Potato leafroll virus (PLRV) and Cucurbit aphid-borne yellows virus (CABYV) (Ashoub, Rohde and Prüfer, 1998; Hwang et al., 2013; Stevens et al., 2005a). Polerovirus genomes also contain three non-coding regions which permit the recombination events frequently seen in the polerovirus family (Moonan et al., 2000; Pagán and Holmes, 2010; Stevens et al., 2005a).

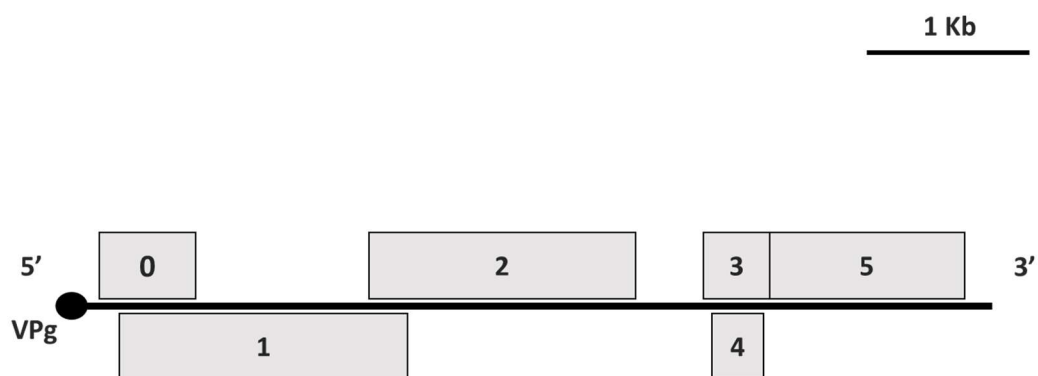


Figure 1.4: schematic diagram showing the shared genomic organisation of Beet mild yellowing virus and Beet chlorosis virus. The 5.5kb single stranded RNA genome (shown as horizontal black line) contains five overlapping open reading frames (ORFs), indicated by grey boxes. These ORFs are named ORF0 through to ORF5 as shown by the number within the grey box. A genome-linked protein (VPg) is associated with the 5' end of the genome and is shown as a black circle. Based on Stevens et al. (2005a).

#### 1.2.4.3 Transmission

Poleroviruses are circulative, non-propagative aphid vectored viruses (Gray and Gildow, 2003; Stevens et al., 2005a). *M. persicae* and *Macrosiphum euphorbiae* are the most efficient vectors and as such the focal point for epidemiological studies and disease control (BBRO, 2023a; Kozłowska-Makulska et al., 2009). Notably, unlike BYV, BMYV and BChV are not transmitted by *A. fabae* (Bjorling and Nilsson, 1966; Kozłowska-Makulska et al., 2009; Russell, 1963). Poleroviruses are phloem limited and as such have long acquisition times (Stevens and Lacomme, 2017). In the case

of BMV, aphids need to feed on an infected host plant for at least 24 hours to acquire the virus and then feed on a healthy plant for at least eight hours to transfer it (Russell, 1962).

#### 1.2.4.4 Symptoms and consequences

##### BMV

Initial infection with BMV is associated with chlorotic areas on fully expanded leaves. As the infection develops, older leaf tissue turns “golden yellow” (Stevens et al., 2004) between the leaf veins and becomes thick and brittle (Harveson et al., 2009). Both root weight and sugar yield are reduced in infected plants, whilst root impurities (which have a detrimental effect on sugar extraction) are increased (Stevens et al., 2004). BMV infection also increases plant susceptibility to *Alternaria* spp. particularly later in the growing season, further compounding yield losses (Smith et al., 1995). Yield losses to BMV can be up to 29% when plants are infected early in the season. Later infection has a reduced impact, with field trials inoculated with BMV in July showing no significant loss of yield (Smith and Hallsworth, 1990). Although virus-infected sugar beet plants have been shown to have reduced rates of net photosynthesis (Hall and Loomis, 1972), the exact way virus infection results in these yield losses has not been conclusively established (Peters, 1988).

##### BChV

BChV infection causes paler interveinal leaf yellowing than the golden yellow seen with BMV (Stevens et al., 2004), and the green leaf veins are more pronounced (Harveson et al., 2009). However, the same thicken brittle older leaves are observed and distinguishing between these viruses in the field is difficult. Overall, BChV has a smaller impact on yield than BMV (up to 24%) but later infections result in higher yield losses than BMV (Stevens et al., 2004).

#### 1.2.5 Beet yellows virus

##### 1.2.5.1 Taxonomy

BYV is a *Closterovirus* in the *Closteroviridae* family and has the species name *Closterovirus Beet yellows virus*. Members of the *Closteroviridae* family are characterised by very long filamentous particles of between 650 to 2200nm and large positive-sense single-stranded RNA genomes (between 13 and 19.3kb). The family is comprised of four genera that are classified based on the

phylogenetic relationship of key proteins, their genome organisation, particle length, and vector species (Fuchs et al., 2020).

BYV particles have a complex molecular structure which has been described as having a 'rattlesnake' like appearance (Agranovsky et al., 1995). The 'body' of the virion encapsidates 96% of the BYV genome and is formed of the major coat protein. The 5' end of the genome is surrounded by the 'tail' which is comprised of at least four proteins including the minor coat protein (Peremyslov et al., 2004). This tail region has a vital role in cell-to-cell movement of the particle, with experimental mutations to the tail proteins inhibiting movement (Napuli et al., 2003).

#### 1.2.5.2 Genomic organisation

The BYV genome is significantly larger than that of the beet poleroviruses. Although some other genera in the *Closteroviridae* family have segmented genomes (where the viral genome is split across two or more RNA molecules), *Closteroviruses* have a monopartite genome of between 14.5 and 19.3kb (Fuchs et al., 2020). Across its 15.5kb genome BYV has nine ORFs, two of which are directly translated from the genomic RNA and the remaining seven in a set of subgenomic messenger RNAs (mRNA) (Agranovsky et al., 1994; Dolja, 2003; He, Rao and Creamer, 1997).

#### 1.2.5.3 Transmission

BYV is transmitted semi-persistently by its aphid vectors. The virus has a wider vector range than the beet poleroviruses with 23 different aphid species known to acquire and transmit the virus (Fuchs et al., 2020; Harveson et al., 2009; Kirk et al., 1991; Summers et al., 1990). Of these *M. persicae* and *A. fabae* are seen as the most important vectors due to their efficiency of transmission and tendency to colonise sugar beet (Heathcote, 1966; Limburg et al., 1997). Acquisition of the virus can occur within five minutes of feeding, and transmission within one hour (Watson, 1946). BYV virus is not as strictly phloem limited as the beet poleroviruses, spreading into mesophyll and epidermis cells during the later stages of infection (Esau et al., 1967; Peters, 1988).

#### 1.2.5.4 Symptoms and consequences

Later symptoms of BYV are very similar to those caused by BMV or BChV infection. However, initial symptoms are distinctive with BYV causing vein clearing, particularly on younger leaves (Wintermantel, 2005). As the infection proceeds, BYV causes leaf yellowing with older leaves

becoming thick and brittle. In advanced infections, necrotic brown/red spots can appear on older leaves (Harveson et al., 2009). As with BMV, the impact of BYV on yield decreases with later infection. 47% losses in sugar yield have been reported for infection occurring in early or mid-season inoculated trials, compared to no significant losses in trials inoculated after the end of July (Smith and Hallsworth, 1990).

### 1.3 Plant virus diagnostics in relation to virus yellows

Currently when a plant becomes infected with virus there is no feasible cure. Yet this does not make knowing exactly which virus is causing disease in a crop a purely academic exercise (Rubio et al., 2020). As previously discussed, different viruses have different transmission mechanisms and therefore different disease control methods may need to be deployed, especially in crops. Likewise, knowing which viruses were present in a previous crop may impact growers' choices of future varieties, particularly if resistant ones are available. Therefore, when working with plant viruses in either a research or agricultural setting, accurate and reliable plant diagnostics are fundamental. Much effort has been given to the development of plant diagnostics, the methodology of some are covered below.

#### 1.3.1 Microscopy

In some cases, the size and shape of a virus particle can be sufficient to identify it. Given virus particles are not visible with the naked eye, the use of microscopy is vital in this approach. The first virus to be seen through an electron microscope was the tobacco mosaic virus (TMV) in 1939 (Kausche et al., 1939). Since then, the electron microscope has been used to identify many plant viruses including BYV. Prepared leaf sap from symptomatic plants consistently showed the characteristic long filamentous particles of BYV, enabling the disease to be diagnosed (Horne et al., 1959). However, this technique does not permit the differentiation of viruses which have similar sized and shaped particles. For example, the beet poleroviruses share the same small icosahedral particle shape making distinguishing them via microscopy alone impossible (Hull, 2009d; Sömera et al., 2021).

Using electron microscopy to identify plant viruses can also be challenging when a single plant is infected with multiple viruses. This was shown during the discovery of BYV, during which spherical particles were also observed in sap preparations containing the filamentous BYV particles (Horne et al., 1959; Nixon and Watson, 1951). These particles could be evidence of a mixed infection with

one or more other viruses. However these particles were also recorded as present in healthy plants, highlighting the difficulty of differentiating virus particles from normal cellular components (Hull, 2009d).

### 1.3.2 Host range techniques

Some plants display characteristic symptoms when infected with a particular virus. These plants are known as indicator hosts and were one of the earliest plant diagnostic techniques. Other plants can be immune to infection by a specific virus, and hence allow identification by their lack of infection (Hull, 2009d).

Unlike electron microscopy, indicator hosts can be used to differentiate BMV and BChV. The host range of BChV is reported as narrower than BMV (Hauser et al., 2002; Stevens et al., 1994a). This characteristic has been utilised to distinguish between these closely related species. BChV causes interveinal reddening on *Chenopodium capitatum* but does not infect *Capsella bursa-pastoris*. Conversely, BMV causes reddening of *C. bursa-pastoris* but does not infect *C. capitatum* (Harveson et al., 2009; Stevens et al., 2005a). *C. capitatum* has also been used as an indicator host for BYV because it produces severe symptoms faster than in sugar beet (Russell, 1960). However the symptoms of BYV in *C. capitatum* are so severe that they often cause premature death and hence *Tetragonia expansa* is favoured for use in laboratory studies (Polák, 1971).

Host range is rarely now used in isolation to identify plant viruses, because there are many drawbacks to the approach. Host range techniques are more time-consuming than serological or molecular approaches, with indicator plants often taking days/weeks to produce any characteristic symptoms. It is also vital to 'back inoculate' from an immune to an indicator host to ensure virus infection was not merely asymptomatic, which further lengthens the diagnostic process. There is also the problem of infection itself, with specific vectors the only way to achieve inoculation of some viruses. Plus there is the containment issues which arise when potentially multiplying plant pathogens and the risk this could cause to nearby crops (Hull, 2009d).

### 1.3.3 Serological based approaches

Serological diagnostics exploit the interaction between protein (antigen) and antibody. Most serological diagnostics are based on antibodies specifically binding the viral coat proteins, though some antibodies instead target protein subunits from disrupted plant viruses or viral proteins expressed during infection. Whichever antigen is targeted, the antibodies used in these diagnostics

are either monoclonal or polyclonal. Monoclonal antibodies target one specific region of protein (called an epitope). Polyclonal antibodies contain a mixture of different antibodies that bind to the different epitopes available on the antigen. Monoclonal antibodies are therefore more specific than polyclonal antibodies. However high specificity may not always be an advantage. For example, a polyclonal antibody may be beneficial when detecting a virus with many strains or variant.

One of the most used serological approaches in plant diagnostics is the enzyme-linked immunosorbent assay (ELISA). ELISA works by using antibodies to specifically bind the virus proteins and then using an enzyme linked antibody to visualise the reaction. There are many variations of the basic ELISA methodology, varying in the number and type of antibodies used. The double-antibody sandwich method (DAS-ELISA) and triple-antibody sandwich method (TAS-ELISA) both used in the detection of virus yellows are outlined in figure 1.4 (D'Arcy et al., 1989; DSMZ, 2023; Polák et al., 1980).

ELISA diagnostics are widely used to identify viral diseases in plants because they are economical in reagent use, adaptable for use with large sample numbers and allow some degree of quantification (Hull, 2009d). However they are also often time consuming, taking up to two days to produce results. ELISA also relies on the successful production of specific antibodies, a process which can be costly and sometimes not possible. This is particularly true for closely related viruses which have largely homologous coat proteins such as the case for the beet poleroviruses. Although an ELISA diagnostic is commercially available for BYV (such as from (DSMZ, 2023)), no specific ELISA diagnostic is currently available to differentiate BMYV and BChV. Attempts have been made to produce such a test, which were successful in the 1990s (Stevens et al., 1994a). However this antibody was subsequently lost and due to high similarity of the two viruses efforts to replace it have been unsuccessful (BBRO, personal communication).



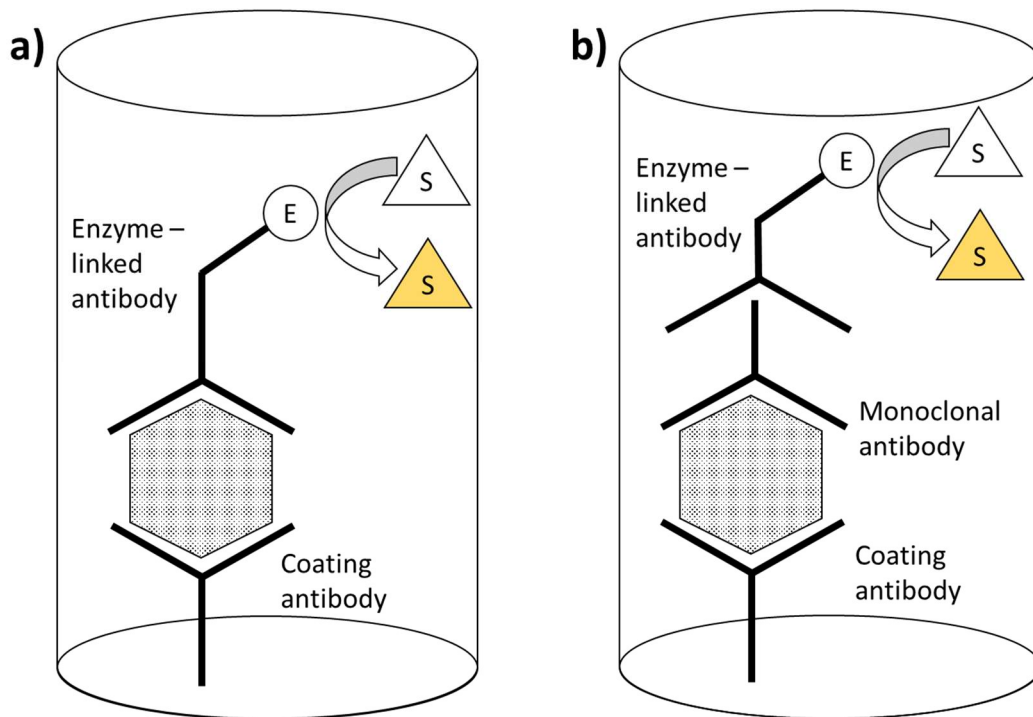


Figure 1.5: schematic diagram showing principle of double antibody sandwich (DAS) and triple antibody sandwich (TAS) enzyme-linked immunosorbent assays (ELISA). In both approaches, polyclonal coating antibody is bound to the microplate well, virus particles (shown as hexagons) then bind to the antibody, before being bound in turn by a monoclonal antibody. In DAS-ELISA (a) this second antibody is conjugated to an enzyme (circle, marked 'E') such as alkaline phosphatase, in TAS-ELISA (b) the monoclonal antibody binds to the virus particle but does not enzyme-linked. Instead, a third antibody with a conjugated enzyme is added which binds to the monoclonal antibody. When a colourless substrate (such as *p*-nitrophenyl phosphate) is added (shown as triangle, marked 'S') in the presence of the bound target virus, the substrate is cleaved by the enzyme and converted into a yellow compound which can be quantified (D'Arcy et al., 1989; DSMZ, 2023).

### 1.3.4 Molecular approaches

A polymerase chain reaction (PCR) is used to amplify specific fragments of DNA. This technique is now ubiquitous in modern laboratories, however is not directly applicable to most plant viruses because of their RNA genomes. Therefore most plant virus diagnostics, utilising PCR, first require a complementary-DNA (cDNA) template to be made via a suitable primer and reverse transcriptase enzyme. Following reverse-transcription PCR (RT-PCR), traditionally, the amplified DNA is visualised using gel-electrophoresis with a DNA 'band' of the expected size indicative of a positive sample.

Such an approach can be highly specific and sensitive, enabling detection of very low virus titres and reliably discriminating between closely related viruses based on their genome sequences.

However, one major disadvantage of conventional RT-PCR diagnostics is the high risk of false positives. The high sensitivity of the technique means that even small amounts of amplified DNA can be detected and every time a test tube containing amplified DNA is opened the risk of such contamination increases (Boonham et al., 2014). Real-time or quantitative PCR (qPCR) goes some way to overcome this issue. qPCR or RT-qPCR involves the association of amplified DNA with a fluorescent signal. This signal can be detected within a 'closed tube' either during the reaction or at the end, therefore greatly reducing the contamination risk. A published RT-qPCR methodology for the detection of BYV is available (Stevens et al., 1997), however to date, no qPCR-based assay able to differentiate between BMV and BChV has been published.

### 1.3.5 Sequencing

The financial and time burden of genome sequencing previously prohibited its adoption as a diagnostic tool in most practical settings. Now, with the continual advance of next generation sequencing technology, sequencing based approaches to plant virus diagnostics are increasing in popularity (Boonham et al., 2014). The ability of these approaches to detect viruses is largely dependent on the type of nucleic acid sequenced. For example, rolling circle amplification can be used to clone circular DNA plant viruses enabling them to be sequenced and identified (Inoue-Nagata et al., 2004; Schubert et al., 2007). Other approaches, such as total messenger RNA (mRNA) sequencing is not limited to one singular virus type and has been successfully used to identify known and previously unknown viruses (Al Rwahnih et al., 2009). However, because of the relative concentrations of host plant mRNA to viral RNA, much of the sequencing data generated by total mRNA sequencing is plant mRNA and not useful in a diagnostic sense (Boonham et al., 2014).

Techniques to improve the ratio of host to virus sequences are therefore often deployed. One such technique is small RNA (sRNA) sequencing, which targets short RNA fragments (Kreuze et al., 2009). These sRNAs are the result of plants naturally occurring RNA interference (RNAi) pathway which combats pathogens by targeting double stranded RNA (dsRNA).

Although the RNAi pathway is not specific to virus infection it is particularly important because of the dsRNA produced by plant viruses during their replication (Muhammad et al., 2019). This dsRNA is recognised by dicer-like (DCL) proteins (specifically DCL2 and DCL4) which cleave the RNA into

short 22 and 21 nucleotide (nt) fragments through their RNase III endoribonuclease activity (Carmell and Hannon, 2004). These sRNAs can be selected for sequencing, however in the natural plant response they are loaded into argonaute (AGO) proteins 1 and 2 (Wang et al., 2011) where they form an RNA-induced silencing complex (RISC) (Hammond et al., 2001). RNA-dependent RNA polymerase (RDR) proteins amplify this silencing function by recognising single-stranded RNAs and synthesising dsRNA which are in turn recognised by DCL proteins, continuing the RNA silencing process (Muhammad et al., 2019).

Sequencing the sRNA produced by the plant RNAi pathway reduces the amount of host RNA sequenced, compared to total mRNA sequencing and permits the detection of both known and novel viruses (Kreuze et al., 2009). For example, Zhang et al. (2017) used sRNA sequencing to identify a previously unknown polerovirus which was causing yellowing symptoms in wheat in China.

## 1.4 Control strategies for virus yellows

Given the impact of virus yellows on sugar beet yield there is a clear need to control the disease to ensure crop profitability. Methods of controlling virus yellows have changed and developed since its discovery in the 1930s (Roland, 1936). Many of these methods, both historically and currently, have centred around the application of a chemical insecticide to kill the aphid vector. However, growing concern over the environmental impacts of these chemicals is placing increasing focus on more sustainable control strategies.

### 1.4.1 Chemical control

Chemicals which target the aphid vector of virus yellows have proved very successful at reducing virus yellows disease incidence. Over time different chemical classes have been introduced and then withdrawn from use in sugar beet either due to safety concerns (e.g. organophosphates such as Schradan (Broadbent, 1957)) or the emergence of aphid resistance (e.g. carbamates such as Aphox, Syngenta (Stevens, 2013)).

One insecticide class of particular importance in the story of virus yellows control in the UK is the neonicotinoids. These chemicals are neurotoxins which act on the nicotinic acetylcholine receptors of insects, causing paralysis and death (Goulson, 2013). Unlike previous insecticides which had to be sprayed onto the crop or applied as granules, neonicotinoids were applied to sugar beet seed.

The chemical was then taken up systemically by the plant as it germinated, providing protection from biting, or sucking insects for up to 14 weeks after planting (BBRO, 2016).

The first neonicotinoid sugar beet seed treatment used in the UK was Gaucho introduced by Bayer CropScience in 1994. Containing imidacloprid, Gaucho was widely adopted by growers with 70% of the crop being Gaucho treated seed by 2006. (Foster and Dewar, 2013). Other neonicotinoid-based seed treatments followed (Cruiser SB containing thiamethoxam and Poncho Beta containing clothianidin by Bayer Crop Science, and Poncho Beta). The treatments provided very effective control against virus yellows, evidenced by the low disease incidence observed during their use (Dewar and Qi, 2021). They were also recommended for protection against other major sugar beet pests such as leaf miner (BBRO, 2016). However following concerns regarding the impact of neonicotinoids on pollinators and other non-target organisms, their use on sugar beet was banned by the European Union in 2018 (The European Commission, 2018a, 2018b). Since 2018, neonicotinoid seed treatments have still been used on the UK sugar beet crop in 2022 and 2023 due to emergency authorisations from the UK government.

Aside from concerns regarding their environmental impacts, chemical control options are also vulnerable to the development of resistance. This is particularly true in the case of combating *M. persicae* which has a remarkable ability to evolve mechanisms through which it can overcome chemical modes of action (Foster et al., 2017). The first chemical resistance reported in *M. persicae* was to organophosphates in 1955 (Anthon, 1955). Since then, resistance mechanisms have been identified against at least seven different chemical classes, including neonicotinoids (reviewed in Bass et al., 2014). Therefore reliance on chemical control strategies is not considered a long term solution to the issue of virus yellows.

#### 1.4.2 Integrated pest management

The use of integrated pest management (IPM), whereby sustainable approaches are prioritised, and chemical applications only used as a last resort, is now seen as the future of agriculture (EASAC, 2023). IPM integrates alternative plant protection methods with an aim of reducing reliance on chemical inputs. In the case of sugar beet, this currently involves removing sources of virus infection, monitoring aphid populations, encouraging natural aphid predators, and ensuring optimum plant populations are achieved.

If these strategies fail to control aphid numbers, applications of foliar insecticides may be required but their use is determined by an economic threshold. This is currently set at one green wingless

aphid per four plants up to the 12-leaf stage, rising to one green wingless aphid per plant between the 12-16 leaves (BBRO, 2023a). This changing threshold reflects the development of mature plant resistance (MPR). Around the 10-leaf stage, sugar beet plants develop a natural resistance to aphids associated with the development of black deposits in the aphid gut, reduced fecundity, and increased mortality (Schop et al., 2022). Agronomic practices which increase the rate of plant development, and the onset of MPR, consequently reduce the risk of virus yellows. Measures such as waiting for optimum weather conditions before planting seed, ensuring optimal fertiliser applications are used, and avoiding herbicide damage are all recommended as part of a sugar beet IPM strategy (BBRO, 2023a).

#### 1.4.3 Monitoring and forecasting

Forecasting and monitoring crop pests is also a crucial part of the IPM system (EASAC, 2023). Accurate predictions of disease risk enable control strategies to be implemented in a timely manner. Sugar beet benefits from a virus yellows forecasting model which estimates the proportion of crop predicted to become infected with virus yellows in the absence of any control strategies (Werker et al., 1998). Forecasting also provides predictions on the first *M. persicae* flight, giving advanced warning on when active crop monitoring should commence.

Since their ban in 2018, emergency authorisations have been granted by the UK government for the use of neonicotinoid seed treatments in 2021, 2022 and 2023. These authorisations were dependent on the virus yellows forecasting model predicting disease incidences above a defined threshold. This threshold was met in 2022 and in 2023, resulting in 70% and 60% of the UK sugar beet crop being treated with neonicotinoids in 2022 and 2023 respectively (BBRO, personal communication).

#### 1.4.4 Virus sources

In isolation, aphids rarely pose a threat to the sugar beet crop. It is only as vectors of viruses that they become economically devastating pests. Therefore, one way to reduce the threat they pose is to reduce the sources of virus in the area surrounding the crop, thereby reducing the chance of an aphid acquiring viruses. This approach has been used ever since the discovery of the viral cause of virus yellows in the 1940s (Watson et al., 1946). Since then our understanding of what species and situations present the biggest viral sources has developed.

Plants which host both aphids and yellowing viruses present the greatest risk as virus sources. Many agricultural weed species are known hosts of virus yellows. A study in 1991 found 3% of weeds collected contained BMV, equating to 2000 infected weeds per hectare (Stevens et al., 1994b). Hence effective weed control programmes can reduce the risk of virus infection. Clamps (the heaps in which roots are stored after harvest) can also act as sources of virus infection. These are particularly problematic because they bridge the gap between the previous years harvest and the subsequent season, providing a site for both aphids and virus to 'overwinter'. One study identified 17% of aphids found feeding on a clamp were carrying BMV, while 10% carried BYV (Heathcote and Byford, 1975). Therefore, it is recommended to destroy or remove clamps (and other overwintering hosts such as cover crops) before sowing any nearby crops the next year (BBRO, 2023a).

#### 1.4.5 Varietal resistance

Although cultural practices, and applications of insecticide when needed, can control the spread of virus yellows it is rarely enough to prevent any yield losses, particularly in epidemic years. The development of varieties which can overcome virus infection is therefore often heralded as the ultimate solution to virus yellows.

There are many different types of virus resistance exhibited by plants. Examples include resistance to virus replication and accumulation, resistance to virus movement between cells, and resistance to virus inoculation. Alternatively, plants may have resistance to the effects of virus infection an ability often termed 'tolerance' (Lecoq et al., 2004). To avoid confusion, precise and consistent terminology is crucial when describing a plants' susceptibility to a pathogen (Oliver and Fuchs, 2011). For consistency the terms susceptible, tolerance and resistance are defined in relation to virus infection below. These definitions are maintained throughout this thesis.

##### *Susceptible*

*Susceptible plants become infected by the virus and the virus replicates uninhibited within them. The symptoms of infection are fully expressed, and resultant yield loss is suffered.*

##### *Tolerant*

*Tolerant plants become infected by the virus and do not inhibit virus replication or accumulation. However, the plant may not express symptoms of the infection as strongly as a susceptible plant, and the resultant yield loss is decreased or negligible.*

## *Resistant*

*Resistant plants inhibit virus replication or accumulation, and therefore resulting symptoms and yield loss are decreased.*

The development of tolerant and resistant varieties has already proved successful in combating some sugar beet diseases. As discussed previously, rhizomania posed a major threat to the UK sugar beet industry in the 1980s. The discovery of the 'Holly' resistance and subsequent resistance genes, now means rhizomania rarely causes yield losses in the UK (Stevens, 2015). All varieties on the 2024 recommended list contain at least the *Rz1* gene, with specialist varieties also containing resistance to the resistance breaking AYPR strain of rhizomania.

Aside from viruses, yield loss from beet cyst nematode (BCN) can now also be combated by using partially resistant sugar beet varieties. Four varieties are included on the 2024 recommended list claiming tolerance to BCN, some of which outperform non-BCN tolerant varieties under BCN free conditions (BBRO, 2023b). This demonstrates that the yield drag, the drop in yield associated with the introduction of specialist traits into a varieties, can be overcome.

Previous studies have identified potential resistance sources from other species within the *Beta* genus. In 2008 Grimmer et al. successfully transferred BMV resistance from garden, fodder and leaf beet into sugar beet cultivars through traditional breeding approaches. For BYV resistance, quantitative trait loci (QTLs) have been identified (Grimmer et al., 2008b). However, for a virus yellows variety to be completely effective it must be able to overcome BMV, BYV and BChV both in isolation and as mixed infections.

The most recent advance in the production of virus yellows-resistant sugar beet varieties is gene editing technology. The gene editing bill, passed by the UK government in 2023, permits the use of genetically-edited crops in the UK market. Gene editing differs from genetic modification in that no genetic material from an unrelated species is incorporated into the target species (UK Government, 2023). This technique has been highlighted as one way of achieving virus yellows-resistant sugar beet varieties, but the timeline for their development and deployment is uncertain and expected to take at least five years (British Sugar, 2023b; Seccombe, 2023).

The development of a virus yellows resistant variety would be environmentally and commercially beneficial. However, currently only one variety, Maruscha KWS has been listed on the UK recommended variety list with recognised tolerance to virus yellows (BBRO, 2021, 2023b). Crucially this variety also only claims tolerance against one of the yellowing viruses, BMV (KWS, 2021), and

suffers a yield penalty compared to other listed varieties when grown in the absence of BMV (BBRO, 2023b).

Currently the BBRO undertakes a large-scale screening programme of both current and future sugar beet varieties to determine their performance under virus infection (Wright and Stevens, 2023). At the start of this PhD project, in 2020, the BBRO routinely tested varieties against BYV and BMV. During this project a UK BChV culture was established and subsequently the BBRO's variety screening has been broadened to include BChV. This screening is conducted via field trials, hand inoculated using viruliferous *M. persicae* aphids reared on the BBRO in-house virus cultures.

The BBRO in-house virus cultures have, however, never been sequenced and little is known about how they compare to 'wild' virus found in the UK crop. Equally, to date, no studies have explored the level of genetic variation seen within BMV, BChV or BYV in the UK, or indeed the impact this variation may have on future varieties. Therefore, determining this variation and its consequences on the durability of future resistant sugar beet varieties is crucial.



## 1.5 Project Aims

This project aimed to categorise the molecular and biological variability of virus yellows disease complex. Through identification of the virus species responsible, and assessment of the intra-species genetic variation, the diversity of virus yellows disease complex in the UK was determined. The effect of this diversity on the durability of future resistant/tolerant sugar beet varieties was then evaluated. Controlled environments were trialled to determine their suitability as a replacement for field trials for the assessment of variety performance under virus infection. However, as no reliable alternative was identified, ultimately a novel field trial was conducted assessing the performance of varieties under multiple virus populations.

# Chapter 2: General materials and methods

## 2.1 Aphid and virus cultures

All virus cultures were maintained within separate Versatile Environment Test Chambers (Panasonic, MLR-352) at the BBRO laboratories. Plants were grown under a 16-hour photoperiod, with light provided by 15 fluorescent tube lights (FL4055 ENW/37, Panasonic). Temperatures were set at 22°C during the light period, and 18°C during the dark period.

Unless otherwise stated, cultures of BMV were maintained on the indicator host *Capsella bursa-pastoris* to ensure they did not become contaminated with any other beet virus (Harveson et al., 2009). Cultures of BChV and BYV were maintained on the sugar beet variety Lightning (SESVanderHave). All plants were grown in commercially available compost such as Premium All Purpose (Miracle-Gro), in 9cm pots (0.36l capacity).

Virus-free *Myzus persicae* aphid cultures were maintained on *Brassica rapa* subspecies *chinensis*. To produce viruliferous aphids, colonies of these aphids were moved onto virus containing plants and allowed to feed. Viruliferous aphids were then transferred to either inoculate experimental plants, or onto fresh plants for culture maintenance. Aphids were removed from cultures using an insecticide (0.1g/L InSyst containing 20% w/w acetamiprid, CertisBelchim). Whenever possible, cultures were kept free of aphids to minimise the risk of contamination.

Whenever aphids were present on the virus cultures, strict contamination control measures were put in place. These included housing the different viruses in separate laboratories whenever possible and avoiding watering different virus cultures on the same day (to prevent aphid transfer from one culture to another).

## 2.2 Production of total RNA

### 2.2.1 Total RNA extraction

Total RNA extraction was completed using Tri Reagent Solution (Invitrogen™, AM9738) following the manufacturers protocol with some adaptations. Plant material was snap frozen in liquid nitrogen and then stored at -80°C prior to extraction.

In a liquid nitrogen environment, 0.4g of sugar beet leaf tissue was ground using a pestle and mortar. The resulting powder was transferred to a 50ml centrifuge tube (Corning, 4558), 4ml Tri Reagent Solution (Invitrogen™, AM9738) added then the solution mixed thoroughly by shaking. The solution was then aliquoted into four microcentrifuge tubes with leak proof lids (Eppendorf, 0030120221), before being vortexed briefly and incubated at room temperature for five minutes. After this, 200µl chloroform (Fisher Scientific) was added to each tube, then mixed gently by inverting. The aliquots were then incubated at room temperature for eight minutes before being centrifuged for 15 minutes at 14000rcf in a 4°C room. The clear top phase (containing the RNA) was then transferred to a clean microcentrifuge tube and 1000µl of 100% ethanol (approximately double the sample volume) added. The microcentrifuge tubes were then incubated at -80°C for two hours.

The supernatant was then removed, and the precipitate washed by adding 980µl of 80% ethanol and centrifuging for three minutes at 7500rcf. This step was repeated, then the pellet dried for five minutes in a 70°C heat block. The pellet was then resuspended in 10µl deionised water, and the sample aliquots recombined.

### 2.2.2 Quantification of total RNA

The concentration of the total RNA produced was determined using a Nanodrop 8000 spectrophotometer (Thermo Fisher Scientific).

The quality of the total RNA was checked by visualising on a 1.5% (w/v) agarose gel in 0.5 X Tris-Borate-EDTA buffer (TBE) with 10mg/ml ethidium bromide (Fisher Scientific). 5µl of Loading Buffer II (Invitrogen™, AM8546G) was added to 1µg of sample RNA and the volume made up to 10µl with deionised water. Gels were run at 110V for 45minutes, then imaged using a Typhoon FLA 9500 (GE Healthcare Life Sciences).

### 2.2.3 Total RNA clean-up

Total RNA samples were cleaned using the mirVana isolation kit (Ambion, AM1560) following the manufacturer's protocol with some modifications. In a microcentrifuge tube, 20µg of total RNA was made up to 50µl with deionised water. Samples which did not yield 20µg of total RNA were still cleaned if at least 5µg of total RNA was available. Then 250µl of Lysis/Binding Buffer was added to the mixture and mixed thoroughly by pipetting, 30µl of miRNA Homogenate Additive was then added, the mixture mixed thoroughly and then kept on ice for 10 minutes. After this, 412.5µl of 100% ethanol was added and mixed thoroughly. The mixture was then passed through a mirVana Filter Cartridge in a Collection Tube by centrifuging for 30 seconds at 10,000rcf. The flow-through was discarded, then 700µl of miRNA Wash Solution 1 was passed through the filter by centrifuging as before. The filter was then washed by adding 500µl of Wash Solution 2/3 and centrifuging for 30 seconds at 10,000rcf. This wash step was repeated, then the empty filter/collection tube assembly centrifuged for one minute at 7500rcf to remove any residual wash solution. The filter was then transferred to a clean collection tube and 50µl of Elution Solution pre-heated to 95°C added. The filter/Elution Solution was left for two minutes (for the Elution Solution to bind to the RNA within the filter) before being centrifuged for 30 seconds at maximum speed. This step was repeated to yield a final volume of 100µl, then 10µl NAOAc (Sigma-Aldrich, S2889) and 100µl 100% ethanol were added to the RNA elute. The mixture was then left to precipitate overnight at -80°C.

The following morning the mixture was centrifuged for 30 minutes at maximum speed in a 4°C room. The supernatant was discarded and 700µl of 80% ethanol was then added directly onto the pellet to wash it. The mixture was then centrifuged for three minutes at 7500rcf and the supernatant removed. The pellet was then centrifuged again for one minute at 7500rcf and as much of the remaining supernatant as possible removed. The pellet was then dried on a 37°C heat block for five minutes, and then resuspended in 16µl nuclease free water.

### 2.2.4 Quantification of cleaned total RNA

The resulting cleaned total RNA was then quantified, and quality checked as for the total RNA (Section 2.2.2).

## 2.3 sRNA library construction and sequencing

### 2.3.1 3' Adapter Adenylation

sRNA libraries were prepared following the protocol published by Xu et al (Xu et al., 2015). This protocol utilises High Definition (HD) adapters which contain four random nucleotides at their ligating ends. The 3' HD adapter was purchased phosphorylated but requires adenylation prior to use. This was achieved using the 5' DNA Adenylation Kit (New England BioLabs, E2610L) following the manufacturers protocol. The reaction was then cleaned up using an Oligo Clean and Concentrator Kit (Zymo Research, D4060) following the manufacturers protocol. The concentration of the resulting 3' adenylated HD adapter was determined using a Nanodrop 8000 spectrophotometer (Thermo Fisher Scientific). The concentration was then made up to 10 $\mu$ M using nuclease free water.

To confirm that the adenylation was successful a 16% urea polyacrylamide gel was run using both the newly adenylated 3' HD adapter and a non-adenylated 3' HD adapter control. If adenylation had been successful, the 3' HD adapter would be heavier than the non-adenylated control and be seen as a higher band on the gel.

For one gel 2.1g of urea (Fisher Scientific, U/0500/63), 1.4ml of deionised water and 0.5ml 5 X TBE were mixed in a 50ml centrifuge tube (Corning, 4558) and heated for 20 seconds in a microwave until the urea had dissolved. The solution was then left to cool before 2ml of 40% acrylamide/bis solution 19:1 (Bio-Rad, 1610144), 2.5 $\mu$ l of Tetramethylethylenediamine (Bio-Rad, 161-0800) and 50 $\mu$ l 10% Ammonium Persulfate solution (Sigma-Aldrich, A3678) was added. The mixture was poured between two 1mm glass plates (Bio-Rad, 1653311 and 1651824) and a 1mm comb added (Bio-Rad, 1653359). The gel was allowed to set, then transferred to a Mini-Protean Tetra Vertical Electrophoresis Cell (Bio-Rad) and 0.5 X TBE added as running buffer.

To 1 $\mu$ l of 3' HD adapter, 2 $\mu$ l of Novex™ Hi-Density TBE Sample Buffer (Invitrogen™, LC6678) was added and the volume made up to 10 $\mu$ l with deionised water. The gel was run at 120V for around 45 minutes or until the Bromophenol blue tracking dye reached the bottom of the gel. The gel was then removed and stained using SYBR™ Gold Nucleic Acid Gel Stain (Invitrogen™, S11494) before being imaged using a Typhoon FLA 9500 (GE Healthcare Life Sciences).

### 2.3.2 Adapter ligation and cDNA synthesis

To 1.5 $\mu$ l of adenylated 3' HD adapter, 2 $\mu$ g of mirVana cleaned sample total RNA was added and the volume made up to 7.5 $\mu$ l with deionised water. The sRNA library was then constructed following

the protocol set out in Xu et al (Xu et al., 2015). Firstly, the adenylated 3' HD adapter was ligated to the sample RNA using a T4 RNA ligase 2 (New England BioLabs, K227Q). An RNA Clean and Concentrator -5 kit (Zymo Research, R1015) was used to clean up the reaction, before de-adenylation using a 5' deadenylase (New England BioLabs, M0331S). The excess 3' adapter was removed using a RecJ exonuclease (New England BioLabs, M0264S), before the 5' HD adapter was ligated to the sample RNA using a T4 RNA Ligase 1 (New England BioLabs, M0204L). The di-tagged RNA was purified again using the RNA Clean and Concentrator -5 kit then cDNA was produced via reverse transcription using High Performance MMLV Reverse Transcriptase (Biosearch technologies, RT80125K).

### 2.3.3 sRNA library PCR and size selection

The resulting cDNA was amplified via PCR as described in Xu et al (Xu et al., 2015), using 10, 12 and 14 cycles of amplification and Phusion™ High-Fidelity DNA polymerase (Thermo Scientific™, F-530).

The PCR products were then separated on an 8% polyacrylamide gel. For one gel, 5ml deionised water, 1.5ml 40% acrylamide/bis solution 19:1 (Bio-Rad, 1610144), and 0.75ml 5 X TBE were mixed together in a 50ml centrifuge tube (Corning, 4558). 75µl 10% Ammonium Persulfate solution (Sigma, A3678-100G) and 3.75µl Tetramethylethylenediamine (Bio-Rad, 161-0800) were then added, and the gel poured between two 1mm glass plates (Bio-Rad, 1653311 and 1651824). A 1mm comb was added (Bio-Rad, 1653359) and the gels left to set. Once set, the gel was transferred to a Mini-Protean Tetra Vertical Electrophoresis Cell (Bio-Rad) and 0.5 X TBE buffer added.

The PCR products were combined with 5µl Novex™ Hi-Density TBE Sample Buffer, 5X (Invitrogen™, LC6678) and loaded into the gel along with 10µl 20 bp DNA ladder (Jenna Bioscience). The gel was run at 120V until the Xylene Cyanol dye had reached the very bottom of the gel. The gel was then stained and the bands of 145-150bp removed as described in Xu et al (Xu et al., 2015).

### 2.3.4 sRNA library sequencing

The constructed sRNA libraries were sent for sequencing where they were first quantified and pooled then sequenced using Illumina NextSeq 550 at a depth of approximately 33million reads by the Quadram Institute (Norwich).

Sequencing was completed in three batches; 2019, 2020 and 2021 samples. In each batch, control libraries from the BBRO cultures (BBRO BMV, BBRO BChV and BBRO BYV) along with non-virus infected sugar beet were also prepared and sequenced.

### 2.3.5 Visualising sequencing data

Raw sequencing data was received and processed by Rocky Payet (UEA). After being trimmed of adapter sequences and mapped to all known virus reference genomes, the resulting data was provided as BAM and BAI files.

The software package Integrative Genomics Viewer (IGV) version 2.3.75 (Thorvaldsdóttir, Robinson and Mesirov, 2013) was used to visualise reads mapping to the reference genomes of viruses of interest. The reference genomes used are outlined in Table 2.1.

*Table 2.1: details of the reference genomes of Beet yellows virus, Beet mild yellowing virus, Beet chlorosis virus, Beet western virus and Turnip yellows virus to which generated sequence data was mapped to.*

Virus	Definition as listed on GenBank	NCBI reference sequence number	Publication reference
Beet yellows virus (BYV)	Beet yellows virus, complete genome	NC_001598.1	(Agranovsky et al., 1994)
Beet mild yellowing virus (BMYV)	Beet mild yellowing virus, complete genome	NC_003491.1	(Guilley, Richards and Jonard, 1995)
Beet chlorosis virus (BChV)	Beet chlorosis virus, complete genome	NC_002766.1	(Hauser et al., 2002)
Beet western yellows virus (BWYV)	Beet western yellow virus, complete genome	NC_004756.1	(Beuve and Lemaire, 2002)
Turnip yellows virus (TuYV)	Turnip yellows virus, complete genome	NC_003743.1	(Veidt et al., 1988)

### 2.3.6 Producing consensus sequences

Consensus sequences were produced by firstly setting the relevant reference genome in IGV. The sample data set was then loaded to show any reads from the sample mapping to the specified reference genome. The IGV copy consensus sequence command was then used to create the consensus sequence for the entire virus genome. The method behind this consensus sequence calculation is taken from (Cavener, 1987), and is based on the frequency of a nucleotide at each position. Briefly, a nucleotide is assigned if it is present at a frequency greater than 50% and more than twice that of the second most frequent nucleotide. A co-consensus nucleotide is assigned when two nucleotides have a combined frequency greater than 75% but neither meet the criteria

of a single nucleotide. When neither of these two criteria are met, or no mapping reads were present at that location, an 'N' is assigned.

## 2.4 Enzyme-linked immunosorbent assay

### 2.4.1 Antibody test kits

Enzyme-linked immunosorbent assay (ELISA) antibody sets were purchased from The Leibniz Institute DSMZ. Details of the specific test kits used, and their specificity are outlined in Table 2.2.

*Table 2.2: details of ELISA antibody test kits used to detect Beet chlorosis virus, Beet mild yellowing virus, Beet western yellows virus, Beet yellows virus and Turnip yellows virus in plant leaf samples.*

Antiserum test name	DSMZ number	Test format	Detects
Beet western yellows virus	RT-0049-0049/1	TAS-ELISA	BWYV, TuYV, BMYV, BChV
Beet yellows virus	RT-0185	DAS-ELISA	BYV

### 2.4.2 TAS-ELISA

#### 2.4.2.1 Coating and blocking

96-well flat-bottomed microplates (Thermo Scientific, 3455 or Star lab, E2996-1600) were coated with 100µl polyclonal immunoglobulin G antibody, diluted 1:1000 in carbonate-bicarbonate with sodium azide coating buffer (Bioreba, 110110). Plates were covered with clingfilm and incubated at 37°C for two hours. Plates were washed with phosphate buffered saline with 0.05% Tween® (Sigma Aldrich, P3563) using an automated plate washer (Wellwash, Thermo Scientific), programmed to wash each well three times with 200µl PBST and then aspirate. To each well, 100µl of 2% dried skimmed milk (less than 1% fat) in PBST blocking buffer was added. The plates were then recovered and incubated at 37°C for 30 minutes. The blocking buffer was then removed and the plates blotted dry.

Plates were either stored at 4°C for use on the same day or stored at -20°C for use within five days.

#### 2.4.2.2 Antigen binding

Two 2.5cm diameter leaf discs were taken from each leaf being tested. Wherever possible these discs were taken from leaf blade, avoiding the midrib. Extraction buffer was prepared by dissolving 2% polyvinylpyrrolidone (Fisher BioReagents, BP431-500) in PBST. The two leaf discs were placed



in a standard extraction bag (Bioreba) with 1ml of prepared extraction buffer and crushed using a Homex 6 semi-automated homogeniser. Of the resulting leaf extract, 100µl was added to the relevant well on the coated plate. Each plate was also loaded with at least two negative and two positive controls. Wherever possible healthy sugar beet leaf tissue was used as a negative control. When it was not possible to guarantee sugar beet had not been contaminated with virus, prepared extraction buffer was used as a negative control. Freeze-dried positive controls were purchased from DSMZ (Table 2.3) and prepared following the manufacturers protocols.

*Table 2.3: details of positive controls used for ELISA tests, purchased from DSMZ.*

<b>Positive control name</b>	<b>DSMZ catalogue number</b>	<b>ELISA test providing positive result</b>
Beet mild yellowing virus	PV-1210	Beet western yellows virus
Beet chlorosis virus	PV-1211	Beet western yellows virus
Beet yellows virus	PC-0981	Beet yellows virus

Plates were covered with clingfilm and incubated at 4°C overnight. Leaf extract was removed from the plate and washed once by hand using PBST. Plates were then washed using the automated plate washer (Wellwash, Thermo Scientific) using the same wash/aspirate programme as described previously (Section 2.4.2.1).

#### 2.4.2.3 Monoclonal antibody

The monoclonal antibody was diluted 1:1000 in conjugate buffer (Bioreba, 110140), and 100µl added to each well. The plate was then covered with clingfilm and incubated at 37°C for 2 hours. The plate was then emptied and washed using the automated plate washer (Wellwash, Thermo Scientific) using the same wash/aspirate programme as described previously (Section 2.4.2.1).

#### 2.4.2.4 Enzyme-linked rabbit anti-mouse antibody

To each well, 100µl of rabbit anti-mouse alkaline phosphatase conjugate antibody, diluted 1:1000 in conjugate buffer (Bioreba, 110140) was then added. The plate was covered with clingfilm and incubated at 37°C for two hours. The plate was then emptied and washed using the automated plate washer (Wellwash, Thermo Scientific) using the same wash/aspirate programme as described previously (Section 2.4.2.1).

#### 2.4.2.5 Substrate addition and absorbance measurement

Substrate was freshly prepared by dissolving one 5mg p-nitrophenyl phosphate tablet (Thermo Scientific, 34047) in 5ml substrate buffer (Bioreba, 110130). Of this prepared substrate, 100µl was added to each well of the plate. The plate was then covered with clingfilm and kept under dark conditions at room temperature. An absorbance microplate reader (Spectrostar Nano, BMG Labtech) was used to measure the absorbance of each well at 405nm. Results were viewed and exported using MARs data analysis software 3.3 (BMG Labtech).

#### 2.4.3 DAS-ELISA

Coating was completed for DAS-ELISA as for TAS-ELISA, with the omission of the blocking step. Leaf extract and antigen binding protocol was also the same as for TAS-ELISA.

Immunoglobulin G alkaline phosphatase conjugate antibody, diluted 1:1000 in conjugate buffer (Bioreba, 110140), was added to each well. The plate was then covered with clingfilm and incubated at 37°C for two hours. The plate was then emptied and washed using the automated plate washer (Wellwash, Thermo Scientific) as per the TAS-ELISA protocol. Substrate was added and plates measured as described in TAS-ELISA protocol above.

## 2.5 Detection of beet poleroviruses by single step RT-qPCR

A single step RT-qPCR diagnostic was developed by Wulf Menzel (DSMZ, personal communication) to identify BMV and BChV infection in plant material. The probe and primer sequences used in this assay are detailed in Table 2.4.

Firstly, total RNA was extracted from the sample leaf tissue as described in section 2.2. Then in a 96-well PCR plate (Star lab, E1403-8200), 1µl of sample RNA was added to 12.5µl TaqMan™ Fast Advanced Master Mix (Applied Biosystems™, 4444556), 0.75µl of forward and reverse primers (10µM), 0.75µl of each probe (10µM), 0.1µl RevertAID RT (Thermo Scientific, 10612191) and 6.9µl of nuclease free water (Invitrogen™, AM9932). The plate was sealed with a MicroAmp optical adhesive film (Applied Biosystems, 4311971) and centrifuged for one minute at 2500rcf. The reaction was carried out in a QuantStudio™ 3 Real-Time PCR System (Applied Biosystems™, A28567) with cycling conditions of; 52°C for 10 minutes, 95°C for 10 minutes for cDNA synthesis, followed by 40 cycles of 95°C for 15 seconds and 60°C for one minute. Results were viewed using QuantStudio Design and Analysis software v1.5.2.

Table 2.4: Primer and probe sequences used in the single step RT-qPCR diagnostic developed by Wulf Menzel (DSMZ, personal communication) for the detection of Beet mild yellowing virus and Beet chlorosis virus in plant tissue.

Type	Shorthand name	Sequence 5'-3'	Target gene
BMV forward primer	BMV_F	GAGTGACGTACACATCTGAGAA	P1
BMV reverse primer	BMV_R	TCACCATCCGACAGAGCAA	P1
BMV probe	BMV_probe	VIC- CCAAACTGGGAAGGAGTTCTTGC	P1
BChV forward primer	BChV_F	AGTCATGACGACGGTTCACA	P1
BChV reverse primer	BChV_R	TTGCCAGTGACGGTTGAGA	P1
BChV probe	BChV_probe	FAM-TGATAACCTGCTCGCATGCTCC	P1

## 2.6 Detection of beet poleroviruses in aphids by RT-qPCR

To identify the presence of Turnip yellows virus (TuYV) and BMV and/or BChV an unpublished two-step RT-qPCR protocol developed at Rothamsted Research (Martin Williamson, personal communication) was used. The probe and primer sequences used in this assay are detailed in Table 2.5. Importantly, this assay was designed to detect both BMV and BChV using the same probe and therefore cannot be used to differentiate between the two viruses.

Table 2.5: primer and probe sequences used in the two-step RT-qPCR protocol for detection of Turnip yellows virus (TuYV) and Beet mild yellowing virus (BMV)/Beet chlorosis virus (BChV) in aphids. Developed by Martin Williamson (Rothamsted Research, personal communication).

Type	Shorthand name	Sequence 5'-3'	Target gene
Reverse transcription primer	VR2	GAACCATTGCCTTTGTAGRGG	P3/4
qPCR forward primer	Pol_F	CGTTTACAGCGTCTTACATCAACG	P3/4
qPCR reverse primer	Pol_R	CCATTGCCTTTGTAGAGGATCCT	P3/4
TuYV qPCR probe	Tu_probe	FAM-TGGTCCTCGGCAAC	P3/4
BMV/BChV qPCR probe	Pol_probe	VIC- TGGTCCTTGGCAACG	P3/4

### 2.6.1 Sample preparation

To each well of a lidded 96-well plate (CytoOne, CC7682-7596), 50µl of phosphate buffered saline (Fisher BioReagents, 12821680) was added, then one aphid was added to each well using a paintbrush. Aphids were homogenised using a 96-well plate multiple homogenizer (Burkard Scientific, BAMH-96), then plates sealed using a clear polypropylene seal (Starlabs, E2796-0793), and plate lids secured. Plates were then incubated in a water bath (Edulab) at 96°C for nine minutes, before centrifuging for two minutes at 2500rcf.

### 2.6.2 cDNA synthesis

To each well of a 96-well PCR plate (Star lab, E1403-8200), 1µl 10µM VR2 reverse transcription primer, 0.5µl 10mMol dNTP and 3.75µl diethyl pyrocarbonate treated water were added. Then 2µl of prepared sample was added, the plate re-sealed and centrifuged for 15 seconds at 2500rcf. The plate was then heated to 70°C for three minutes in a thermocycler (SimpliAmp, Applied biosystems) to anneal the primer to the RNA template. The plate was then placed on ice.

Following this, 0.15µl RevertAID reverse transcriptase (200U/µl) and 2µl RT buffer (provided as set from Thermo Scientific, 10612191), 0.30µl RNaseOUT recombinant ribonuclease inhibitor (10777019) and 0.30µl DEPC treated water was added to each well. The plate was then sealed with a fresh clear polypropylene seal and centrifuged for one minute at 2500rcf. DNA polymerisation and enzyme deactivation was carried out in the thermocycler; 42°C for 45 minutes, 50°C for 15 minutes, 70°C for 10 minutes. The plate was then placed on ice.

### 2.6.3 qPCR

To each well of a new 96-well PCR plate, 5µl TaqMan™ Fast Advanced Master Mix (Applied Biosystems™, 4444556), 0.2µl 10µM TuYV-2 probe, 0.2µl 10µM BMVYV-2 probe, 0.08µl 100µM polero-forward-primer-2, 0.08µl 100µM polero-reverse-primer-2 and 2.44µl DEPC treated water was added. After this, 2µl of synthesised cDNA was added to each well and the reaction mixed by pipetting. The plate was then sealed using a MicroAmp optical adhesive film (Applied Biosystems, 4311971), and centrifuged for one minute at 2500rcf. The reaction was completed in a QuantStudio™ 3 Real-Time PCR System (Applied Biosystems™, A28567), cycling conditions were a pre-read hold at 60°C for 30 seconds, UNG incubation hold at 50°C for two minutes, polymerase activation hold at 95°C for 20 seconds. Then 40 cycles of 95°C for one second and 60°C for 20 seconds, followed by a post-read hold at 60°C for 30 seconds. Results were viewed and exported in Microsoft Excel using QuantStudio Design and Analysis software v1.5.2.

# Chapter 3: Occurrence and prevalence of yellowing viruses in the UK 2019-22

## 3.1 Chapter Contributions

Leaf samples were collected by a combination of the BBRO network, British Sugar and Belchim Crop Protection. I conducted the molecular work myself. Rocky Payet processed the raw sequencing data, completed the initial bioinformatic analysis (preparing the data into BAM and BAI files, as outlined in section 2.3.5) and conducted the sequencing alignment (to determine the sample total read number and the number of sample reads mapping to each virus). Subsequent sequencing data analysis using Integrative Genomics Viewer, I completed myself.

## 3.2 Introduction

Virus yellows is a global issue, affecting sugar beet crops worldwide (Hossain et al., 2021; Kaya and Yilmaz, 2016; Kyriakou et al., 1983; Yoshida and Tamada, 2019). The prevalence of the viruses which cause the disease, however, varies between countries (Hossain et al., 2021; Stevens et al., 2005b). In the UK, virus yellows is caused by three viruses: BMV, BYV and BChV (Stevens et al., 2005b). Previous studies, undertaken prior to the widespread use of neonicotinoid seed treatments, identified BMV as the predominant cause of virus yellows in the UK sugar beet crop. BChV in comparison, was found to only account for around 10% of diseased plants in 1990 (Stevens et al., 2005b).

However, such studies become significantly rarer following the widespread adoption of neonicotinoid seed treatments. One UK survey from 2003, referenced in Stevens et al. (2005b), confirmed BMV was still the dominant beet polerovirus in the UK finding only 25% of polerovirus infected sugar beet leaves had BChV. However, as the methodology of this study is unpublished and the sample size unknown this result must be interpreted with caution. Nevertheless, the prevailing opinion of the UK sugar beet industry for the last thirty years has been that the lower

incidence of BChV, compared to BMV, and its smaller yield penalty (Stevens et al., 2004) make BChV of lesser importance to UK sugar beet growers (BBRO, 2017; Bowen, 2020). This is evidenced by the first virus yellows tolerant variety to appear on the UK recommended list only having recognised tolerance to BMV (BBRO, 2021; KWS, 2021).

The European ban on neonicotinoid seed treatments in 2019 increased the need for virus yellows surveillance (The European Commission, 2018a, 2018b). In 2021 Hossain et al. published a large-scale study of 10 European countries, including the UK, assessing virus yellows between 2017 and 2019. From symptomatic leaves sampled in the UK, between 3.6% and 25.8% were found to be infected with polerovirus. A subset of these polerovirus positive leaves were further analysed via qPCR to identify the specific polerovirus present. This analysis identified BChV present in between 33% to 100% of polerovirus positive leaves, suggesting an increasing occurrence of BChV in the UK (Hossain et al., 2021). However, the samples size was small with only one polerovirus positive sample being tested via qPCR in 2018. It is also important to note that in two of the three years this study was conducted over (2017 and 2018) the UK sugar beet crop was grown with neonicotinoid seed treatments which may have influenced virus epidemiology.

BYV incidence is much more variable than the beet poleroviruses (Hossain et al., 2021; Stevens et al., 2005b). The most recently published survey found 56.8% of symptomatic sugar beet leaves sampled from the UK were infected with BYV in 2017, compared to only 4.8% in 2018 (Hossain et al., 2021). This varying occurrence is perhaps one reason why resistance breeding efforts have prioritised BMV over BYV in the UK, despite BYV having a larger impact on yield. Alternatively, it may be due to difficulties in identifying BYV resistance sources.

BYV, BChV and BMV are not the only viruses known to cause yellowing symptoms in beet. BWYV, though present in America and Asia, has not yet been isolated in the UK or Europe (Beuve et al., 2008; Stevens et al., 2005a; Xiang et al., 2008). There is also continued confusion around Turnip yellows virus (TuYV). In 2002 the International Committee on Taxonomy of Viruses (ICTV) reclassified European isolates of Beet western yellows virus (BWYV) which do not infect sugar beet to a new species in the Polerovirus genus named TuYV (Mayo, 2002). Despite this, some isolates of TuYV have been found to infect sugar beet and recent studies claim to have identified TuYV from surveyed symptomatic sugar beet plants (Newbert, 2016; Puthanveed et al., 2023). Newbert (2016) inoculated sugar beet plants with three separate isolates of TuYV, identifying one isolate (Cau74-R) which infected sugar beet plants. Meanwhile survey work in Sweden conducted by (Puthanveed et al., (2023) found two field grown sugar beet plants infected with a mixed polerovirus infection of BMV, BChV and TuYV. In both studies, the identification of TuYV in sugar beet was confirmed

through sequencing however to date no evidence of naturally occurring TuYV infection in UK sugar beet has been found.

The availability of reliable and accurate diagnostics has also played an important role in our understanding of the distribution of yellowing viruses in the UK. Early studies on the distribution of yellowing viruses relied on basic serological methods which could not discriminate between closely related viruses (Duffus and Russell, 1970; Russell, 1963). Advances in molecular techniques have improved the reliability of virus detection and discrimination firstly via enzyme-linked immunosorbent assay (ELISA) and now increasingly by quantitative real-time polymerase chain reaction (qPCR) (D'Arcy, Torrance and Martin, 1989; Lemaire et al., 1995; Stevens et al., 2005a). Although an improvement in diagnostic specificity compared to ELISA, one downside of qPCR diagnostics is that it requires the generation of specific primers for each virus of interest (Stevens et al., 2005a). One novel technique which overcomes this need is the production and sequencing of small RNA (sRNA) libraries (Pooggin, 2018). An important benefit of this technique is that, theoretically, any virus can be identified from a single sample data set removing the need for multiple separate diagnostic assays (Kreuze et al., 2009). The sequencing data can also be stored and used in the future to determine if currently unknown viruses were present in these now historical samples.

In this chapter I aim to update our knowledge on the prevalence of yellowing viruses, establishing if BMV remains the most prevalent and important of the virus yellows complex in the UK. Across three years (2019 to 2021) I have determined the frequency of each yellowing virus, sampling symptomatic fodder and sea beet as well as sugar beet. Using novel sRNA library techniques and sequencing for virus detection, as well as utilising established ELISA and qPCR diagnostics. I also searched for evidence of poleroviruses which are yet to be found in sugar beet in the UK, namely BWYV and TuYV.

## 3.3 Materials and methods

### 3.3.1 Sample collection

Symptomatic sugar, fodder and sea beet were collected between August and October 2019 to 2021. Fully expanded leaves showing virus symptoms were selected by British Sugar contract managers, agronomists and other industry professionals and sent to the BBRO laboratory.

### 3.3.2 Virus diagnostics – 2019

In 2019, leaf samples were stored at 4°C prior to testing via ELISA with the BWYV and BYV antibody sets to confirm infection with either a beet polerovirus or BYV as outlined in Chapter 2 (section 2.4). Leaves were then freeze dried and stored at -80°C prior to the project start date. Unfortunately, due to Covid-19 laboratory closures, work on these samples was delayed and they remained in storage until June 2020. In June 2020 clean total RNA was extracted from ELISA positive leaves (as per Chapter 2, section 2.2). sRNA libraries were then produced and sequenced as outlined in Chapter 2 (section 2.3). In total sRNA libraries were produced via 15 leaves from 11 locations from samples collected in 2019 (Figure 3.1).

### 3.3.3 Virus diagnostics – 2020

As in 2019, in 2020 sampled leaves were initially stored at 4°C prior to determining their polerovirus and BYV infectivity via ELISA. Samples of leaf tissue from ELISA positive leaves were snap frozen in liquid nitrogen and stored at -80°C. Cleaned total RNA was extracted from this tissue and used to produce sRNA libraries. In total sRNA libraries were made from 14 leaves from the 12 locations in 2020 (Figure 3.1).

### 3.3.4 Virus diagnostics – 2021

In 2021, 338 leaves were received from 96 locations (Figure 3.1). These leaf samples were also used to create new virus cultures as detailed in Chapter 5 and as such, samples needed to be maintained as fresh as possible to permit aphid feeding. To achieve this, the leaf petioles were trimmed, and the remaining leaf placed upright in a beaker of deionised water at room temperature out of direct sunlight.

As in 2019 and 2020, all 2021 sampled leaves were first tested via ELISA. Polerovirus positive leaves were then randomly sub-sampled for further testing to determine if either BMYV or BChV was present. To do this RNA was extracted from sampled leaves via total RNA extraction (Chapter 2 section 2.2), then the single-step qPCR BMYV and BChV assay developed by Wulf Menzel (DSMZ, personal communication) was used (Chapter 2, section 2.5).

From the leaves determined polerovirus positive by ELISA, an additional sub-sample of 16 leaves was taken from which sRNA libraries were made. An additional sRNA library was also produced from one leaf collected from Fochabers, Scotland which exhibited yellowing symptoms but tested negative via ELISA and qPCR diagnostics. These libraries were produced predominantly for the



genetic variation investigation detailed in Chapter 5; data are included here for determining the presence of viruses other than BMYV, BChV and BYV. The controls sequenced with the 2021 library set are included to assist with determining positive thresholds. An aliquot of the extracted RNA that was used for creating these sRNA libraries was then tested using the single-step qPCR BMYV and BChV assay.

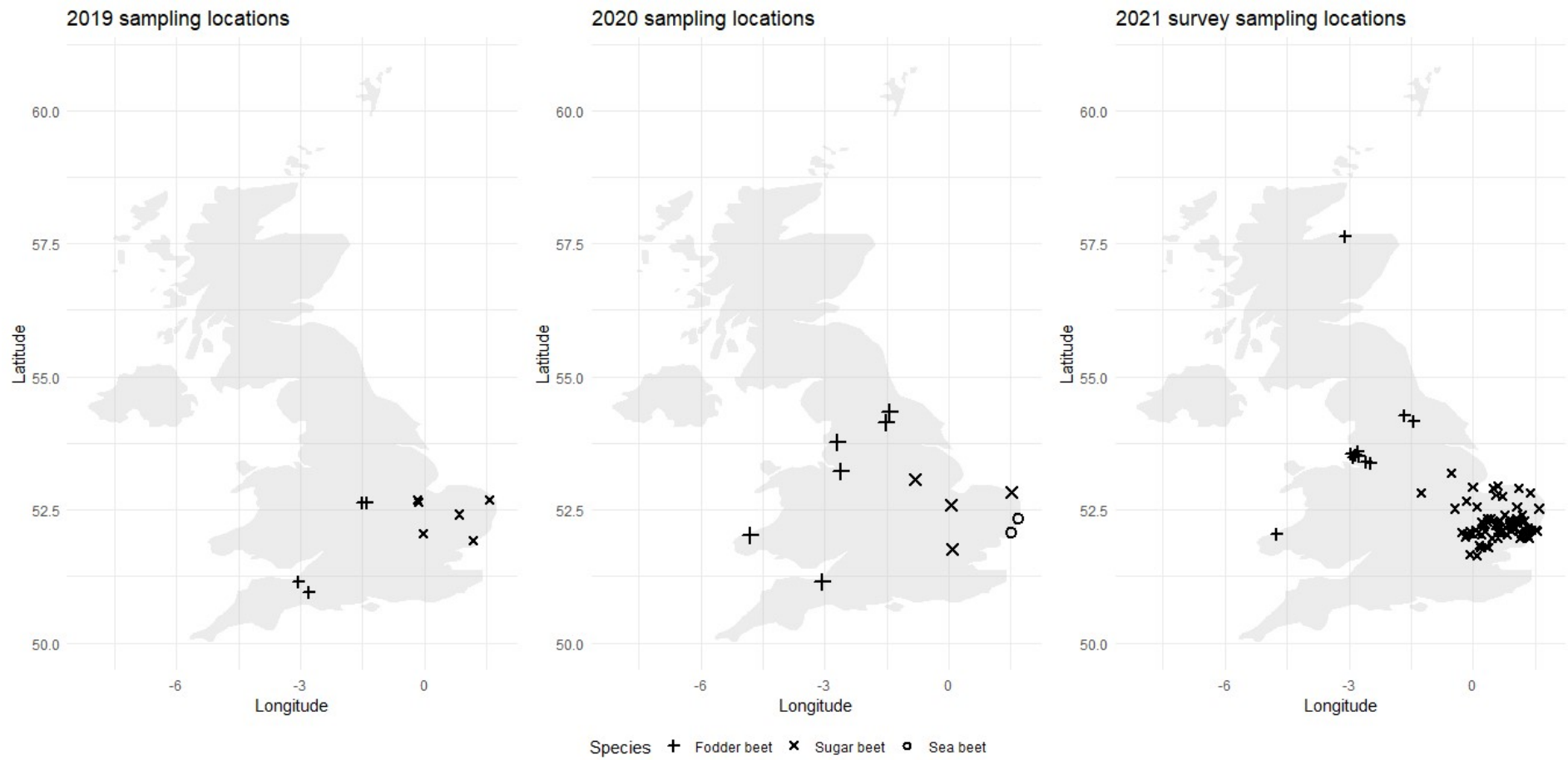


Figure 3.1: map showing sugar beet, fodder beet and sea beet sampling locations in the United Kingdom in 2019, 2020 and the larger scale survey sites in 2021.

### 3.3.5 Determining virus infection using sRNA library sequencing data

#### 3.3.5.1 Calculating the number of reads mapping to viruses of interest

The total reads for each sRNA library and the number of reads mapping to each virus were provided by Rocky Payet (UEA). To normalise these data, and enable comparisons between libraries with different total read numbers, the number of reads mapping to each virus of interest was divided by the total read number for that sample. To aid interpretation this value was then converted into a percentage.

#### 3.3.5.2 Calculating the percentage of a virus genome present in sample library

Firstly, Integrative Genomics Viewer (IGV) was used to produce a consensus sequence for the virus of interest (Chapter 2 section 2.3.6). This sequence was exported into Notepad++ (v8.4.9) and the number of 'N' nucleotides counted using the find/count function. To determine if an 'N' had been assigned due to high read variation at a location, or because no reads mapped at that location, the mapped reads were checked manually using IGV. Genome locations where reads differed from the consensus sequence are highlighted to enable quick identification. All instances where an 'N' had been assigned in the consensus sequence were due to no reads mapping at that location.

The percentage of the virus genome mapped by at least one read in the sample data set was then determined using the formula:

$$\left( \frac{100}{\text{Total nucleotides in reference genome}} \right) \times \left( \text{Total nucleotides in reference genome} - \text{Number of Ns in consensus sequence} \right)$$

#### 3.3.5.3 Defining ORF0, 1 and 2 genome regions

To permit comparisons between genome regions of different polioviruses, standardised genome regions were defined spanning open reading frames (ORF) 0, 1 and 2 based on the ORF locations as detailed by Hauser et al (2002). ORF0 region refers to nucleotides (nt) 30-750, ORF1 region to 160-2130nt and ORF2 region to 1540-3400nt.

#### 3.3.5.4 Filtering sequencing reads to ORF0 genome region

IGV was used to determine the percentage of the ORF0 region of a virus genome present in sample libraries. After loading the reference sequence as detailed in Chapter 2 section 2.3.5, the region 30-

750nt was specified and a consensus sequence produced for this specific region. The percentage of the region present in the sample was then determined as above.

#### 3.3.5.5 Percentage identity

Similarity of poleroviruses was determined by percentage identity calculated using the multiple sequence alignment tool Clustal Omega 2.1 (Sievers et al., 2011).

## 3.4 Results

### 3.4.1 BMYV and BChV

#### 3.4.1.1 Normalised read data

Very few reads from any sample sRNA library mapped to BChV, with the percentage of mapping reads ranging from 0-1.6% (Figure 3.2). Low mapping read numbers were also seen in the positive controls, with both having less than 1.25% BChV mapping reads. Although the negative controls had fewer BChV mapping reads than the positive controls, the difference between the closest negative and positive controls was small (0.2%). Overall, no obvious grouping of samples or controls was present.

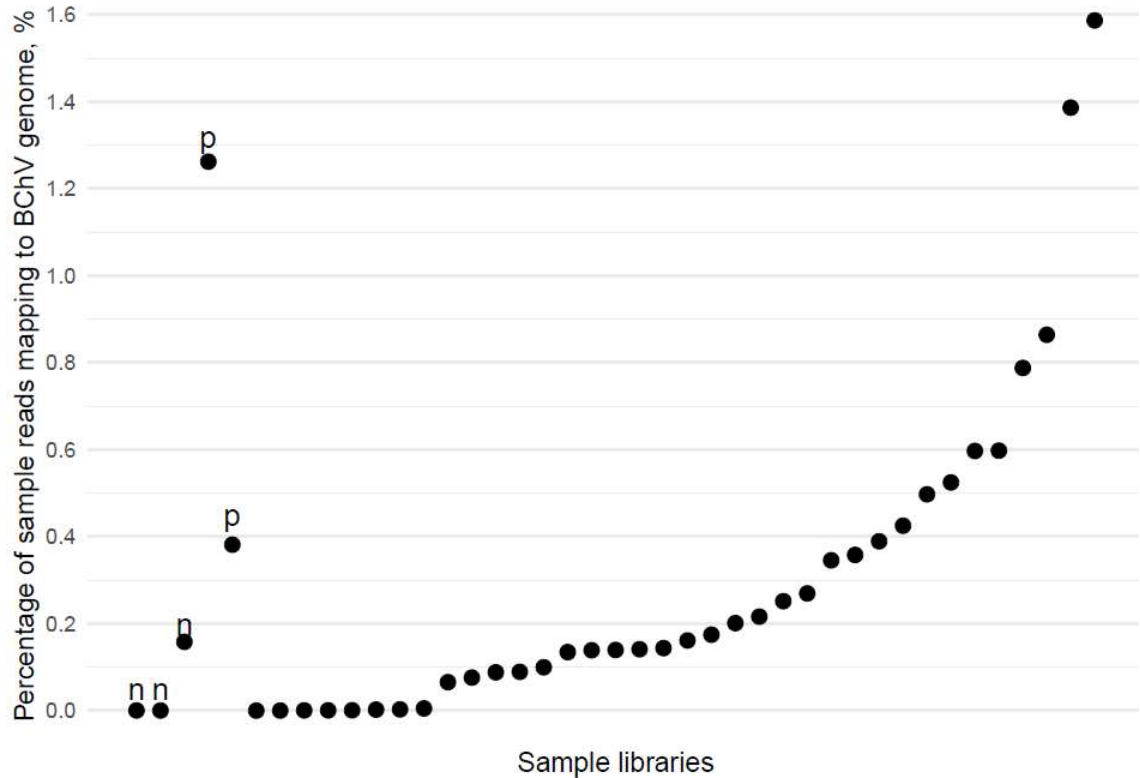


Figure 3.2: scatter plot showing the percentage of total small RNA (sRNA) reads mapping to the Beet chlorosis virus (BChV) reference genome for each of the 2019 and 2020 sample sRNA libraries, alongside the negative control libraries (indicated by 'n') and BChV positive control libraries (indicated by 'p').

mapping to BMV (Figure 3.3). Again, the overall abundance of mapped reads was low with a maximum of 2.8% of reads mapping to BMV. The BMV positive controls grouped more closely than the BChV controls, ranging from 0.5-0.8%. However, the negative controls showed a greater variation of between 0-0.97%. Two outlying samples were identified which had more than twice the number of reads mapping to BMV compared to the mean of the positive controls. One of these samples (with 2.77% BMV mapping reads) was from a plant known to contain both BMV and BYV. The other sample (with 2.57% BMV mapping reads) was a BYV positive control. Given these relatively small percentages and large variation amongst the controls, an alternative way to interpret this data was needed.

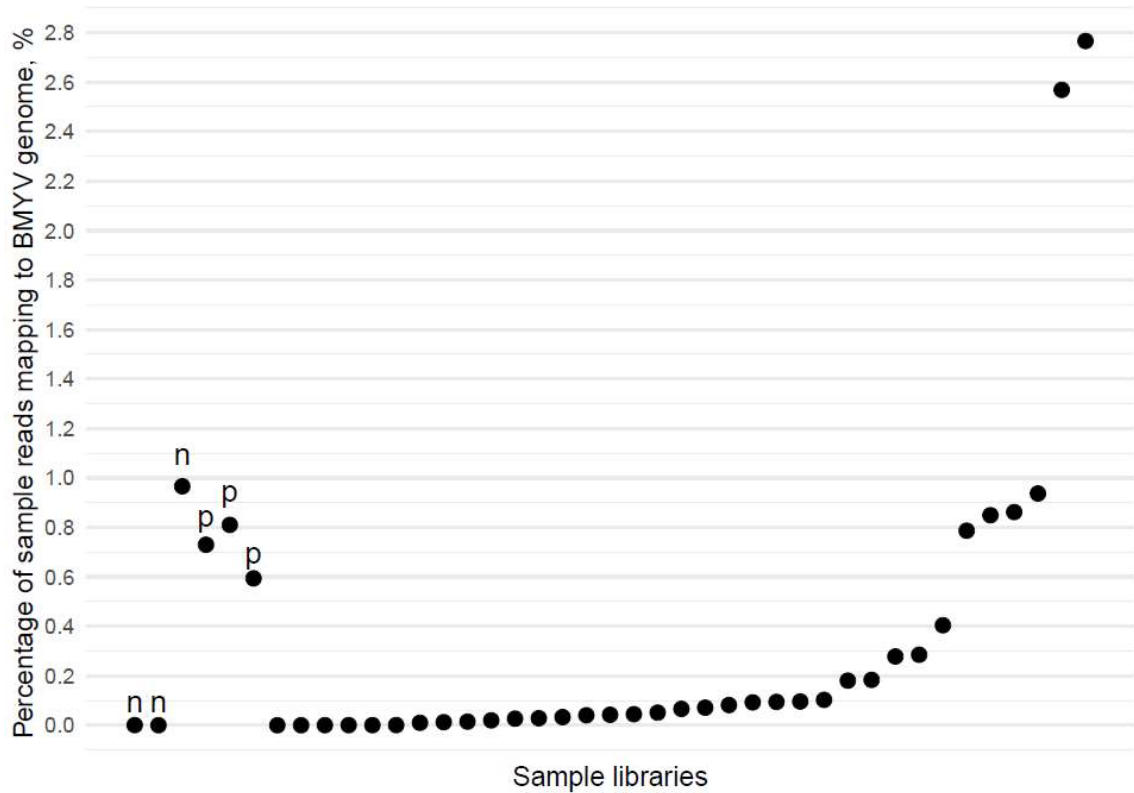


Figure 3.3: scatter plot showing the percentage of total small RNA (sRNA) library reads mapping to the Beet mild yellowing virus (BMV) reference genome for each of the 2019 and 2020 sample sRNA libraries, alongside the negative control libraries (indicated by ‘n’) and BMV positive control libraries (indicated by ‘p’).

3.3.1.2 Refined read data

Polerovirus reference genomes were compared to ascertain an infection threshold which could be applied to the survey data (Table 3.1). The highest percentage identity was found between BMV and BWYV, at 73.99% and therefore a value of 74% was set as a threshold above which samples were deemed positive here.

Table 3.1: whole Beet chlorosis virus (BChV), Beet mild yellowing virus (BMV), Beet western yellows virus (BWYV) and Turnip yellows virus (TuYV) genome percentage identity matrix produced using Clustal Omega 2.1 multiple sequence alignment.

	BChV	BMV	BWYV	TuYV
BChV	100.00			
BMV	67.03	100.00		
BWYV	66.89	73.99	100.00	
TuYV	66.50	68.26	68.55	100.00

sRNA library reads were filtered to only include reads mapping to the virus of interest. The percentage of the BMV genome present in the sRNA library of each sample is shown in Figure 3.4. This approach produced a clear difference between positive and negative controls, with the three BMV positive controls ranging from 88.12-88.68% and negative controls between 9.4-26.69%. Survey samples fell into two loose groups: below 45% and above 60%. Using the 74% threshold, 7 samples were classed as positive for BMV. Two of the BYV controls and one control known to be infected with both BMV and BYV also passed this threshold.

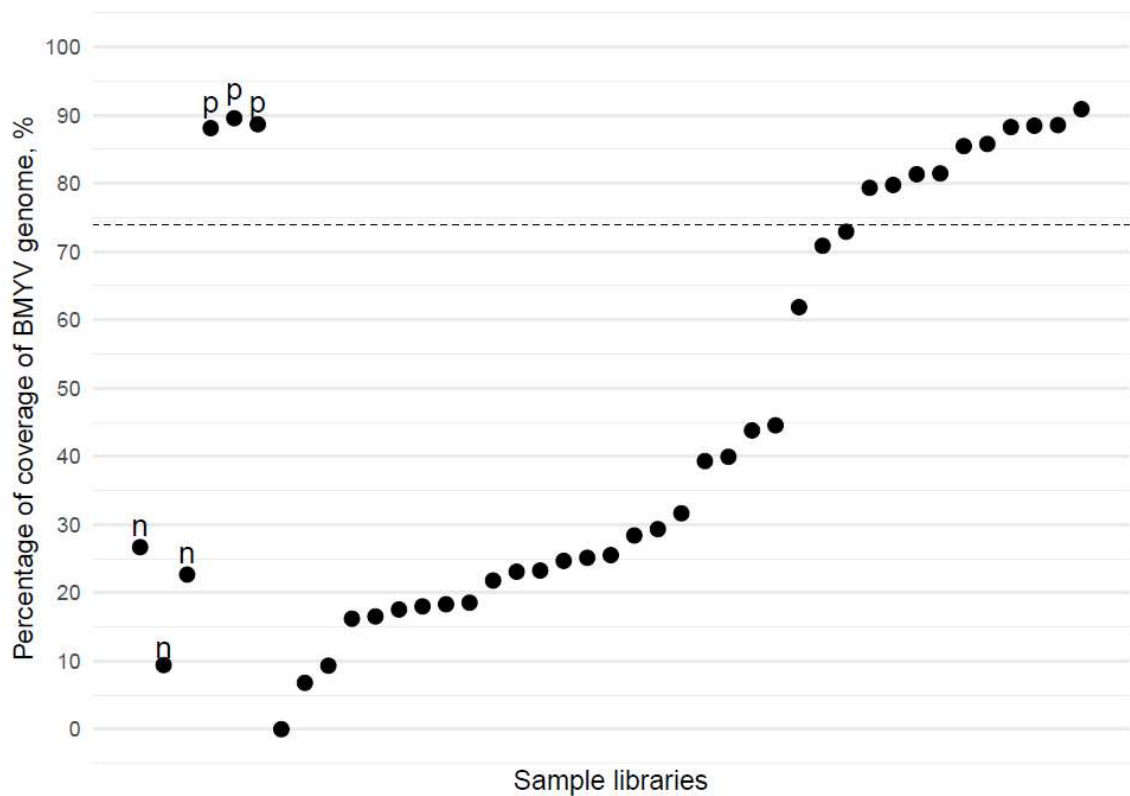


Figure 3.4: scatter plot showing the percentage coverage of the Beet mild yellowing virus (BMV) genome of each of the 2019 and 2020 sample small RNA libraries. p indicates BMV positive control libraries. n indicates negative control libraries. The dotted line marks threshold value of 74%.

The same clear difference between positive and negative controls was also seen when focusing on reads mapping to BChV. Both BChV positive controls had more than 90% of the BChV genome present, compared to the negative controls which all had less than 40%. None of the BMV or BYV positive controls reached the 74% threshold. However, unlike BMV, no grouping of survey samples was observed as shown in Figure 3.5. Using the 74% threshold, 17 samples were classed as positive for BChV.

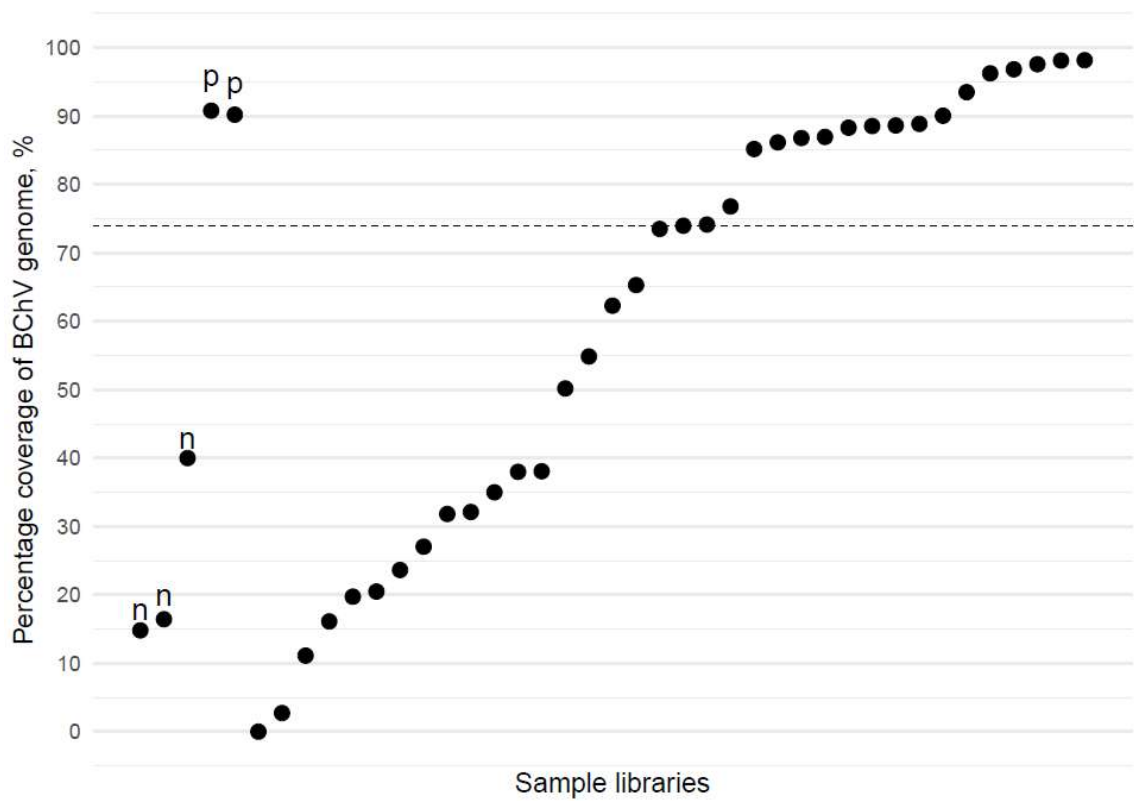


Figure 3.5: scatter plot showing the percentage coverage of the Beet chlorosis virus (BChV) genome of each of the 2019 and 2020 sample small RNA libraries. *p* indicates BChV positive control libraries. *n* indicates negative control libraries. The dotted line marks threshold value of 74%.

### 3.3.1.3 Read data mapping to the ORF0 region

Analysis of the 5' reading frames of BMV, BChV, TuYV and BWYV identified the genome region spanning ORF0 as the least homologous, with lower percentage identities across all virus pairings compared to ORF1 and ORF2 (Table 3.2, 3.3 and 3.4).



Table 3.2: percentage identity matrix of 30-750nt region (open reading frame 0) of Beet chlorosis virus (BChV), Beet mild yellowing virus (BMYV), Beet western yellows virus (BWYV) and Turnip yellows virus (TuYV) produced by Clustal Omega 2.1 multiple sequence alignment. Overall mean percentage identity = 46.045%

	BChV	BMYV	BWYV	TuYV
BChV	100.00			
BMYV	43.34	100.00		
BWYV	42.20	56.50	100.00	
TuYV	43.71	43.65	46.77	100.00

Table 3.3: percentage identity matrix of 16-2130nt region (open reading frame 1) of Beet chlorosis virus (BChV), Beet mild yellowing virus (BMYV), Beet western yellows virus (BWYV) and Turnip yellows virus (TuYV) produced by Clustal Omega 2.1 multiple sequence alignment. Overall mean percentage identity = 50.945%

	BChV	BMYV	BWYV	TuYV
BChV	100.00			
BMYV	47.24	100.00		
BWYV	47.60	61.31	100.00	
TuYV	46.99	49.97	52.56	100.00

Table 3.4: percentage identity matrix of 1540-3400nt region (open reading frame 2) Beet chlorosis virus (BChV), Beet mild yellowing virus (BMYV), Beet western yellows virus (BWYV) and Turnip yellows virus (TuYV) produced by Clustal Omega 2.1 multiple sequence alignment. Overall mean percentage identity = 63.178%

	BChV	BMYV	BWYV	TuYV
BChV	100.00			
BMYV	59.79	100.00		
BWYV	61.42	70.82	100.00	
TuYV	60.32	62.98	63.74	100.00

The percentage of sample reads mapping to the ORF0 region of BMYV is shown in Figure 3.6. The percentage of this genome region found in both the positive and negative controls was slightly lower than the percentage of the whole genome. Despite this, both sets of controls still grouped together and were clearly separated from each other with the negative controls ranging 6.0-24.3% and the positives 82.5-85.9%. The survey samples fell into at least two, possibly three, discrete

groups: less than 31% (classed as negative), above 80% (classed as positive) and between 31-80% which were likely to contain a polerovirus but could not be reliably called as BMVYV infected.

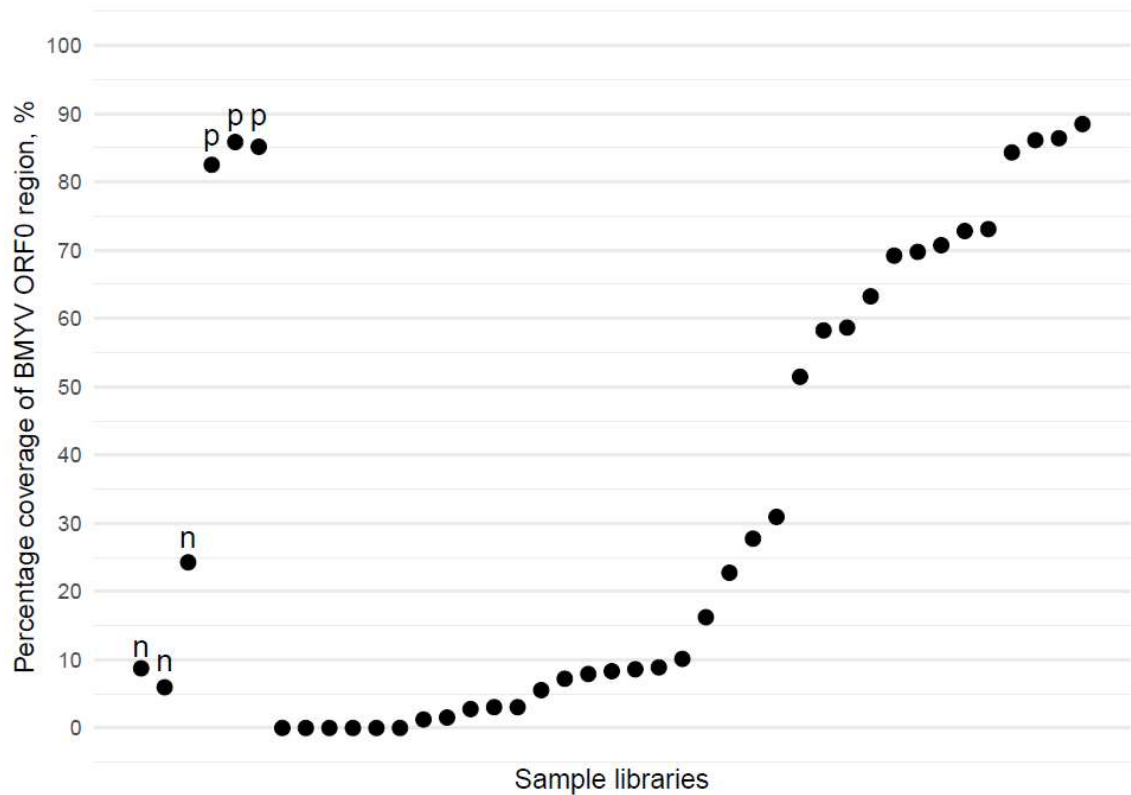


Figure 3.6: scatter plot showing the percentage coverage of the Beet mild yellowing virus (BMVYV) open reading frame (ORF) 0 genome region of each of the 2019 and 2020 sample small RNA libraries. p indicates BMVYV positive control libraries. n indicates negative control libraries.

The grouping of samples was less obvious when assessing the percentage of sample reads mapping to the ORF0 region of BChV (Figure 3.7). As with BMVYV, positive and negative controls were clearly distinguishable with ranges of 99.6-97.8% and 0-31.8% respectively. Of the 29 survey samples, 18 contained more than 80% of the ORF0 BChV genome region and were determined to be BChV positive. Samples with less than 32% of the genome region present were considered negative based on the grouping seen with the BMVYV data and the BChV negative controls. The three BMVYV controls contained 11.65, 15.12 and 55.2% of the BChV ORF0 region, compared to 0-8.6% of the BMVYV ORF0 found in the BChV positive controls. As such, samples with between 32-80% of the BChV ORF0 region were classed as polerovirus positive but not specifically BChV positive.

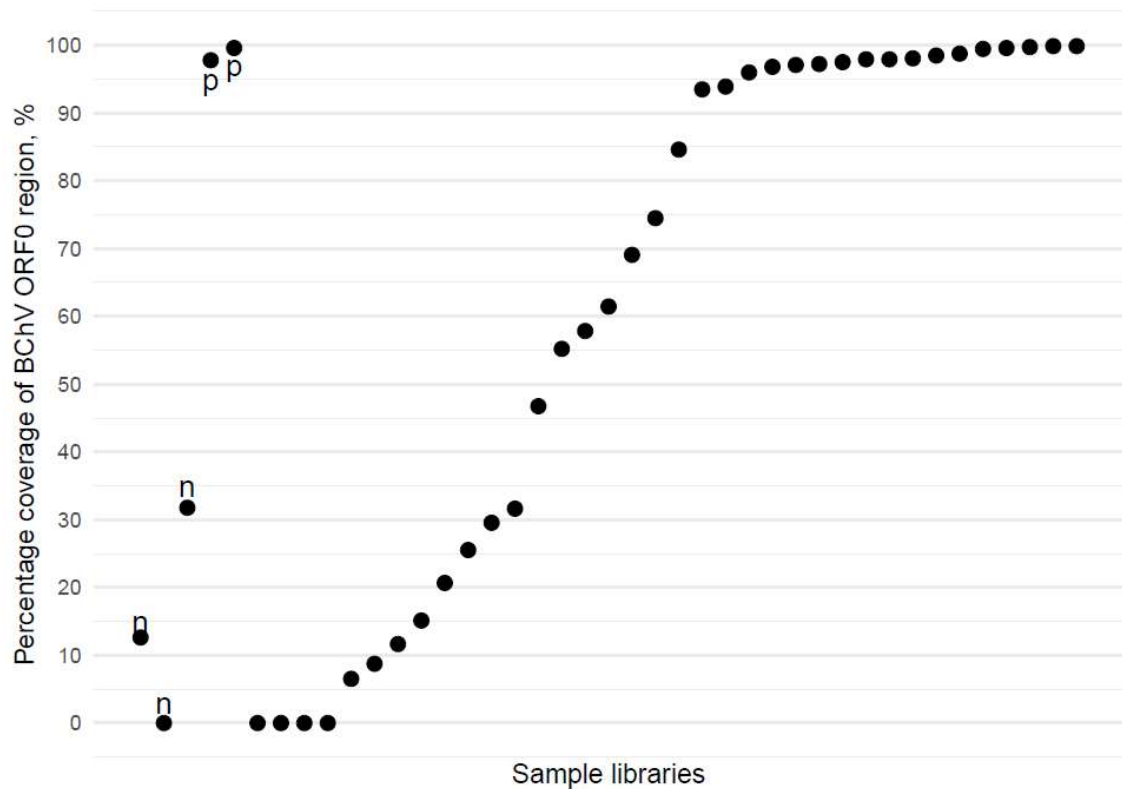


Figure 3.7: scatter plot showing the percentage coverage of the Beet chlorosis virus (BChV) open reading frame (ORF) 0 genome region of each of the 2019 and 2020 sample small RNA libraries. *p* indicates BChV positive control libraries. *n* indicates negative control libraries.

### 3.4.2 TuYV and BWYV

As with BMV and BChV the percentage of sRNA library reads mapping to TuYV was low, between 0-0.46% (Figure 3.8). The negative controls contained between 0-0.15% of reads mapping to TuYV, however as no TuYV positive controls were used it was not possible to compare these results to a known positive. Two outliers were observed in the data set having 0.46% and 0.43% of reads mapping to TuYV and could be considered positive for TuYV as they had more than twice the number of mapping reads compared to the mean of the negative controls. These two samples were the same samples identified as outliers when investigating the percentage of reads mapping to BMV.

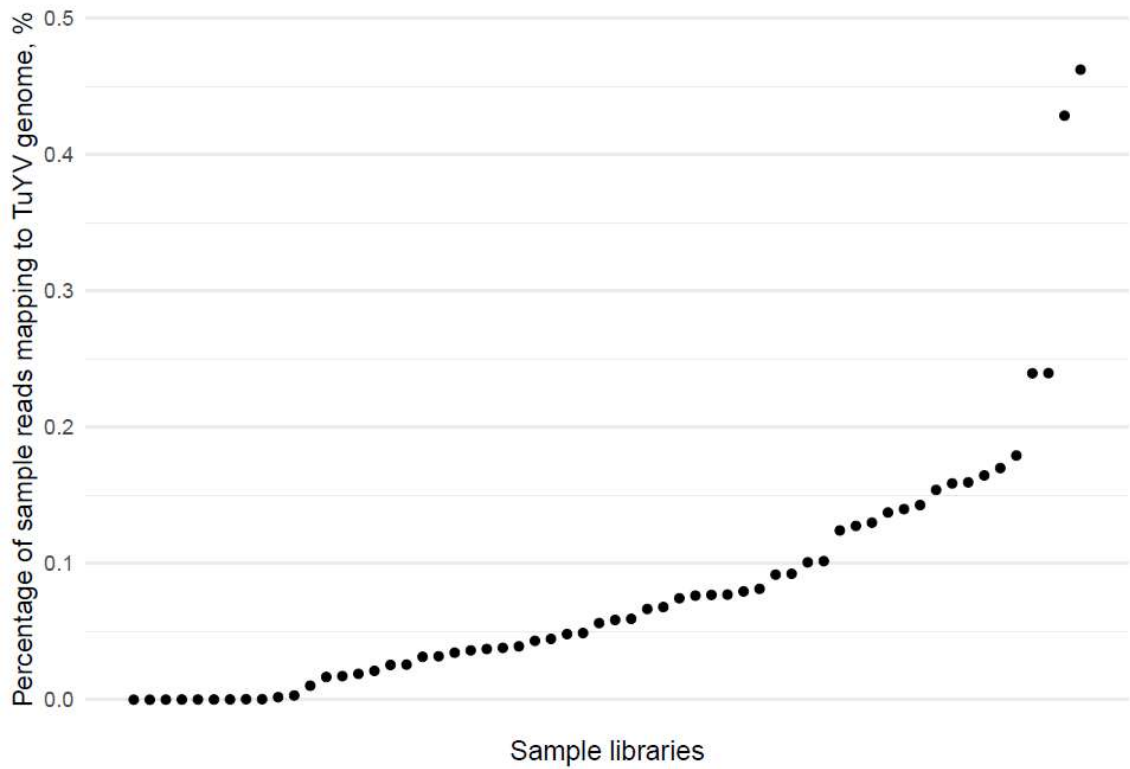


Figure 3.8: scatter plot showing the percentage of total small RNA (sRNA) library reads mapping to the Turnip yellows virus (TuYV) reference genome for each of the 2019, 2020 and 2021 sample sRNA libraries.

When refining the read data to measure the percentage of the TuYV genome found within the sRNA libraries, two distinct groups of samples emerged: those with less than 30% of the TuYV genome present and those with more than 40% (Figure 3.9). Two of the negative controls were in the <30% group, but one negative control had 44.7% of the TuYV present. Overall, the percentage of TuYV genome present was lower than that seen for both BMYV and BChV genomes with no samples containing more than 55% of the TuYV genome.

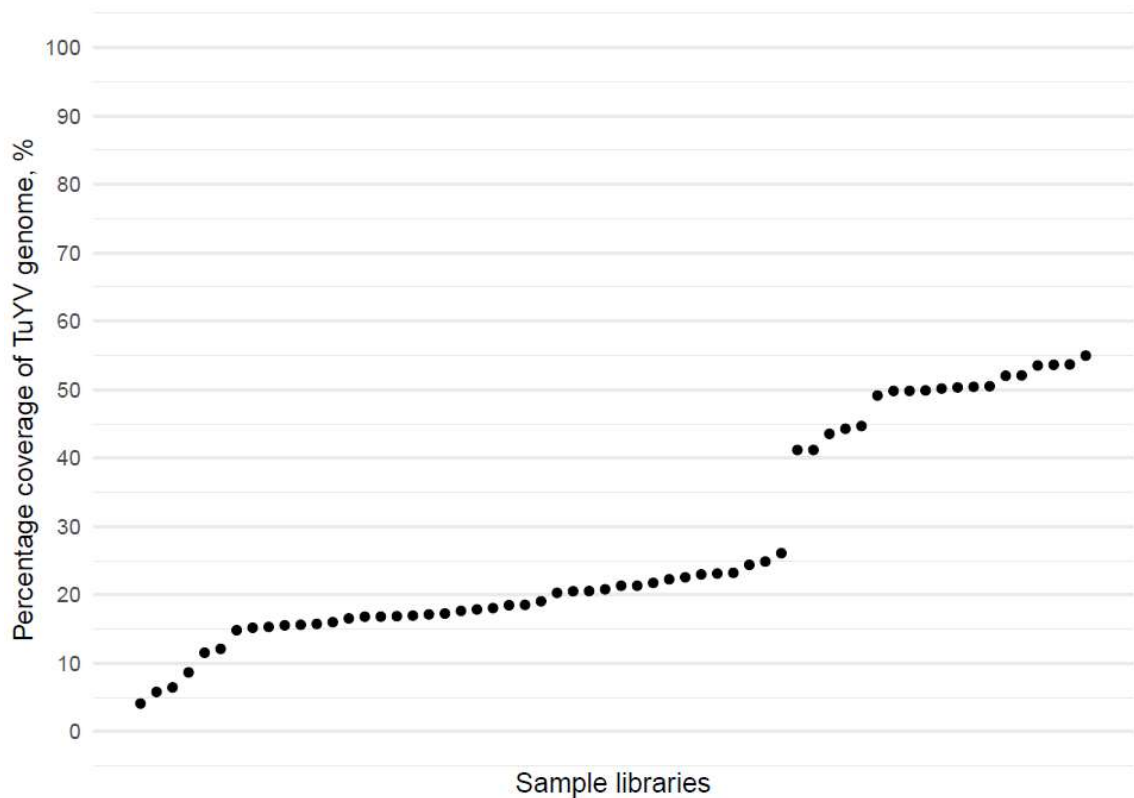


Figure 3.9: scatter plot showing the percentage coverage of the Turnip yellows virus (TuYV) reference genome of each of the 2019, 2020 and 2021 sample small RNA libraries.

By focusing on the ORF0 region of the TuYV genome it became clear that none of the survey samples were infected with TuYV (Figure 3.10). None of the samples had more than one read mapping to this region, including the two outlying samples from the first analysis approach. The results of reads mapping to BWYV was very similar to that of TuYV, with up to 57% of the BWYV genome present in some sample sRNA libraries. However, as no reads were found in any sample to map to the ORF0 region of BWYV all samples were determined negative for BWYV.



Figure 3.10: scatter plot showing the percentage coverage of the Turnip yellows virus (TuYV) open reading frame (ORF) 0 genome region of each of the 2019, 2020 and 2021 sample small RNA libraries.

### 3.4.3 BYV

A much larger proportion of sample sRNA library reads mapped to BYV than to the poleroviruses (Figure 3.11). There was also a much greater distribution of samples with survey libraries ranging from 0 -71.8% reads mapping to BYV. There was a clear discrimination between positive and negative controls with BYV positive controls having between 5.3-31.9% of BYV mapping reads, whilst all three negative controls contained 0.0%. Given the clear negative results, any survey sample containing more reads mapping to BYV than the lowest positive control (5.3%) was considered infected with BYV.



these samples contained the three highest percentages of the ORF0 region in the data set, ranging from 67.1 to 71.4%.

*Table 3.5: comparison of qPCR call and percentage coverage of open reading frame (ORF) 0 region of Beet mild yellowing virus (BMV) and Beet chlorosis virus (BChV) present in each sample.*

Sample number	BChV qPCR call	BMV qPCR call	Percentage coverage of BChV ORF0 region	Percentage coverage of BMV ORF0 region
21-071	Positive	Negative	98.34	26.21
21-039	Positive	Negative	97.92	20.11
21-278	Positive	Negative	96.39	12.07
21-303	Negative	Negative	69.07	10.68
21-187	Positive	Negative	98.47	10.54
21-196	Positive	Negative	96.26	9.15
21-111	Positive	Negative	98.47	8.88
21-024	Positive	Negative	99.58	5.41
21-020	Positive	Negative	97.78	0.00
21-294	Positive	Positive	99.72	67.13
21-254	Negative	Positive	19.83	69.07
21-056	Positive	Positive	98.06	71.43
21-179	Positive	Negative	96.95	2.77
21-121	Positive	Negative	99.03	13.31
21-097	Negative	Negative	35.51	65.19
21-290	Positive	Negative	99.31	0.00
21-256	Positive	Negative	95.84	53.81

## 3.5 Discussion

### 3.5.1 Prevalence of BYV

Using sRNA library sequencing to identify viruses is a novel approach to plant diagnostics and has not been exploited previously for studying the virus yellows complex in sugar beet. In this study, initially the percentage of total sRNA library reads, mapping to the virus of interest, was calculated. This methodology proved successful in determining BYV positive samples using the lowest value of the BYV positive controls as a threshold above which survey samples were deemed BYV positive.



However, one sample from 2020 narrowly missed the threshold value, having 4.5% of reads mapping to BYV, compared to the threshold value of 5.3%. This result may be a false negative, perhaps due to a low BYV titre in the sample leaf. Alternatively, it may be a true negative with mapping reads caused by contamination during the sRNA library preparation, particularly as some of the other samples had such a high level of BYV present. However even when treating this sample as negative there were a higher number of BYV positive samples in 2020 compared to 2019.

In 2019, 26.6% of samples were found to be positive for BYV compared to 57.1% in 2020. In 2019 the leaf samples used were freeze dried and stored for much longer than those from 2020. As the lower incidence of BYV was detected in the 2019 samples the impact of longer storage cannot be ruled out; degrading the viral RNA and therefore reducing the abundance of BYV RNA present in the sRNA library. Nevertheless, this variation in prevalence between years fits with findings of previous studies (Hossain et al., 2021; Stevens et al., 2005b). The 2021 larger scale survey also showed variation in BYV prevalence between years, with 34.6% of samples testing positive for BYV via ELISA. This is a decrease of 22.5% compared to the 2020 BYV prevalence (Figure 3.12).

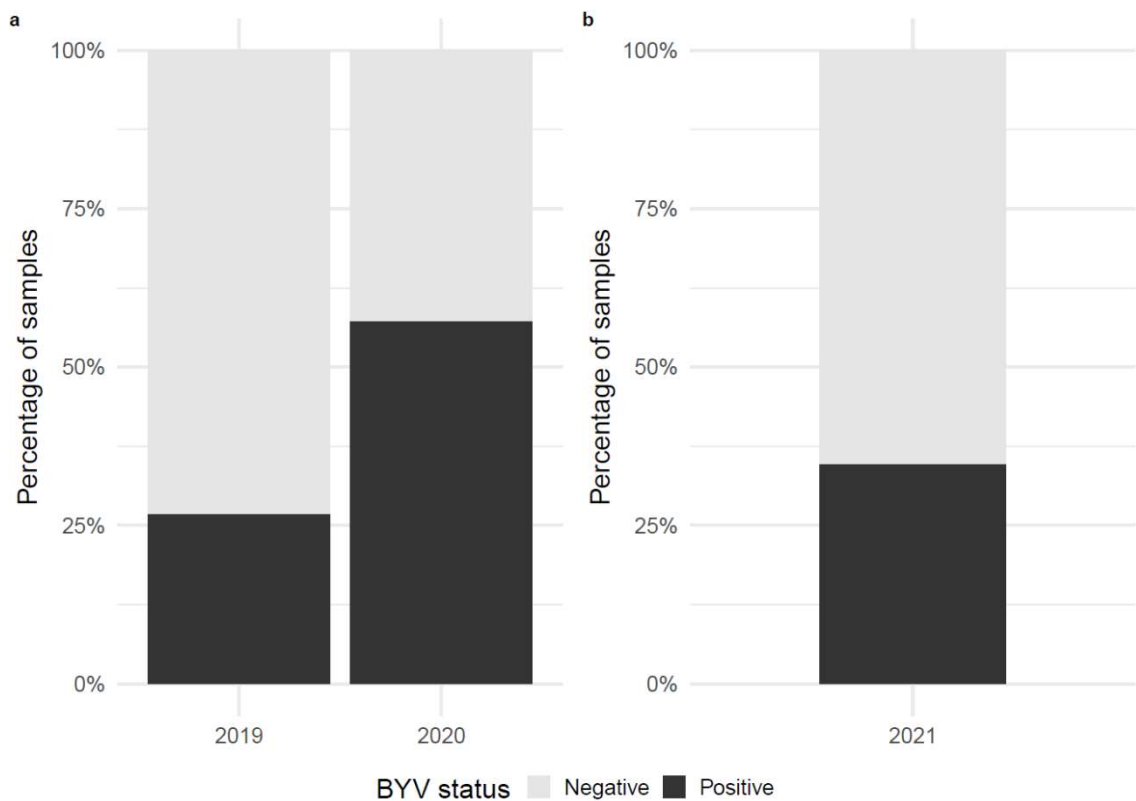


Figure 3. 12: bar plots showing prevalence of Beet yellows virus (BYV) in 2019, 2020 and 2021. a – percentage of samples determined to be positive for BYV in 2019 and 2020 based on the percentage of total small RNA library reads mapping to the BYV reference genome, based on a threshold value of 5.3%. b – percentage of 2021 survey samples containing BYV determined by enzyme-linked immunosorbent assay (ELISA) result.

The reasons for the variation in BYV prevalence between years has not yet been ascertained. One possible explanation is the impact of winter weather conditions on the survival of virus host species and therefore the abundance of virus sources. BYV is reported to have a narrower host range than BMV and BChV and does not survive harsh winter conditions in many plant species other than *Beta* species (Hani, 1988; Stevens et al., 2005b). Therefore, harsh winters are more likely to reduce sources of BYV than the beet poleroviruses, leading to lower relative prevalence of BYV in the subsequent sugar beet crop. The mild winter of 2019/20 followed by the higher BYV prevalence in 2020 would support this hypothesis. However, it is still not clear what, if any, legacy effect the use of neonicotinoid seed treatments may be having on virus epidemiology.

Although the interpretation of sRNA library sequencing data was straightforward in determining the presence of BYV, the approach still took much longer than the established BYV ELISA method and did not provide any major benefits. The DSMZ BYV antibody set has no known cross-reactivity

with other plant viruses, making it as reliable, yet cheaper and faster than the sRNA sequencing approach (DSMZ, 2023). Therefore, when solely diagnosing a suspected BYV infection sRNA library sequencing is not the best diagnostic to use.

### 3.5.2 Prevalence of BMV and BChV

Compared to BYV, only a very small proportion (<2.8%) of sample sRNA library reads mapped to either the BMV or BChV genomes. There was also no clear differentiation between the positive or negative controls, hence this approach did not appear to be a suitable method to determine virus infection. One explanation for this is that unlike BYV, both BMV and BChV are phloem limited (Peter et al., 2009) and are unlikely to be found at high titres within the leaf blade used for RNA extraction and subsequent sRNA library production. Some isolates of BMV have also been shown to have RNA silencing suppression activity which would further reduce the abundance of BMV viral sRNA within the sample sRNA library (Kozłowska-Makulska et al., 2010).

Given the low read numbers mapping to the beet poleroviruses the methodology was refined to focus on the percentage of the BMV and BChV genomes present within the sample libraries. This approach provided clearer differentiation between positive and negative controls and enabled partial grouping of samples. Using a threshold value of 74%, 13.3% of samples in 2019 and 35.7% of samples from 2020 were deemed positive for BMV compared to 46.7% and 71.4% for BChV in 2019 and 2020. However, this approach has a high risk of yielding false negative results because it is possible sampled leaves were infected with one of these viruses but by chance the sRNA library produced did not include more than 74% of the virus genome. Conversely, reducing this threshold increases the risk of false positives due to the short read length and high sequence homology between poleroviruses. This makes it likely for reads originating from one virus to cross map to other viruses.

To further improve the methodology the less homogenous 5' end of the polerovirus genomes was targeted. Focusing specifically on the 30-750nt region surrounding ORF0, which had the lowest percentage identity of the 5' ORFs, reduced the likelihood of reads cross-mapping between poleroviruses. This approach identified more samples in both 2019 and 2020 as positive for BChV than BMV. Using the conservative 80% threshold, 62.1% of samples had BChV compared to only 3.4% with BMV (Figure 3.13 a). This trend was still seen if the lower 31% BMV, and 32% BChV threshold was used with 79.3% of samples determined BChV positive and 34% BMV positive (Figure 3.13 b).

The qPCR assay run in 2021 for BMV and BChV was run using the same extracted RNA that the sRNA libraries were made from, enabling a direct comparison between the two diagnostic approaches (Table 3.5). The results of the BChV qPCR assay supported the use of the 80% ORF0 threshold as used to interpret the sRNA sequencing data. Results were more contradictory for BMV. The 80% ORF0 threshold was not supported by the qPCR results, with the three BMV-positive qPCR samples not surpassing the 80% threshold. The BMV qPCR assay has been tested against BChV, BWYV, TuYV, Beet leaf yellowing virus (BLYV), Brassica yellowing virus (BrYV), Potato leafroll virus (PLRV) and Cucurbit aphid-borne yellows virus (CABYV) along with healthy plant tissue including beta vulgaris (Wulf Menzel personal communication). Therefore, the results are unlikely to be false positives caused by cross-reactivity with the plant tissue or other poliovirus.

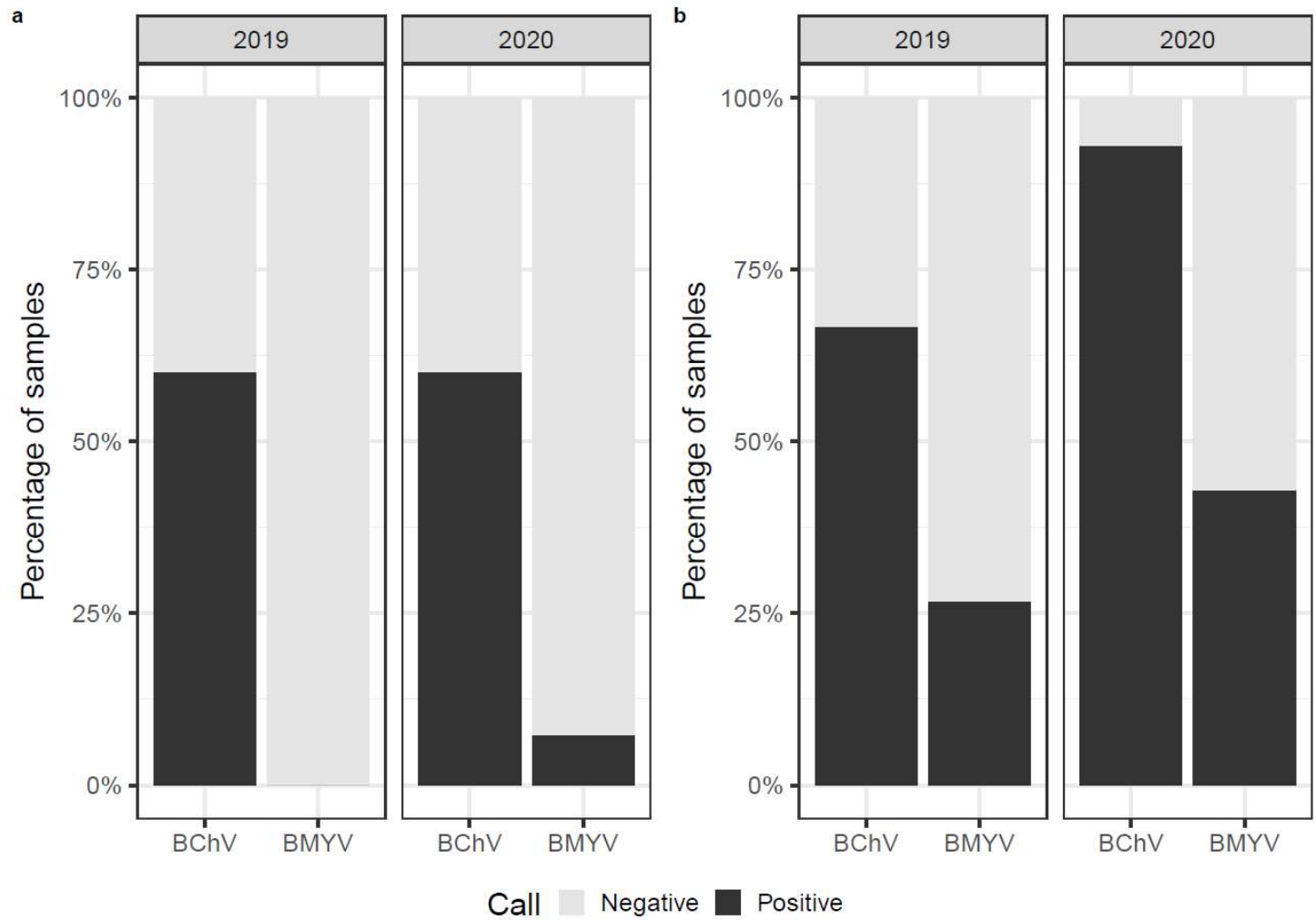


Figure 3. 13: Prevalence of Beet mild yellowing virus (BMV) and Beet chlorosis virus (BChV) in 2019 and 2020 determined by percentage of open reading frame (ORF) 0 region present in small RNA library. a - using conservative threshold of 80% above which samples were considered positive, b - using lower threshold of 31% for BMV and 32% for BChV above which samples were considered positive.

The higher prevalence of BChV compared to BMVYV was also observed in the larger survey conducted in 2021. 46.2% of the 338 sampled leaves were determined polerovirus positive by ELISA (Figure 3.14 a). Of the subset of these positive leaves which were tested via qPCR, 60.0% were found BChV positive, compared to 18.6% BMVYV positive (Figure 3.14 b). Only a randomly stratified sample of leaves which were polerovirus positive (as determined by ELISA) were analysed via qPCR. It is possible that the polerovirus positive leaves which were not qPCR tested were all BMVYV positive, however this is unlikely especially given the supporting results from 2019 and 2020.

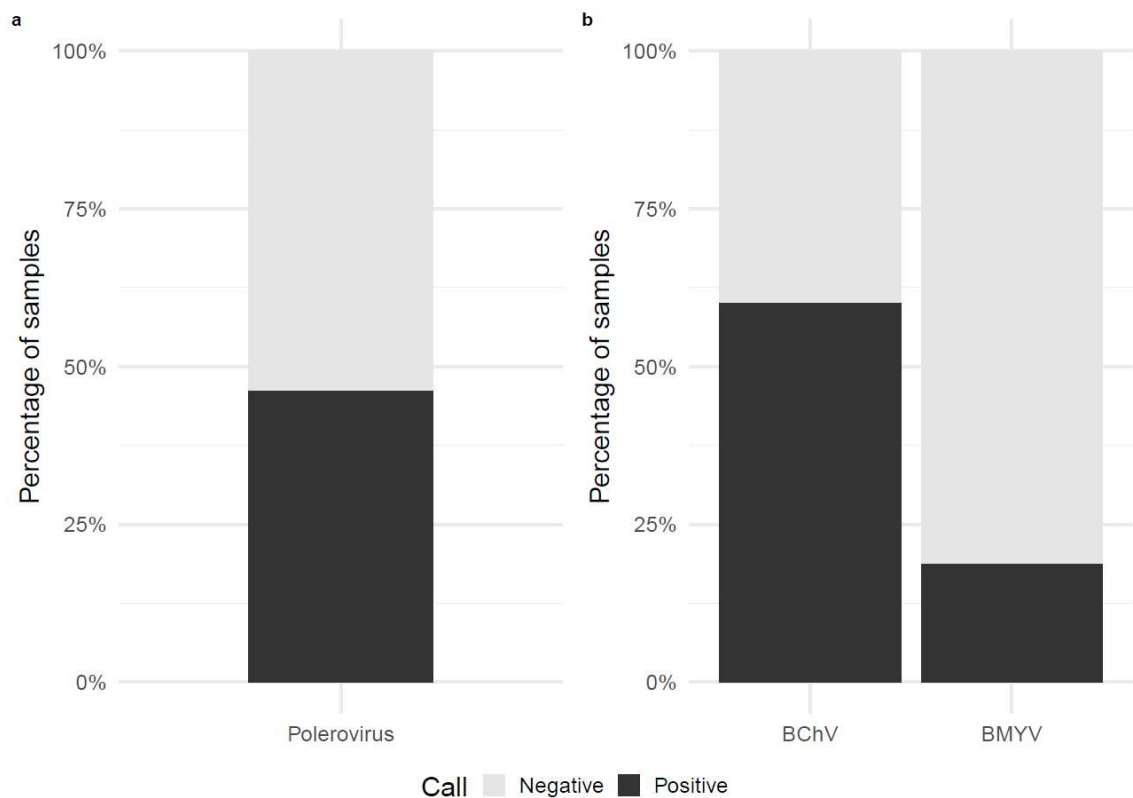


Figure 3.14: bar charts showing prevalence of Beet chlorosis virus (BChV) and Beet mild yellowing virus (BMVYV) amongst samples collected during the 2021 larger scale survey. a - percentage of samples with polerovirus based on enzyme-linked immunosorbent (ELISA) assay result. b - percentage of samples with BChV and BMVYV as determined by qPCR.

sRNA library sequencing is an improvement on the currently available ELISA diagnostics in its greater ability to discriminate between BMVYV and BChV. However, because of the low read numbers, and high homogeneity between poleroviruses it was still not always possible to determine a sample as BMVYV or BChV positive, or indeed a mixed BMVYV and BChV infection. In 2020 at the start of this project, no qPCR assay for reliable BChV and BMVYV detection was available. Although this project could have aimed to design such an assay it was decided that trialling the

sRNA sequencing approach would yield more information on potential other poleroviruses present; consequently this was the approach taken.

In December 2020 Wulf Menzel shared a yet unpublished qPCR assay developed to differentiate between BMYV and BChV. This diagnostic produced clearer results than the sRNA library approach and was also more cost and time efficient. qPCR assays rely on the specificity of primers, and the homogeneity of those primer target sequences across the targeted virus species. There is a chance that some virus positive samples were determined negative via qPCR owing to sequence variation in the primer target site. Twenty samples which had tested positive for polerovirus via ELISA, were negative with the qPCR assay. There are a number of possible explanations for these results. Firstly, the samples could be ELISA false positives, made more likely given the high throughput approach. Alternatively, they may be infected with isolates of BMYV and/or BChV which do not contain the primer target sequence. Or they may contain a polerovirus which is not BMYV or BChV, such as BWYV or TuYV, though this is not supported by the sRNA library sequencing data.

The higher incidence of BChV compared to BMYV goes against the findings of previous UK virus yellows surveys. Historical surveys identified BMYV as the most prevalent polerovirus in the UK (Stevens et al., 2005b). Nevertheless, the results presented here do align with the findings of (Hossain et al., 2021) for France, where BChV was also identified as more prevalent than BMYV. It is not apparent whether these findings indicate a stable shift towards BChV or if they are a snapshot in a longer-term cycle of alternating polerovirus abundance. To understand this interaction, further studies should be undertaken, ideally annually, so that long-term prevalence data can be collected and changes in virus epidemiology reliably identified. These results do, however, highlight the importance of developing sugar beet varieties which are resistant to BChV not only BMYV.

### 3.5.3 Occurrence of TuYV and BWYV in UK beet

This survey did not identify TuYV or BWYV in *Beta vulgaris* sp. in the UK in 2019, 2020 or 2021. Although 14.6% sRNA libraries contained more than 50% of the TuYV genome, all these libraries each also contained more than 70% of either the BMYV or BChV genome. The same situation was seen with BWYV, with the 14.6% of sRNA libraries containing more than 50% of the BWYV each also containing more than 80% of either the BMYV or BChV genome. These results could be interpreted as showing mixed infections of TuYV or BWYV with BMYV or BChV. Yet given only one read from one sample mapped to the 30-750nt region of TuYV or BWYV, it is more likely the mapping reads originated from either BMYV and/or BChV and were cross-mapping to TuYV and BWYV.

Some studies have identified isolates of TuYV which infect sugar beet, suggesting a broadening of the TuYV host range and potential risk to the sugar beet crop (Newbert, 2016; Puthanveed et al., 2023). However to date, no TuYV has been isolated from commercially grown sugar or fodder beet in the UK. This is despite a known high incidence of TuYV in *Myzus persicae* and *Macrosiphum euphorbiae* aphids entering UK sugar beet crops. The yellow water pan network run by the BBRO, catches *M. persicae* and *M. euphorbiae* aphids entering UK sugar beet crops between April and July each year. The aphids caught in these traps are tested via qPCR for BMYV/BChV and TuYV. Over the last seven years (2017 to 2023), on average 81.3% of tested aphids have been carrying TuYV (BBRO personal communication). Given this high pressure, if TuYV was entering UK sugar beet plants it seems likely we would have identified at least one infected plant during this survey, or indeed see a much higher incidence of symptomatic sugar beet plants.

Reliable differentiation between these closely related poleroviruses is challenging. Sequencing approaches, such as the sRNA library sequencing used here, theoretically permit this differentiation. However in practice, when working with short read lengths and potentially mixed infections, reliably identifying each and every polerovirus present within a sample is still difficult. The main focus of this survey was to understand the prevalence of BMYV, BChV and BYV in the UK and as such no TuYV or BWYV positive control sRNA library was produced or sequenced. Future efforts to identify if these two viruses are present in the UK sugar beet crop must include such positive controls if a reliable and accurate methodology is to be devised. Were these viruses to be isolated from sugar beet, host range and transmission studies would be needed to better understand the risk posed to the UK sugar beet crop. For example, if TuYV were to be transmissible from one sugar beet plant to another the potential consequences would be much more severe than if sugar beet were to be a 'dead-end' host to the virus.

#### 3.5.4 Sea and fodder beet implications

This survey also included symptomatic sea beet and fodder beet leaves. Identifying sources of virus infection is important in predicting virus risk to the sugar beet crop. The British Beet Research Organisation (BBRO) monitors virus yellows in the UK sugar beet crop however fodder beet falls outside of its remit. As such levels of virus yellows in fodder beet crops are largely unknown, despite fodder beet often being grown in close proximity to sugar beet and being a potential source of virus yellows. BMYV, BChV and BYV were all identified in fodder beet samples, confirming their risk as virus sources.



Only two symptomatic sea beet leaves were found and tested, one of which was positive for BMV. Future virus studies on sea beet should include samples not exhibiting virus infection. *Beta maritima* has been used as a source of disease resistance and bred into commercial sugar beet lines (Asher et al., 2001). Therefore, it is possible that some virus-infected *B. maritima* plants do not express symptoms. Expanding sampling to include non-symptomatic plants would provide a more thorough dataset on the prevalence of virus in *B. maritima* plants.

Although only a limited number of sea beet leaves were tested, the results illustrate the potential risk posed by non-agricultural plant species to the UK sugar beet industry by providing sources of virus infection. In this study only *Beta* species were tested, however all three viruses within the virus yellows complex are known to infect other plant species (Hani, 1988; Hauser et al., 2002; Stevens et al., 1994b). These plants can act as a 'green bridge' maintaining virus from one sugar beet growing season to the next. The relative abundance of non-sugar beet hosts may also impact virus yellows epidemiology from year to year. For example, if winter weather conditions favoured the survival of BYV host species over BMV host species this may result in higher BYV prevalence in the sugar beet crop. Expanding virus prevalence surveys, such as this one, to include known non-beet hosts would improve our understanding of the risks posed by such species to the sugar beet crop and potentially allow for targeted control of those species in field margins, for example.

### 3.5.5 Diagnostic technique development

Three different diagnostic techniques were used to determine virus infection during this survey. ELISA is a long-established method for virus detection and continues to be used in agricultural diagnostics thanks to it being high throughput and relatively low cost. However, there is currently no antibody which can differentiate BMV and BChV. qPCR overcomes this issue, discriminating between BMV and BChV infection but the current protocol is not as high throughput as ELISA. In 2021, testing all 338 sampled leaves by total RNA extraction followed by RT-qPCR would have taken a considerable amount of time and was not financially viable.

Neither qPCR or ELISA provides sequencing data and can only be used to detect the virus for which the specific diagnostic was used. The sRNA libraries produced in this study could be used to identify any virus, including those yet to be identified. This provides a powerful tool for future epidemiological research. sRNA library sequencing is, however, more expensive and time consuming compared to qPCR, and therefore unlikely to be widely adopted.

# Chapter 4: Phenotyping varietal virus responses under controlled conditions

## 4.1 Chapter Contributions

All work presented in this chapter was completed by me.

## 4.2 Introduction

Phenotyping is a crucial tool in sugar beet variety development through which advantageous traits can be identified and increased (Bosemark, 2006). When assessing a variety's performance under pest or disease pressure, variety trials are either left to become naturally infected or are purposely inoculated. In the case of developing varieties to combat virus yellows disease, future varieties ideally need to overcome all three of the major UK yellowing viruses: BMV, BChV and BYV. Therefore sugar beet breeding lines need to be assessed for their performance under each virus.

Assessment of sugar beet varieties response to virus infection, is currently largely based on field trial performance. Such trials can be conducted on a large scale, with multiple replicates, and provide reliable data on how varieties will perform when affected with virus in a field environment. However, these types of trials have several disadvantages. Fundamentally, being located outdoors, variety field trials are at the mercy of the weather. This can have direct consequences for plant health, for example late frosts killing off vulnerable seedlings, but also makes comparisons of data across multiple years and trial locations difficult. The same is true for naturally arising pests and diseases, such as foliar fungal infections or beet leaf miner which may be present in a trial one year but absent the next.

When assessing variety performance under virus infection specifically, there is also the difficulty of successfully inoculating test plants with virus whilst minimising the risk of contaminating the wider environment. Where multiple viruses are being introduced into the same field, this issue of containment is exacerbated with contamination risks not only to the wider environment but also between virus treatments. In an outdoor field trial setting, all these challenges are combined with the need to prevent 'wild' aphids entering the trial and introducing other viruses or transferring

virus from one treatment to another. These problems can be mitigated (as discussed in Chapter 6), however, it raises many practical challenges and ultimately increases the cost of such research.

Even when all the above factors are considered, and controlled as much as they can be, field trials can still only be conducted once a year with seedlings being sown in the spring and harvested in the autumn/winter. This creates a significant delay in the generation of data on which seed breeders base their variety development upon, increasing the time it takes for resistant or tolerant varieties to come to market. There is, therefore, a clear need for an alternative to field trials for phenotyping variety virus responses.

Controlled environment facilities could provide a solution to many of these challenges. Hence, the research presented in this chapter aimed to develop a method of simultaneously phenotyping multiple viruses and varieties under controlled environment conditions. In this chapter I trialled three different controlled environment facilities to identify which, if any, could provide a viable alternative to variety field trials. A summary of the rationale behind choosing these three facilities is given below.

#### 4.2.1 MLR growth chamber

The Versatile Environment Test Chambers (Panasonic, MLR-352) is a plant growth chamber used frequently in laboratory studies. Regarding sugar beet research specifically, the MLR-352 chambers have been used to rear virus yellows cultures at the BBRO since 2014. BMVYV, BChV and BYV can all be maintained in MLR-352 chambers, as confirmed via ELISA and qPCR diagnostic testing (BBRO, personal communication). Anecdotally, although yellowing symptoms are observed in sugar beet plants in the BBRO BYV culture, rarely are symptoms of either beet polerovirus seen. Nevertheless, no published trials quantifying phenotypic response to virus yellows have been conducted in MLR-352 growth chambers, and therefore the lack of symptom expression needs to be confirmed.

#### 4.2.2 Glasshouse

One benefit of glasshouse facilities over most reach-in controlled environment chambers is that they are generally larger providing greater growing space. This enables trials to be conducted with a larger sample size and therefore yield more reliable results. Glasshouse facilities have been used successfully to identify Turnip yellows virus (TuYV) resistance in oilseed rape varieties (Ibrahim et al., 2023), as well as sources of resistance genes in sugar beet (Asher et al., 2001). However, both these studies used viral titre (determined via qPCR (Ibrahim et al., 2023) and ELISA (Asher et al.,

2001)) to determine resistance and did not provide information on crop yield or visual symptom expression. As defined in Chapter 1 section 1.4.5, true tolerant sugar beet varieties become infected by the virus but do not express symptoms of infection and suffer no resultant yield loss. Consequently, tolerant varieties cannot be distinguished from susceptible varieties based on viral titre data alone and other phenotypic characteristics need to be assessed. A standard protocol for phenotyping sugar beet leaf yellowing to virus infection, under glasshouse conditions, has not been published to date.

One disadvantage of using glasshouse facilities for phenotyping work is that the environmental conditions are more variable than controlled environment chambers. The glasshouse facility available for this project to use at UEA, for example, is made of polycarbonate which is partially whitewashed. Although supplementary lighting and temperature control fans are present, the external weather conditions can have a large influence on both the available light and temperature inside the glasshouse. Nevertheless, the higher sample size permitted by glasshouse facilities could outweigh this reduced environmental control.

#### 4.2.3 Conviron growth chamber fitted with LED lights

In June 2021 the UK government announced a ban on fluorescent light bulbs effective from September 2023 (Department for Business Energy and Industrial Strategy, 2021). This has triggered a move away from fluorescent light bulbs in controlled environment chambers, and the increased use of LED bulbs. LED bulbs have a lower power usage than fluorescent bulbs resulting in lower running costs, whilst still permitting full control of light levels. The GEN1000 reach-in growth chamber (Conviron) is one controlled environment chamber which utilises LED light (Conviron, 2020a). Positioned above the plant growth shelves, these LED bulbs provide broad-spectrum white light with a maximum intensity of 700  $\mu\text{mol}/\text{m}^2/\text{sec}$ . Importantly the GEN1000 chambers can be programmed to ramp up and down light intensity, mimicking natural diurnal light fluctuation (Conviron, 2020b).

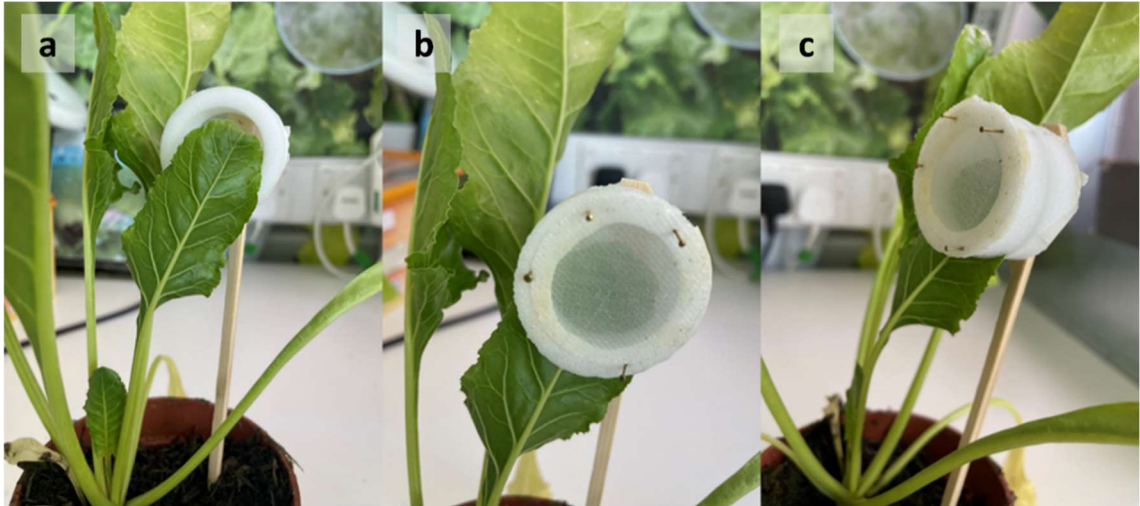
Given the increased plant growth area (compared to the MLR chambers) and the more advanced environmental controls (compared to both the MLR chamber and glasshouse facility), the GEN1000 controlled environment has strong potential for virus/variety phenotyping studies.

## 4.3 Materials and methods

### 4.3.1 Clip cage virus inoculation

Clip cages (BDC1540-12, BugDorm) can be used to contain viruliferous aphids on plants such as described by (Davis et al., 2005; Gray, 1991). Crucial to their successful use is achieving a sufficiently tight seal between the foam of the cage and the leaf, ensuring that aphids placed within the cage do not escape. Based on the BugDorm manufacturer's instructions, staples are used to secure the cage to the leaf. However previous testing had found this method did not produce a sufficient seal on sugar beet leaves (personal communication, BBRO). As such an adapted fixing method was developed using fine brass pins (such as Hemline Brass Craft Pins 450 Pack, Hobbycraft).

Plants were inoculated approximately two weeks after transplantation, at the two-true leaf stage. One half of the aphid cage was secured to a flat bamboo skewer (such as 123912940, Sainsbury's), and the skewer then positioned in the plant pot. This adaption meant that the weight of the cage was supported by the skewer rather than the young seedling. 10 wingless aphids were then gently transferred using a paintbrush into the second half of the aphid cage. This cage was then placed on the underside of a true-leaf and secured in place using the brass pins (Figure 4.1). Aphids were allowed to feed for 48 hours, before the cages were carefully removed and all aphids finger crushed. After the cages were removed, and to ensure all aphids were destroyed, an insecticide spray was applied to all plants aphicide (0.1g/L InSyst containing 20% w/w acetamiprid, CertisBelchim). Experiments were concluded after four weeks (pilot study in Panasonic MLR-352) or eight weeks (glasshouse and LED study).



*Figure 4.1: images showing the set-up of the adapted aphid cages. Image a shows the clip cage connected to the support positioned alongside the adaxial surface of a first true leaf. Images b and c show the second half of the clip cage, containing the 10 wingless aphids positioned on the abaxial surface of the leaf and attached to the support cage via fine pins.*

## 4.3.2 Phenotype measurements

### 4.3.2.1 Canopy area and symptom expression

To quantify the size of plant canopy, plants were photographed from above using a PowerShot G7X camera (Canon) positioned on a tripod against a black background with a measuring tape. Images were imported into the image analysis software, ImageJ (version 1.53t) and the scale set using the known distance of the measuring tape. The 'colour threshold' tool was then applied to select the leaf area by adjusting the hue, saturation, and brightness filters (Figure 4.2). Once optimum filter settings had been determined (selecting only non-sensing canopy), based on three randomly selected plants, the canopy area was calculated using a batch process macro that measured the area of the filtered image (Wright et al., 2018).

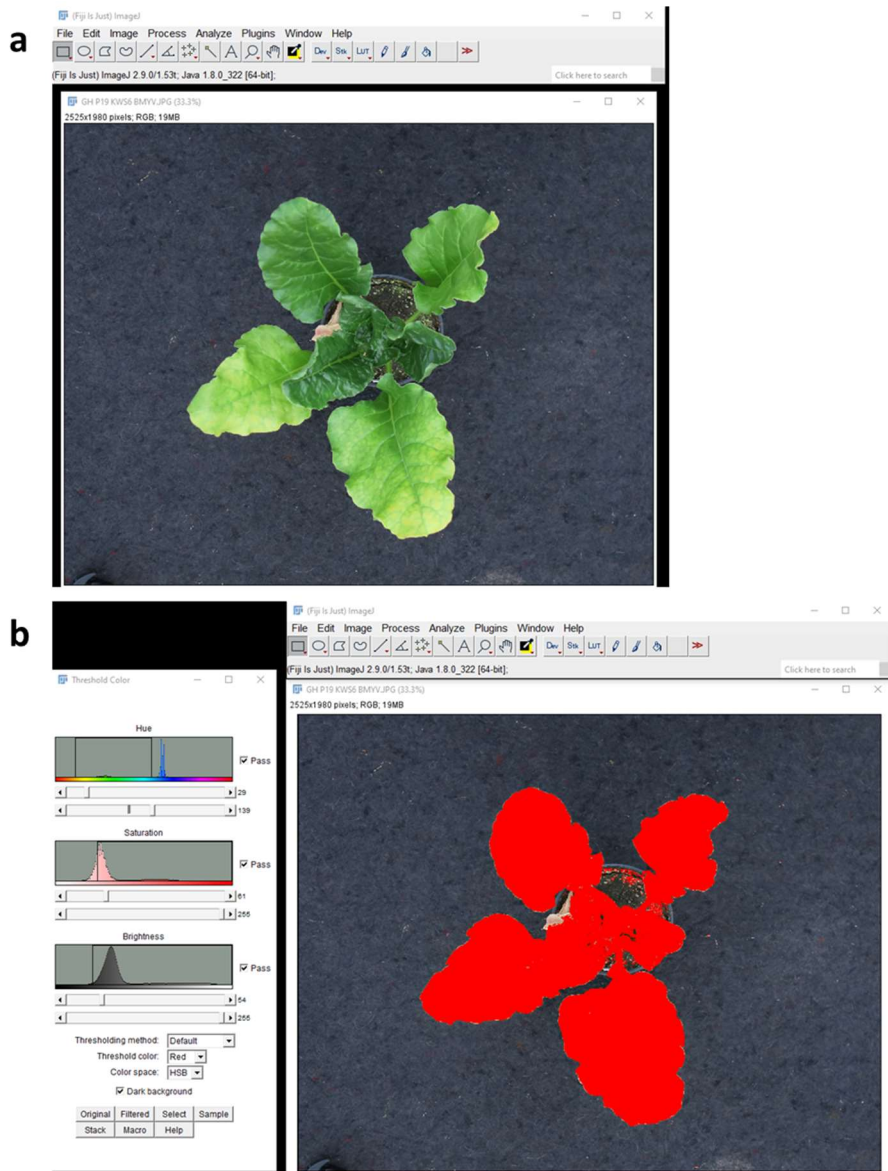


Figure 4.2: ImageJ screen shots showing user interface whilst conducting the canopy area selection process. Image a shows an example experimental image loaded into the ImageJ software. Image b shows the hue, saturation and brightness filters adjusted to select all non-senescent leaf tissue, whilst discounting the image background.

The same images were used for visual assessment of symptom expression. Symptom expression was compared to those observed in field-grown sugar beet, namely interveinal leaf yellowing and leaf thickening such as described by (Harveson et al., 2009).

#### 4.3.2.2 Leaf weight and ELISA sampling

After photographing, all leaves were removed from the plant at the point where the leaf petiole joined the root crown. Leaves were weighed ('entire fresh weight') and then leaf disc samples

removed for ELISA testing. The remaining leaf was then reweighed ('fresh weight of sample'), and placed in a paper bag (PAP7B, polybags.co.uk). Leaves were dried at 80°C in an oven (MK53 climate chamber, Binder) until their weight no longer decreased. All samples were then reweighed in the paper bag ('dry weight including bag'). Three new, unused empty paper bags were also dried for the same time as the leaf samples to establish the weight of the dried paper bag.

To determine the total dry leaf weight the following calculation was used:

$$\textit{Weight of dried sample} = \textit{Dry weight including bag} - \textit{mean dry bag weight}$$

$$\textit{Total dry leaf weight} = \frac{\textit{Weight of dried sample}}{\textit{Fresh weight of sample}} \times \textit{Entire fresh weight}$$

ELISA testing was conducted using the beet western yellows virus antibody sets (DSMZ) as described in Chapter 2 section 2.4.2.

#### 4.3.2.3 Root weight

Roots were removed from the pots and carefully washed to detach any soil or compost. During the pilot study it became clear that removing all the compost from the root hairs was not a viable method. As such it was decided to remove the root hairs, leaving the tap root which could be cleaned of compost more consistently. This method was used for all subsequent experiments.

After washing the roots were blotted dry on paper towel and then weighed and placed into gusseted paper bags (PAPBG2, polybags.co.uk). Any larger roots were cut into smaller pieces to quicken the drying process. Roots were dried under the same conditions as the leaves, 80°C until their weight no longer decreased alongside three new empty paper bags. After drying was completed the roots were reweighed in their bags, and the mean empty dry bag weight removed to determine the dry root weight for each sample.

#### 4.3.3 Pilot study

The pilot study was conducted using two varieties, KWS-1 and KWS-5 (further variety details included in Appendix A). Variety KWS-5 was understood to be susceptible at the time the trial was conducted, however was later described by the seed breeder as having medium resistance to



BMV. KWS-1 had high resistance to BMV infection. The study comprised of two treatments, inoculation with BMV infective aphids, and inoculation with non-viruliferous aphids (total of 16 plants, four plants per variety/treatment). Seed from each variety was germinated in compost (Premium All Purpose, Miracle-Gro), 10 days after sowing (when most seedlings had expanded cotyledons) individual seedlings were transplanted into separate 9cm diameter pots (0.36l capacity) filled with compost. The trial was split across two Versatile Environment Test Chambers (Panasonic, MLR-352), arranged in a randomised block design comprising of four blocks. The compost contained a pre-mixed granular fertiliser, and therefore no additional fertiliser was applied.

The plants were grown under a 16-hour photoperiod, with the chamber set to 'full light' provided by 15 fluorescent tubes (FL4055 ENW/37, Panasonic), arranged to provide light from three sides. During this phase, the temperature was set to 22°C. An 8-hour dark period followed during which the temperature was set to 18°C.

Plants were inoculated with aphids, as described above, using BMV infective aphids reared in the BBRO BMV virus culture on *Capsella bursa-pastoris* and non-viruliferous aphids reared in the BBRO 'healthy' aphid culture on *Brassica rapa* subsp. *pekinensis* (Chapter 2, section 2.1).

The trial was concluded four weeks after inoculation. Using the methodologies described above, ELISA testing was conducted on the oldest non-senescent leaf and measurements were taken to assess canopy coverage, leaf dry weight and root dry weight.

#### 4.3.4 Glasshouse study

The glasshouse study contained varieties KWS-1, KWS-2, KWS-3, KWS-4, KWS-6, and KWS-7 (see Appendix A for further variety details), which were trialled under non-inoculated, BChV inoculated, BMV inoculated and combined BChV and BMV inoculation. Seed from each variety was germinated in compost (Premium All Purpose, Miracle-Gro), in a climate-controlled glasshouse. Ten days after sowing, individual seedlings were transplanted into separate 1L (11cm diameter) pots filled with a sand-soil mix (Baileys of Norfolk). Approximately three days after transplanting 50ml ammonium sulphate fertiliser (2.9g/l) equating to 0.03g nitrogen was applied directly to the soil of each plant. A second application of fertiliser was given at the same rate one week later. Plants were arranged across four trays in a randomised block design with each tray containing one replicate of each treatment.

Plants were grown in a glasshouse at 18-22°C with additional supplementary lighting to provide a 16-hour photoperiod. Plants were inoculated with aphids, as described in section 4.3.1. The BChV

treatment was inoculated with ten viruliferous BChV infective aphids reared in the BBRO BChV virus culture on *Chenopodium capitatum*. The BMV treatment was inoculated with ten viruliferous BMV infective aphids reared in the BBRO BMV virus culture on *C.bursa-pastoris*. For the mixed infection, plants were inoculated with ten BMV and ten BChV viruliferous aphids. No aphids or clip cages were applied to the uninoculated plants.

The glasshouse study was concluded 8 weeks after inoculation. Using the methodologies described previously, measurements were taken of canopy coverage, leaf dry weight and root dry weight. As in the pilot study ELISA testing was conducted, however in this study leaf five and leaf ten from each plant was tested rather than the oldest non-senescent leaf.

#### 4.3.5 LED light study

The LED light study contained varieties KWS-1, KWS-2, KWS-3, KWS-4, KWS-6, and KWS-7, under three test treatments; BMV inoculated, BChV inoculated and uninoculated. Seed from each variety was germinated in compost (Premium All Purpose, Miracle-Gro) before being transplanted into 1L pots of soil-sand mix (Baileys of Norfolk). As with the glasshouse study, two doses of ammonium sulphate fertiliser (2.9g/l) were applied to the soil of each plant; the first dose approximately three days after transplanting and the second one week later. The trial was arranged across two Gen1000 reach-in chambers (Convion), in a randomised block trial design comprised of four blocks each containing one replicate of each treatment.

Plants were inoculated as described in section 4.3.1. Each week following inoculation plants were removed from the reach-in chamber and photographed as described in section 4.3.2.1 so that canopy growth could be measured. The trial was concluded 8 weeks after inoculation, with measurements being taken as per the glasshouse study of ELISA absorbance for leaves five and ten, root and leaf dry weight, and final canopy coverage.

The plants were grown under a 16-hour photoperiod, with the chamber set to 400 $\mu$ mol light at canopy level provided by broad spectrum/white light LED (12 watt T5 LED fixture) arranged above the plants. During this phase, the temperature was set to 22°C. An 8-hour dark period followed during which the temperature was set to 18°C.

### 4.3.6 Statistical analysis

Data from all experiments were analysed using GenSTAT 22<sup>nd</sup> edition (VSN International). All graphs were produced using RStudio version 2022.02.1+461 "Prairie Trillium" released for Windows (RStudio Team 2022).

#### 4.3.6.1 Pilot and Glasshouse study

In the pilot study two-way analysis of variance (ANOVA) in randomised blocks were conducted using the GenSTAT 'General Analysis of Variance' statistical test. The experiment design was set to "General analysis of variance", and the Y-variate to the relevant dependent variable (ELISA result, leaf area, leaf dry weight or root dry weight). The treatment structure was defined using the fixed factors 'Variety x Treatment' and the block structure to the randomised block plants had been assigned to. The least significant difference (LSD) at 5% significance calculation was included in the ANOVA.

#### 4.3.6.2 LED study

In the LED study, two-way ANOVA in randomised blocks were conducted as detailed in section 4.3.6.1 for the ELISA, leaf area, leaf dry weight and root dry weight results. In addition, leaf area data were analysed using the GenSTAT 'repeated measures ANOVA' to assess how the treatment effects developed. The Y-variate was set as the leaf area data sets at successive time points, and the treatment structure defined using the fixed factors 'Variety x Treatment'. The block structure was set to the randomised block plants had been assigned to.

## 4.4 Results

### 4.4.1 Pilot study

#### 4.4.1.1 ELISA results

ELISA results from the oldest non-senescing leaf showed significant differences between the plants inoculated with BMVY viruliferous aphids and those inoculated with non-viruliferous aphids, (Figure 4.3, ANOVA  $p = 0.008$ ). The absorbance of the ELISA negative controls ranged from 0.204 to 0.492, resulting in a threshold of 0.793 above which a sample would be called positive. The mean absorbance for the non-viruliferous treatment was 0.27. None of the non-viruliferous treatment plants had ELISA results above the positive threshold indicating no contamination occurred between the treatments.

Although the mean absorbance for the BMVYV inoculated plants was 0.93, and therefore above the positive threshold, the range of ELISA results for this treatment varied widely from 0.148 to 1.948. There was no significant difference in ELISA results between the varieties and no significant difference in the way the varieties responded to the treatments.

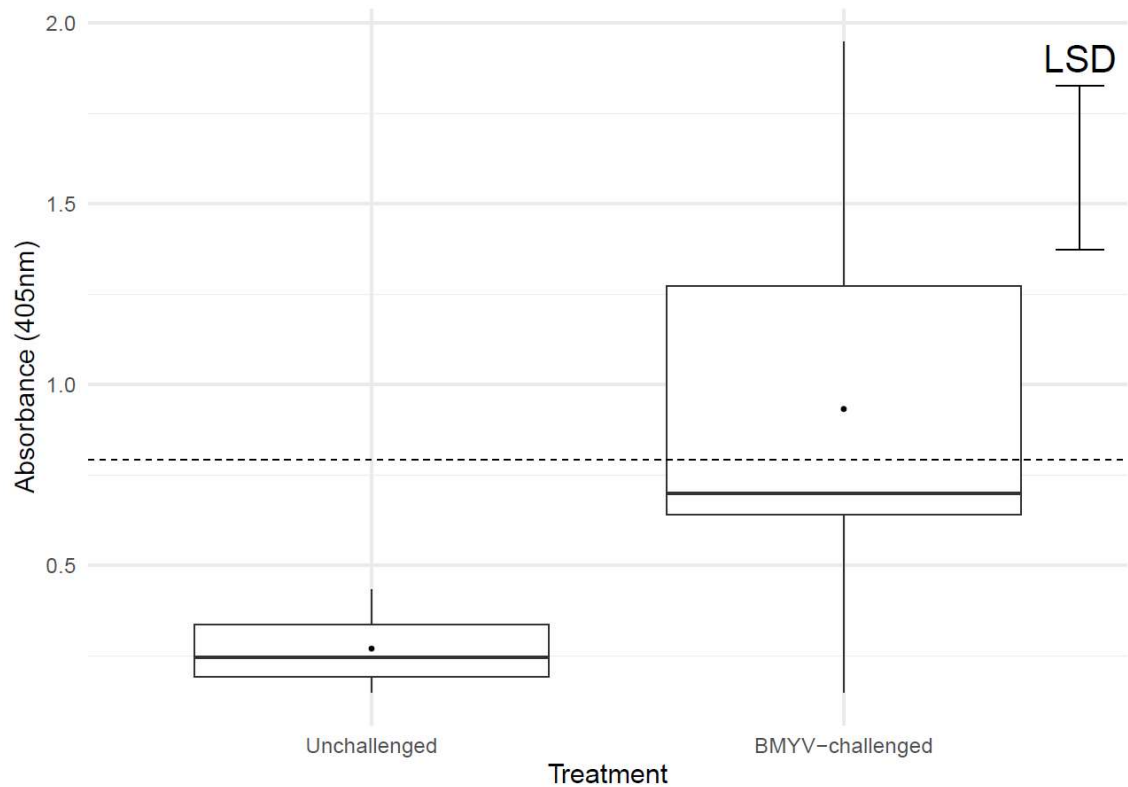


Figure 4.3: boxplot showing the combined enzyme-linked immunosorbent assay (ELISA) absorbances of varieties KWS-1 and KWS-5 of the Beet mild yellowing virus (BMVYV)-challenged (inoculated with BMVYV viruliferous *Myzus persicae* aphids) and unchallenged (inoculated with non-viruliferous *M. persicae* aphids) treatments. The mean absorbance is indicated for each treatment by the black circular point. The dashed horizontal line indicates the ELISA absorbance value above which a sample is deemed positive (calculated by the mean of the negative controls plus three times the standard deviation), set at 0.793. Error bar shows least significant difference (LSD) at 5% significance between treatments (analysis of variance (ANOVA),  $p = 0.008$ ,  $n = 16$ ).

#### 4.4.1.2 Symptom expression

Despite significant differences in ELISA absorbance between the virus challenged and unchallenged treatments, no typical yellowing symptoms were observed in either variety (Figure 4.4). There were also no other visual phenotypic differences between the BMVYV-challenged and unchallenged treatments for either variety.



Figure 4.4: photos taken from above of every plant in the pilot study at the end of the trial (4 weeks after inoculation) for both the Beet mild yellowing virus (BMV)-challenged ((inoculated with BMV viruliferous *Myzus persicae* aphids) and unchallenged (inoculated with non-viruliferous *M. persicae* aphids) treatments. a = variety KWS-5 unchallenged, b = variety KWS-5 BMV-challenged, c = variety KWS-1 unchallenged, d = variety KWS-1 BMV-challenged.

#### 4.4.1.3 Leaf area and dry weight

As quantitatively observed, the canopy area at the end of the study (4 weeks after inoculation) did not differ significantly between treatment (ANOVA,  $p = 0.705$ ) or variety (ANOVA,  $p = 0.915$ ). The mean canopy area of KWS-5 was slightly larger in the infected treatment than the uninfected ( $48.0\text{cm}^2$  compared to  $45.0\text{cm}^2$  respectively). The opposite was seen with KWS-1 which had a larger canopy when uninfected compared to infected (mean of  $50.9\text{cm}^2$  compared to  $43.4\text{cm}^2$  respectively). None of these results were statistically significant.

The dry weight of the uninfected plants was higher, on average than the infected (0.671g compared to 0.570g respectively), however this difference was not statistically significant (ANOVA,  $p = 0.411$ ). Neither was there any significant difference between varieties (ANOVA,  $p = 0.203$ ), or in how the varieties responded to the treatments (ANOVA,  $p = 0.358$ ).

#### 4.4.1.4 Root weight

As shown in Figure 4.5, KWS-5 had a lower mean dry root weight when inoculated with BMV than in the uninoculated treatment. Conversely, KWS-1 had a higher mean dry root weight when inoculated with BMV. However, none of these differences were significant (ANOVA,  $p = 0.131$ ), and the spread of these data were large.

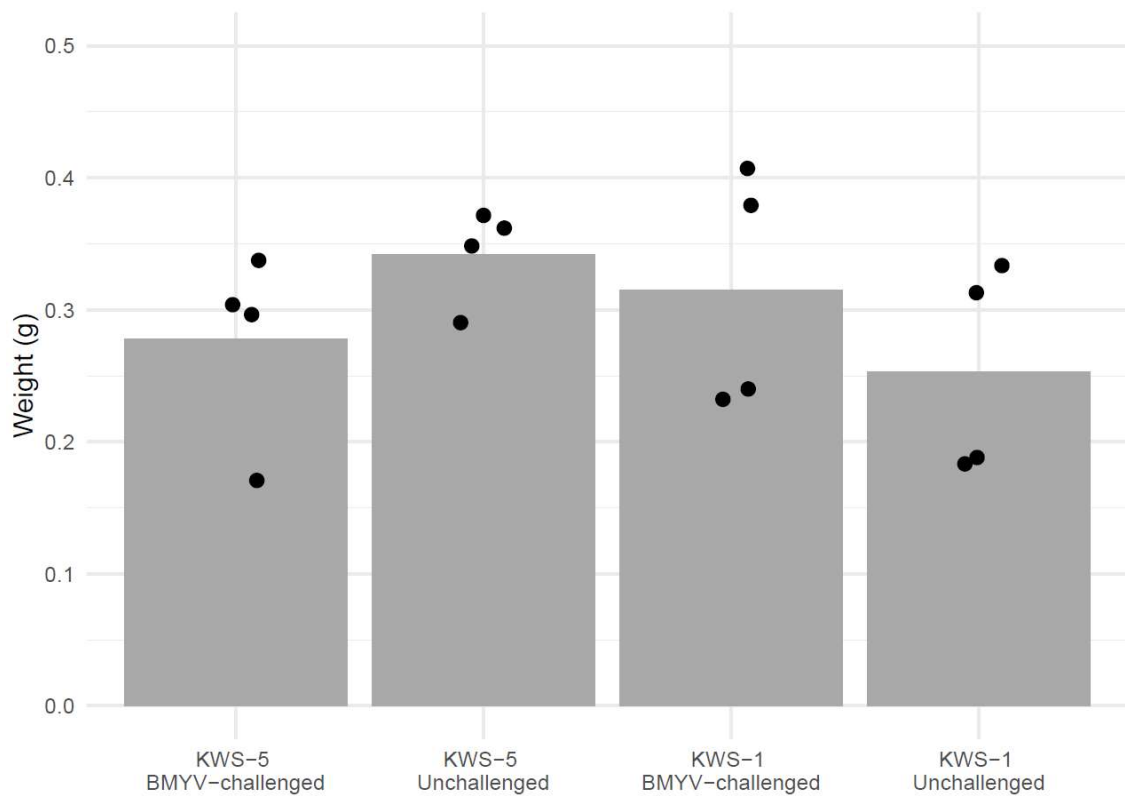


Figure 4.5: bar chart showing the mean dry root weight of each variety under Beet mild yellowing virus (BMV) challenged (plants inoculated with BMV viruliferous *Myzus persicae* aphids) and unchallenged (plants inoculated with non-viruliferous *M. persicae* aphids) conditions. Scatter points overlaid show individual data point and the variation seen. No significant differences in dry root weight were found between the different varieties or between the virus treatments (analysis of variance (ANOVA),  $p = 0.131$ ,  $n = 16$ ).

## 4.4.2 Glasshouse study

### 4.4.2.1 ELISA results

Both leaf 5 and leaf 10 were sampled for ELISA testing in the glasshouse study. ELISA results from both leaves found that the mean ELISA absorbance of the virus inoculated plants was above the threshold set by the negative controls and significantly higher than the mean absorbance of the uninoculated plants (Figure 4.6). The mean ELISA results for inoculated plants were higher in leaf 5 than leaf 10, with an average absorbance of 2.32 from leaf 5 compared to 0.99 for leaf 10.

Based on the ELISA results of leaf 5, only one virus-inoculated plant failed to pass the positive threshold (0.457). The plant was KWS-7, BMV-inoculated, and had a leaf 5 ELISA absorbance of 0.109 compared to the other KWS-7 BMV-inoculated plants which had leaf 5 ELISA results of 1.405, 1.868 and 2.773. This plant also had a low leaf 10 ELISA result of 0.173, which was also below the positive threshold (0.321). As such the results from this plant were removed from subsequent analysis. Other virus inoculated plants were below the positive threshold for leaf 10, however none failed to pass the threshold for leaf 5 and so all other plants were kept in the data analysis.

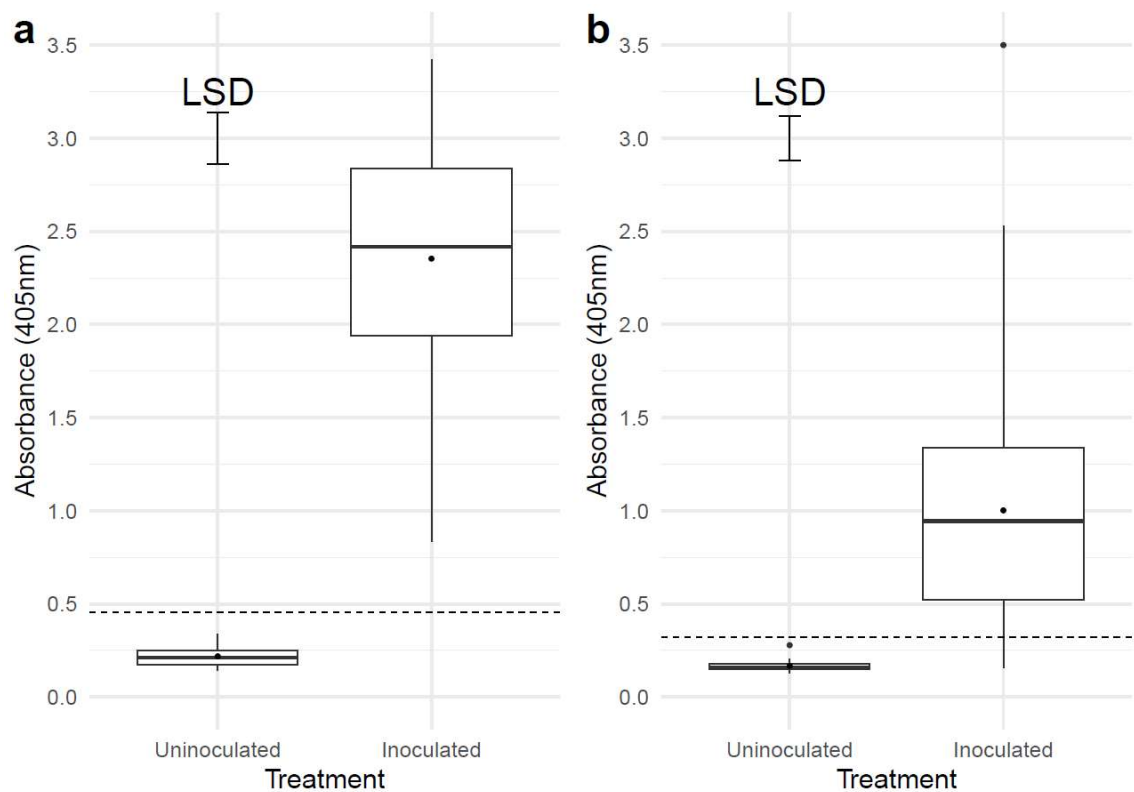


Figure 4.6: boxplots showing enzyme-linked immunosorbent assay (ELISA) absorbances of the uninoculated treatment and inoculated with viruliferous aphid treatments (Beet mild yellowing virus (BMV), Beet chlorosis virus (BChV) and combined BMV and BChV treatments) across all varieties. a = Leaf 5 ELISA results, b = Leaf 10 ELISA results. The mean absorbance is indicated for each treatment by the black circular point. The dashed horizontal line indicates the ELISA absorbance value above which a sample is deemed positive (calculated by the mean of the negative controls plus three times the standard deviation), set at 0.457 for leaf 5 and 0.321 for leaf 10. Error bars show least significant difference (LSD) at 5% significance between treatments (analysis of variance (ANOVA),  $p < 0.001$  for leaf 5,  $p = 0.001$  for leaf 10,  $n = 95$ ).

The ELISA results of leaf 5 were broadly similar between all virus inoculated treatments (data not shown), with no statistically significant differences identified (ANOVA excluding uninoculated,  $p = 0.162$ ). Significant differences were seen between the virus inoculated treatments in the ELISA results of leaf 10. The combined BMV and BChV treatment had a significantly higher mean ELISA absorbance of 1.276 compared to either BMV (0.765) and BChV (0.951) treatments (ANOVA excluding uninoculated,  $p = 0.008$ ).

Focusing on the virus inoculated treatments, varieties differed significantly in their leaf 5 ELISA absorbances (Figure 4.7). Combining the data from the virus inoculated treatments (BMV, BChV



and combined BMV and BChV treatment), the “high resistance” varieties KWS-1 and KWS-2, and the “low resistance” variety KWS-6, had significantly lower mean ELISA values (ANOVA excluding uninoculated,  $p = 0.002$ ) than “medium resistance” varieties KWS-3 and KWS-4. No significant difference was found between KWS-1 and KWS-2 and the “low resistance” varieties KWS-6 and KWS-7. These differences were not seen in the leaf 10 ELISA results; analysis identified no significant differences between varieties under virus inoculation (ANOVA excluding uninoculated,  $p = 0.874$ ).

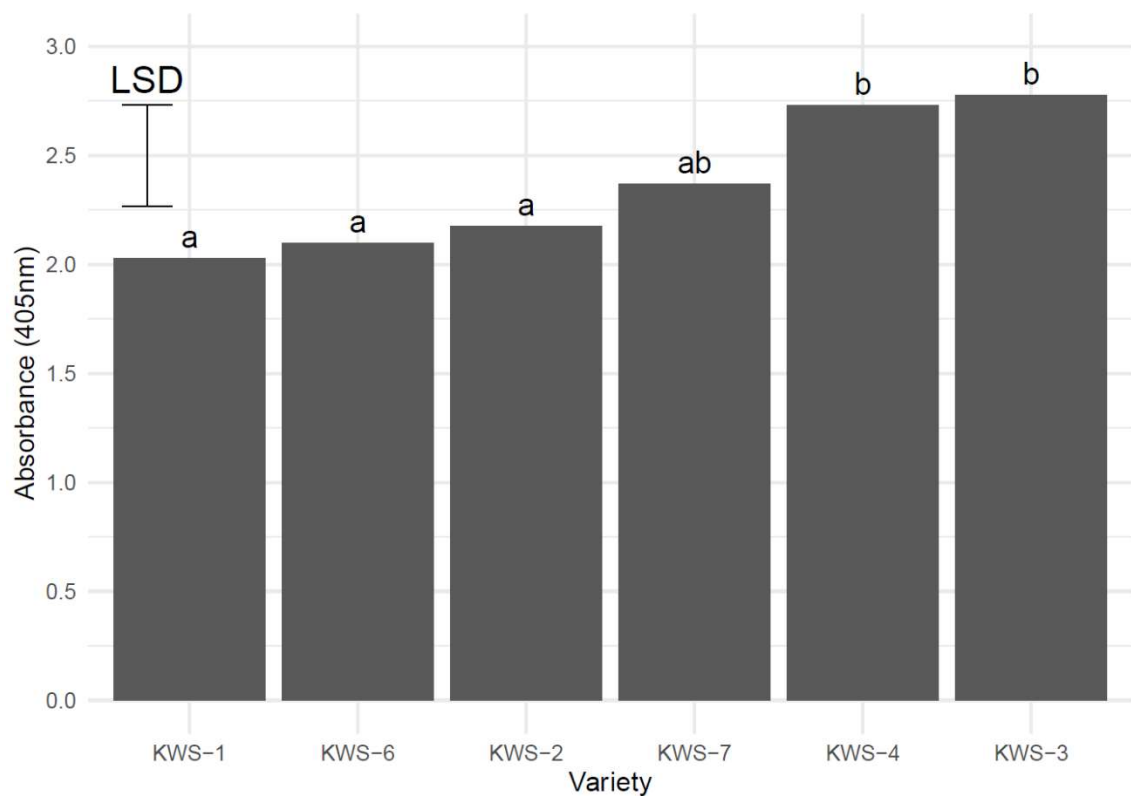


Figure 4.7: mean enzyme-linked immunosorbent assay (ELISA) absorbance of leaf 5 across all virus-inoculated treatments (Beet mild yellowing virus (BMV), Beet chlorosis virus (BChV) and combined BMV and BChV inoculated treatments) for each variety. Error bar shows least significant difference (LSD) at 5% significance between varieties (analysis of variance (ANOVA), excluding uninoculated  $p = 0.002$ ,  $n = 71$ ). Varieties KWS-1, KWS-2 and KWS-6 differed significantly from KWS-3 and KWS-4, shown by the differing letters above each bar in the graph.

#### 4.4.2.2 Symptom expression

Unlike in the pilot study conducted in the MLR-352 growth cabinets, some virus infected plants in the glasshouse study did exhibit yellowing symptoms. The strongest symptoms were observed in variety KWS-7, and for this variety it was possible to distinguish the uninoculated and virus inoculated treatments based on visual symptoms (Figure 4.13). However, for all other varieties

symptoms expression was not consistent, and no clear differences could be identified between treatments or varieties (Figures 4.8 to 4.12). Some leaf paling/yellowing was also visible in the uninoculated controls, despite these plants testing negative for virus via ELISA. Plants across all varieties and treatments also exhibited a more elongated growth habit compared to field grown sugar beet. This was particularly noticeable in the length of leaf petioles.



*Figure 4.8: photos taken from above of each test plant of variety KWS-1 in the glasshouse study, 8 weeks after inoculation. a = uninoculated, b = Beet mild yellowing virus (BMV)-inoculated, c = Beet chlorosis virus (BChV)-inoculated, d = combined BMV and BChV-inoculated.*



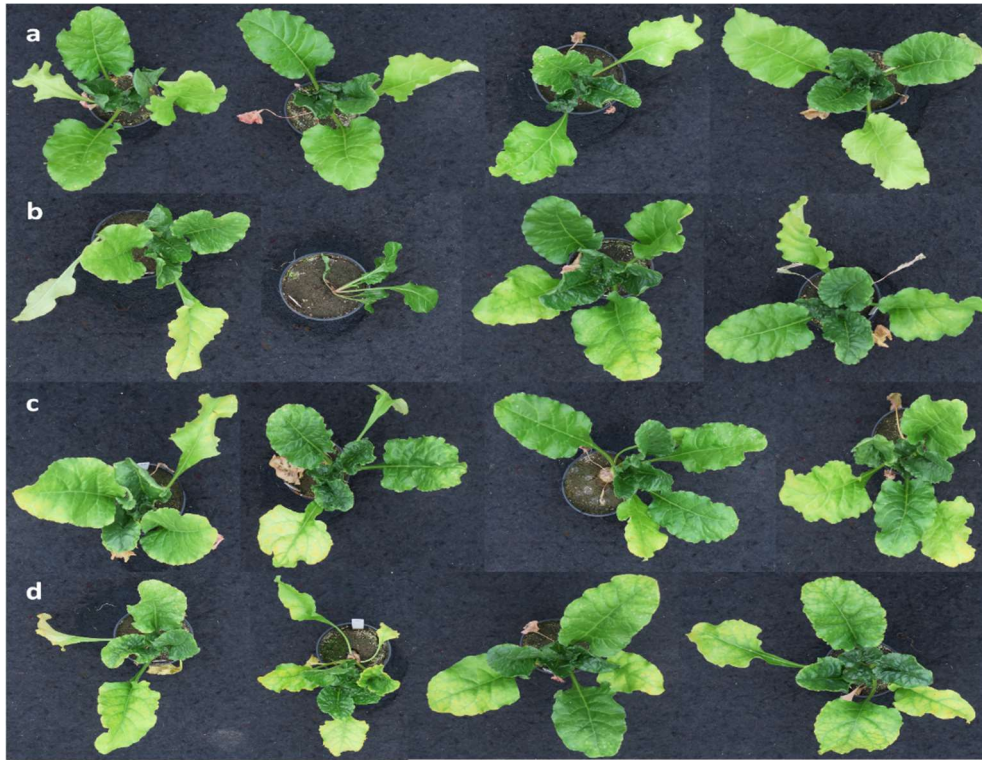
*Figure 4.9: photos taken from above of each test plant of variety KWS-2 in the glasshouse study, 8 weeks after inoculation. a = uninoculated, b = Beet mild yellowing virus (BMV) -inoculated, c = Beet chlorosis virus (BChV) -inoculated, d = combined BMV and BChV-inoculated.*



Figure 4.10: photos taken from above of each test plant of variety KWS-3 in the glasshouse study, 8 weeks after inoculation. a = uninoculated, b = Beet mild yellowing virus (BMV)-inoculated, c = Beet chlorosis virus (BChV) - inoculated, d = combined BMV and BChV-inoculated.



*Figure 4.11: photos taken from above of each test plant of variety KWS-4 in the glasshouse study, 8 weeks after inoculation. a = uninoculated, b = Beet mild yellowing virus (BMV) -inoculated, c = Beet chlorosis virus (BChV)-inoculated, d = combined BMV and BChV-inoculated.*



*Figure 4.12: photos taken from above of each test plant of variety KWS-6 in the glasshouse study, 8 weeks after inoculation. a = uninoculated, b = Beet mild yellowing virus (BMV) -inoculated, c = Beet chlorosis virus (BChV)-inoculated, d = combined BMV and BChV-inoculated.*



Figure 4.13: photos taken from above of each test plant of variety KWS-7 in the glasshouse study, 8 weeks after inoculation. a = uninoculated, b = Beet mild yellowing virus (BMV)-inoculated, c = Beet chlorosis virus (BChV)-inoculated, d = combined BMV and BChV-inoculated.

#### 4.4.2.3 Leaf area and weight

Virus treatment did not have a significant impact on leaf dry weight (ANOVA,  $p = 0.870$ ). All treatments showed a large range, for example the uninoculated plants had leaf dry weights ranging from 1.8 to 5.5g compared to 2.6 to 5.3g for the mixed BMV and BChV infection. Variety did have a significant impact on leaf dry weight (ANOVA,  $p < 0.001$ ), with the greatest leaf dry weight found in KWS-4 and the lowest in KWS-6 (Figure 4.14). However, no differences were identified in how the varieties responded to virus inoculation was found (ANOVA, treatment variety interaction,  $p = 0.370$ ). Canopy area was also not affected by virus treatment (ANOVA,  $p = 0.428$ ), or variety (ANOVA,  $p = 0.109$ ).

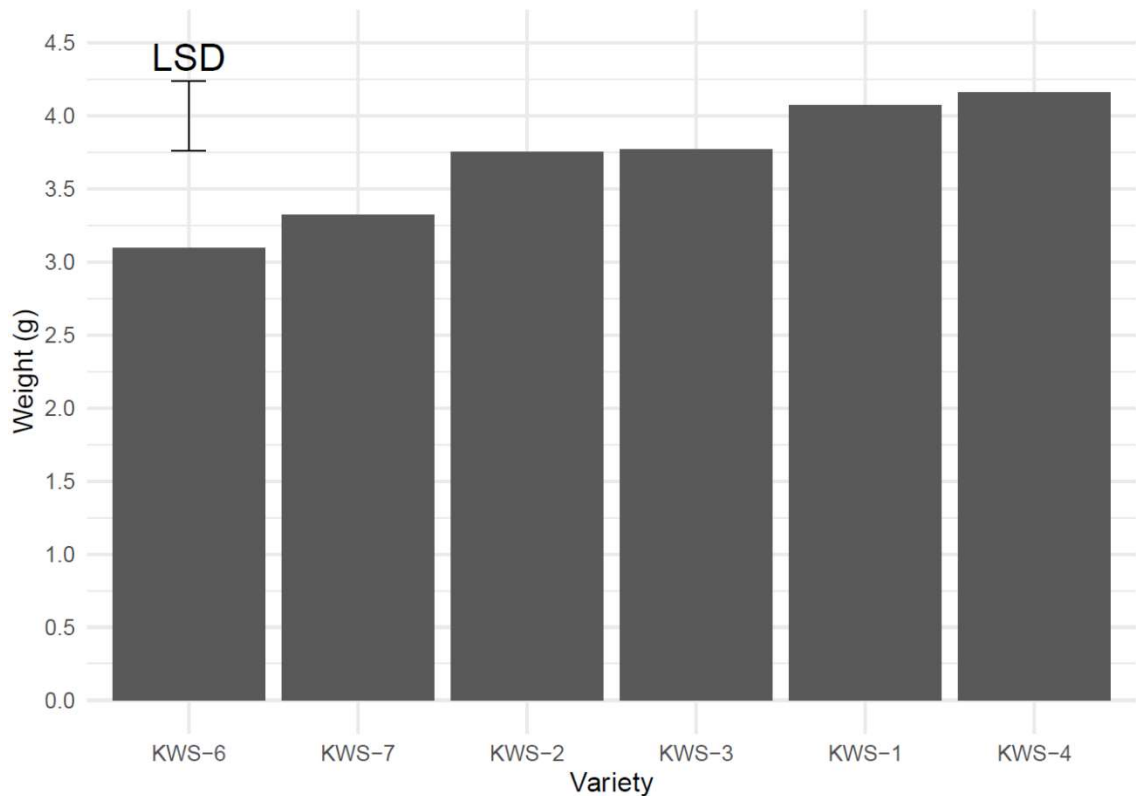


Figure 4.14: bar chart showing mean dry leaf weight of each variety across all treatments (uninoculated, Beet mild yellowing virus (BMV)-inoculated, Beet chlorosis virus (BChV)-inoculated and combined BMV- and BChV-inoculated). Error bar shows least significant difference (LSD) at 5% significance between varieties (analysis of variance (ANOVA),  $p < 0.001$ ,  $n = 95$ ).

#### 4.4.2.4 Root yield

The uninoculated treatment produced a significantly larger mean dry root weight than the virus inoculated treatments (ANOVA,  $p = 0.013$ ). Although there was no significant difference between the different virus treatments, the mixed BMV and BChV inoculation gave the lowest mean dry root weight (2.79g) and the BChV treatment the highest (3.28g), (Figure 4.15). Focusing on the virus inoculated treatments, significant differences were found between the varieties (ANOVA without uninoculated,  $p = 0.005$ ), (Figure 4.16). KWS-7 produced the smallest mean dry root weight, significantly lower than all other varieties apart from KWS-2. All varieties showed a high level of variation in root weight with variety KWS-6 being the most variable ranging from 0.16 to 4.87g.



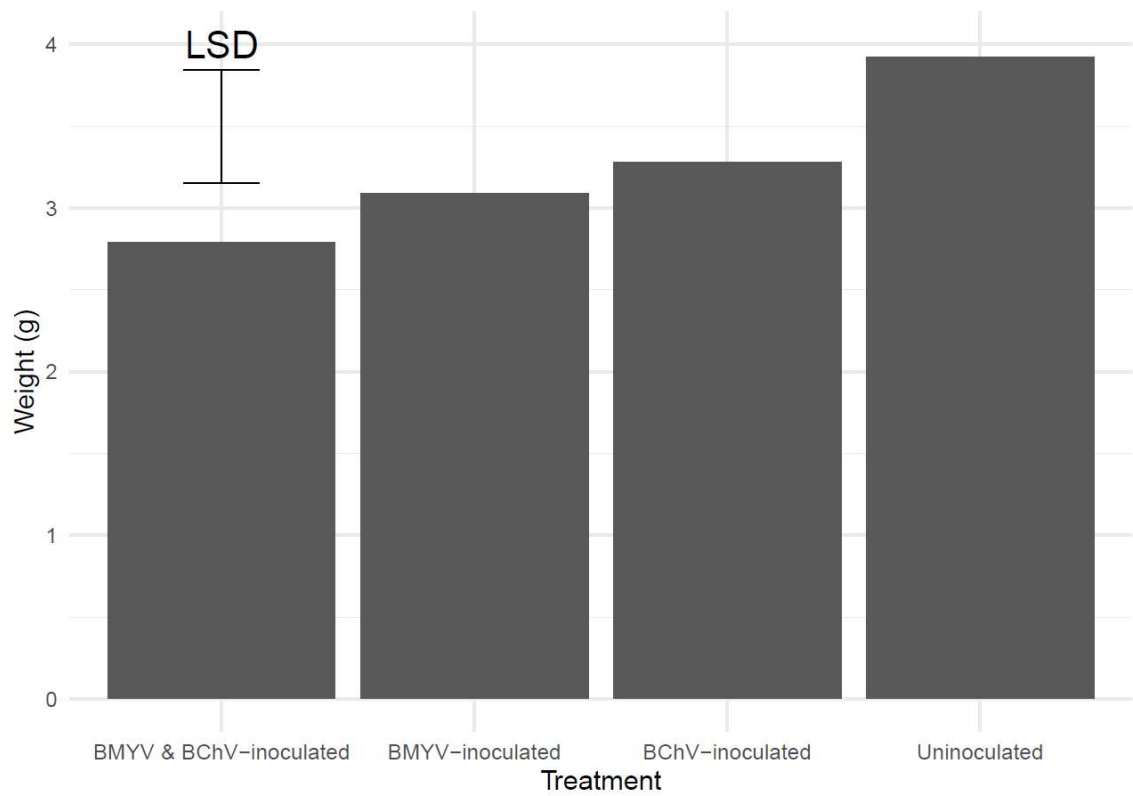


Figure 4.15: bar chart showing effect of virus inoculation on mean root dry weight across all varieties (KWS-1, KWS-2, KWS-3, KWS-4, KWS-6, KWS-7). Error bar shows least significant difference (LSD) at 5% significance between treatments (analysis of variance (ANOVA),  $p=0.013$ ,  $n = 95$ ).

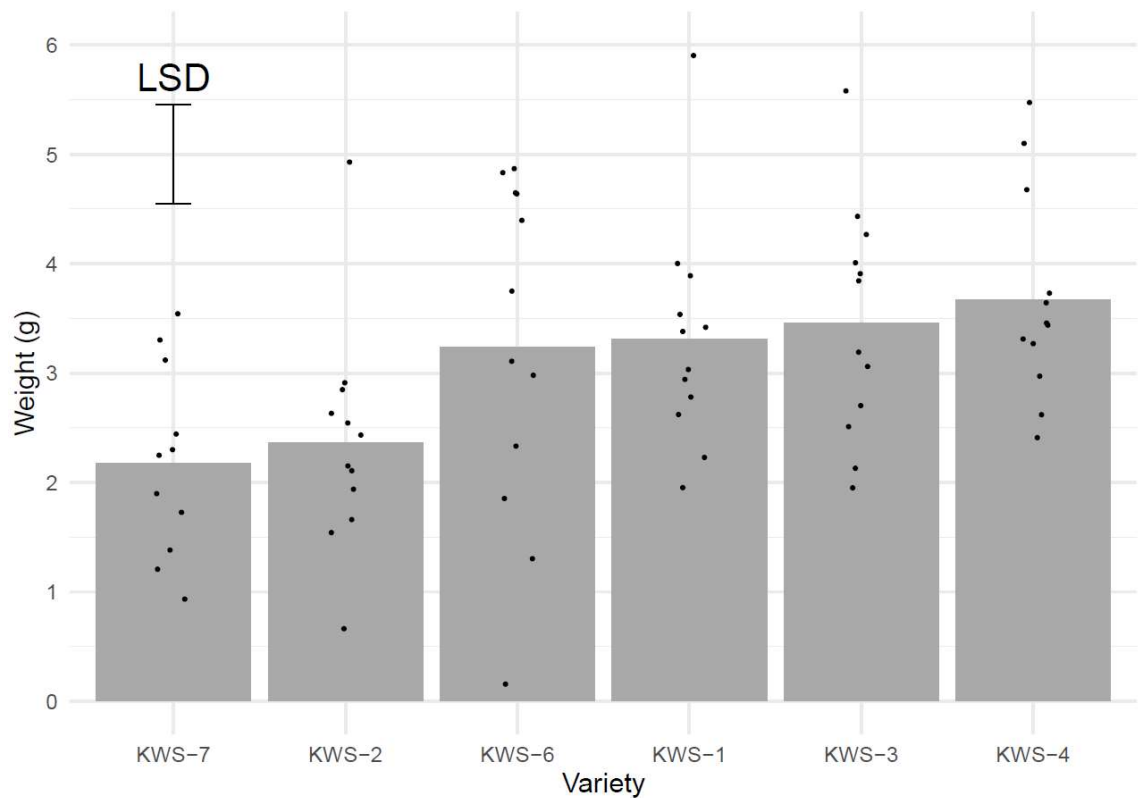


Figure 4.16: bar chart showing the mean dry root weight of each variety under the virus inoculated treatments (Beet mild yellowing virus (BMV)-inoculated, Beet chlorosis virus (BChV)-inoculated and combined BMV and BChV-inoculated). Overlaid points are the individual data point showing the large range in results. Error bar shows least significant difference (LSD) at 5% significance (analysis of variance (ANOVA),  $p < 0.001$  for leaf 5,  $p = 0.001$  for leaf 10,  $n = 71$ ).

#### 4.4.3 LED study

##### 4.4.3.1 Symptoms

The plants in this study, grown under LED light, had an overall shorter canopy architecture and more typical petiole length compared to the plants grown in the glasshouse study. Nevertheless, the plants still had a different growth habit to field grown sugar beet, with the leaves twisting in towards the crown rather than opening out. Many plants, including from the uninoculated treatment, exhibited leaf yellowing making assessment of virus symptoms difficult (Figure 4.17 to 4.22). No obvious differences were present between the uninoculated and virus treatments for any variety.



Figure 4.17: photos taken from above of each test plant of variety KWS-1 in the LED study, 8 weeks after inoculation. a = uninoculated, b = Beet mild yellowing virus (BMV)-inoculated, c = Beet chlorosis virus (BChV) -inoculated.

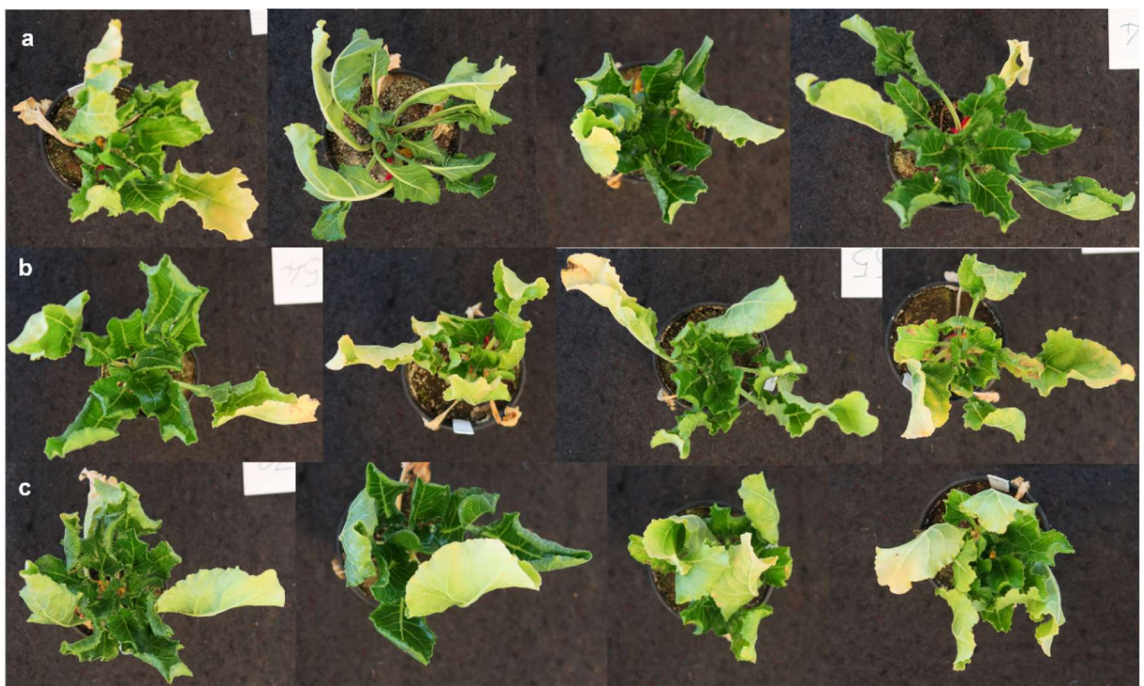


Figure 4.18: photos taken from above of each test plant of variety KWS-2 in the LED study, 8 weeks after inoculation. a = uninoculated, b = Beet mild yellowing virus (BMV)-inoculated, c = Beet chlorosis virus (BChV) -inoculated.

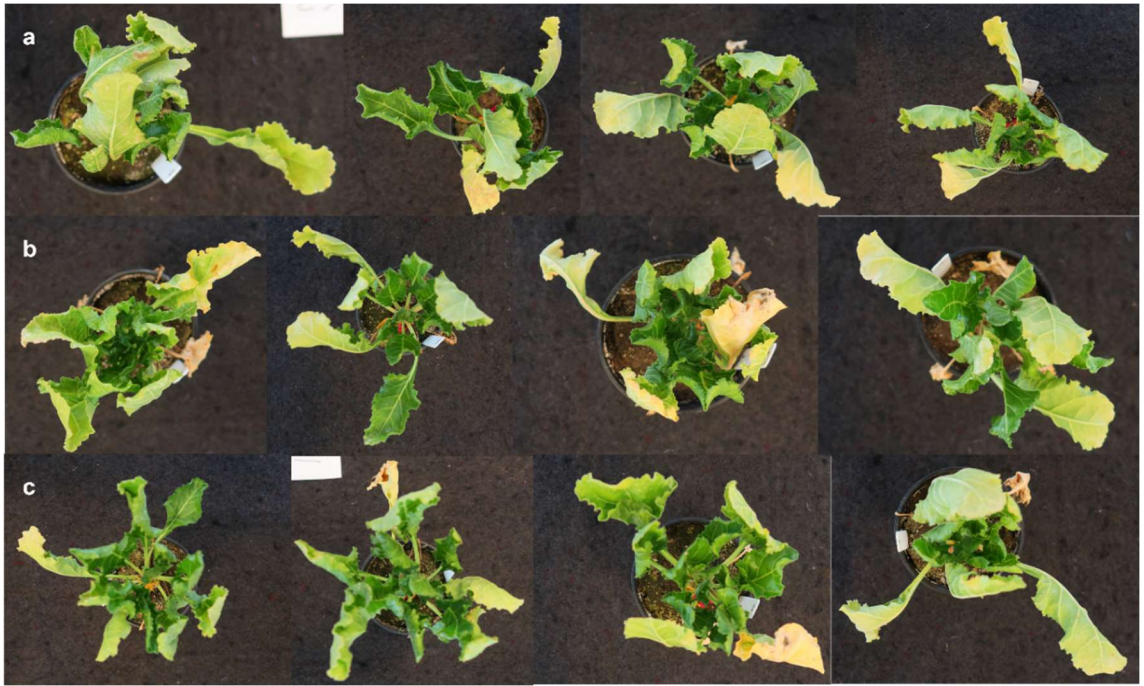


Figure 4.19: photos taken from above of each test plant of variety KWS-3 in the LED study, 8 weeks after inoculation. a = uninoculated, b = Beet mild yellowing virus (BMV)-inoculated, c = Beet chlorosis virus (BChV) -inoculated.

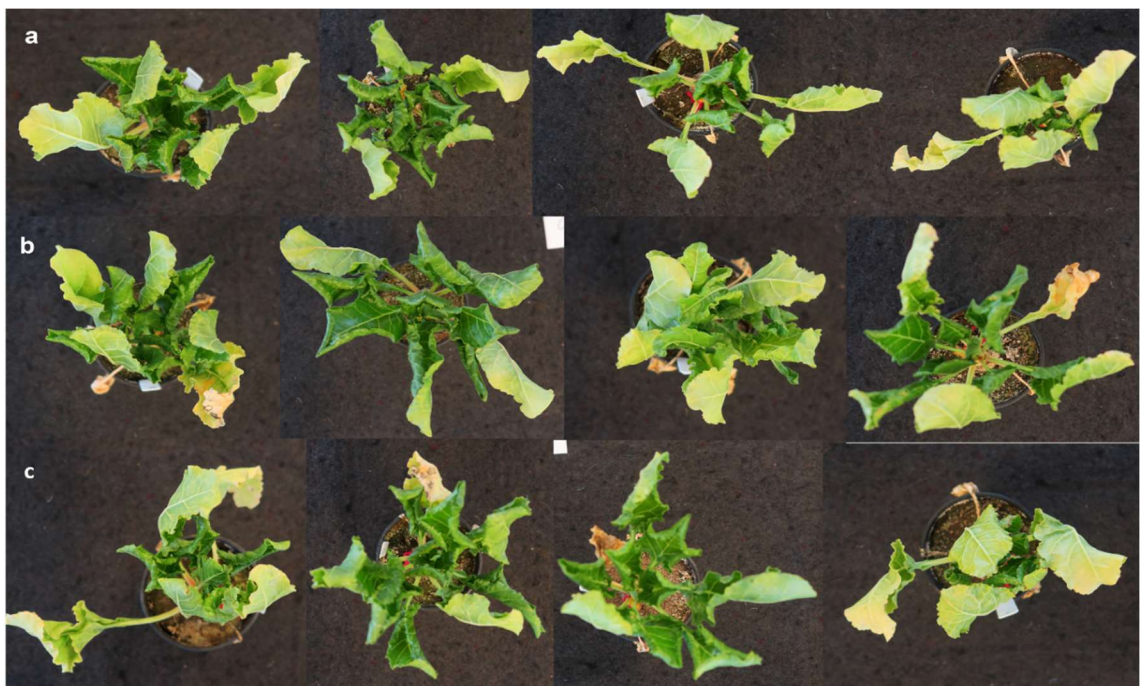


Figure 4.20: photos taken from above of each test plant of variety KWS-4 in the LED study, 8 weeks after inoculation. a = uninoculated, b = Beet mild yellowing virus (BMV)-inoculated, c = Beet chlorosis virus (BChV) -inoculated.

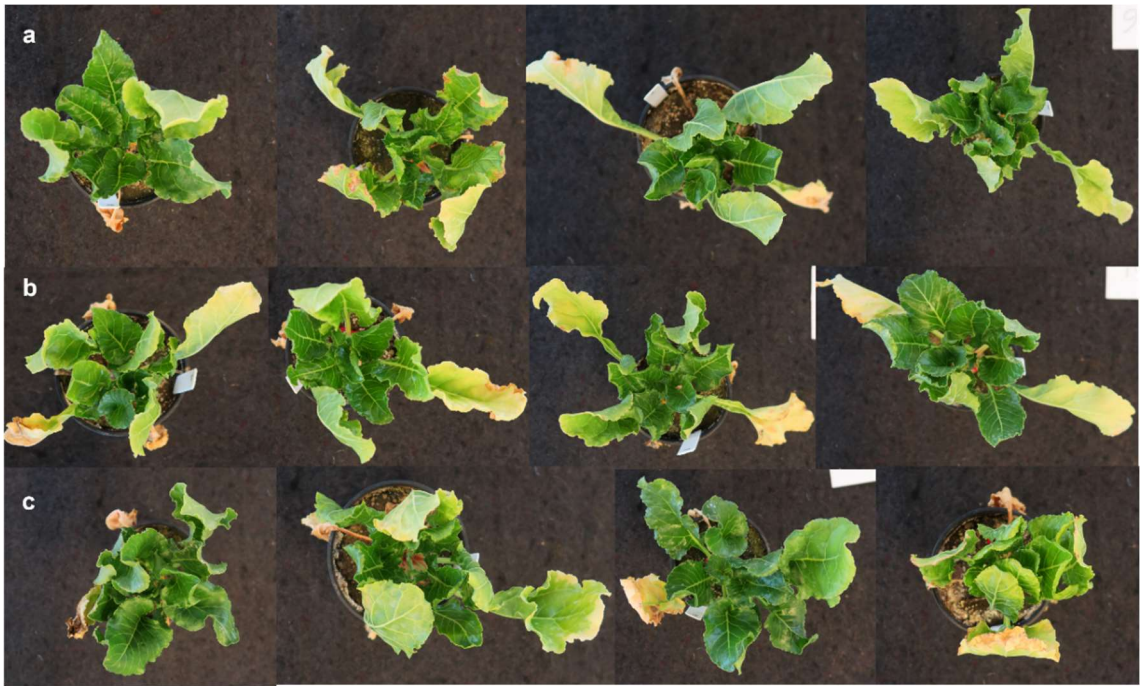


Figure 4.21: photos taken from above of each test plant of variety KWS-6 in the LED study, 8 weeks after inoculation. a = uninoculated, b = Beet mild yellowing virus (BMV)-inoculated, c = Beet chlorosis virus (BChV) -inoculated.



Figure 4.22: photos taken from above of each test plant of variety KWS-7 in the LED study, 8 weeks after inoculation. a = uninoculated, b = Beet mild yellowing virus (BMV)-inoculated, c = Beet chlorosis virus (BChV) -inoculated.

#### 4.4.3.2 ELISA results

The ELISA results from leaf 5 were much more variable across all varieties than leaf 10 (Figure 4.23). The threshold, as determined by the negative controls, above which a sample was classed as positive was 0.533. Leaf 5 results did not indicate a successful inoculation, with 68.8% of virus inoculated plants failing to pass this threshold. ELISA results from leaf 10 were much more consistent, with clear differences between inoculated and uninoculated plants. Based on the leaf 10 ELISA results, only 17% of inoculated plants did not pass the positive threshold, whilst none of the uninfected plants were deemed positive. The overall mean ELISA result for leaf 10 of the uninoculated plants was 0.224, this was significantly lower than the mean for the inoculated plants of 1.215, (ANOVA  $p < 0.001$ ). No significant difference in the ELISA results of leaf 10 was found between the BMV and BChV treatments (ANOVA,  $p = 0.417$ ).

As in the glasshouse experiment, when focusing on the virus-inoculated treatments KWS-1 had the lowest mean ELISA result at 0.858 followed by KWS-6 at 0.911. KWS-7 had the highest mean ELISA result at 1.781, however none of these differences were statistically significant (ANOVA,  $p = 0.051$ ). No differences were found in how the varieties responded to the different virus treatments (ANOVA,  $p = 0.388$ ).

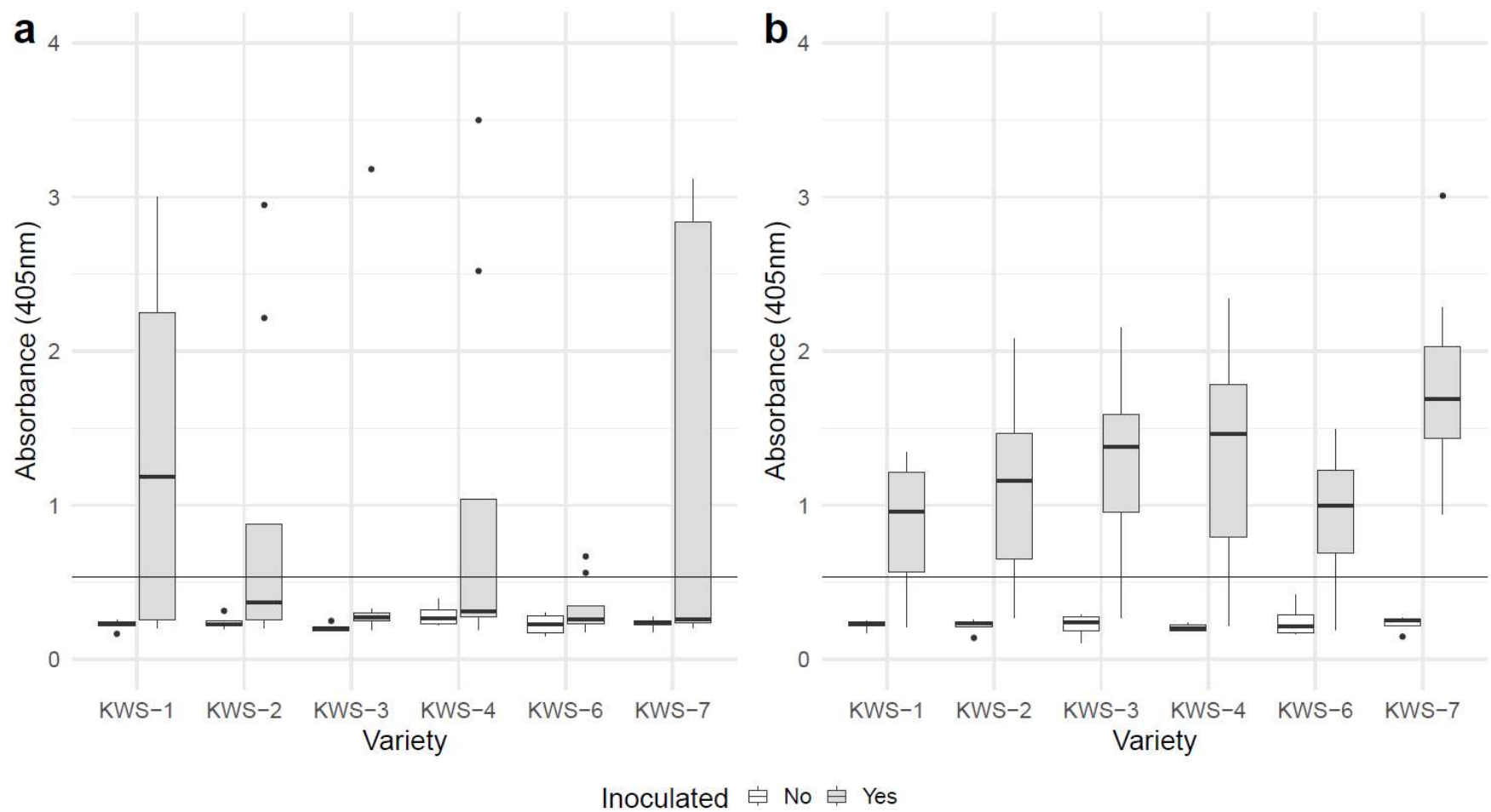


Figure 4.23: box and whisker plots showing enzyme-linked immunosorbent assay (ELISA) results for each variety in the LED study when uninoculated and inoculated with viruliferous aphids (Beet mild yellowing virus (BMV)-inoculated and Beet chlorosis virus (BChV)-inoculated treatments). a – ELISA results of leaf 5, b – ELISA results of leaf 10.

#### 4.4.3.3 Leaf weight and canopy area

Although time had a significant impact on canopy cover (repeated measures ANOVA,  $p < 0.001$ ), canopy cover did not increase linearly (Figure 4.24). All varieties and treatments decreased canopy area six weeks after inoculation, before increasing again. The one exception to this was variety KWS-2 under BMV infection which also reduced in canopy area between seven and eight weeks after inoculation.



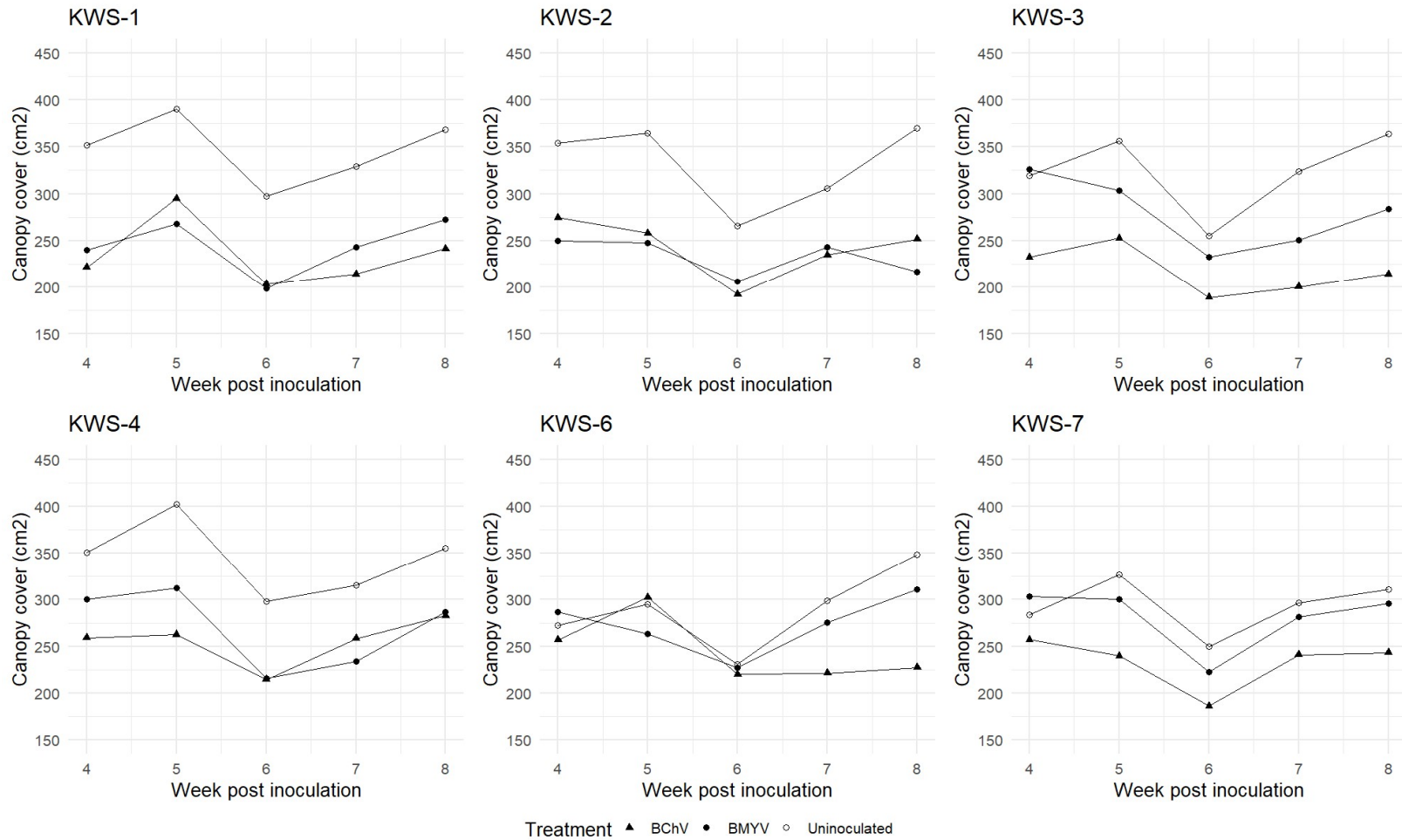


Figure 4.24: line charts showing change in canopy area in each variety in the LED study, under different virus treatments (Beet mild yellowing virus (BMV)-inoculated, Beet chlorosis virus (BChV)-inoculated and uninoculated), between four weeks after inoculation and harvest at eight weeks after inoculation.

Despite these changes in canopy cover over time, virus infection was found to have a significant impact on canopy area (repeated measures ANOVA,  $p = 0.003$ ), with the BMV and BChV-inoculated plants having a significantly smaller canopy area than the uninoculated (Figure 4.25). Between the BMV and BChV treatments no significant differences in canopy area were found. There were also no significant differences between varieties or in how the varieties interacted with virus treatments identified at any time point.

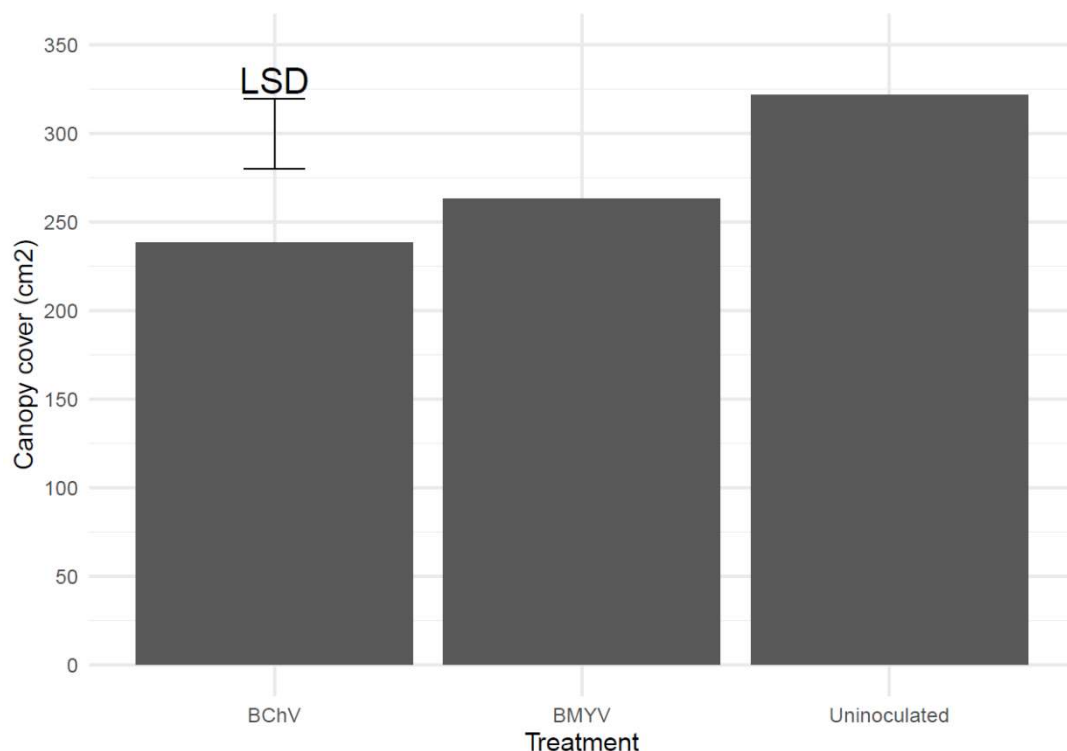


Figure 4.25: bar chart showing effect of virus treatment (Beet chlorosis virus (BChV) inoculated, Beet mild yellowing virus (BMV)-inoculated and uninoculated) on mean canopy cover across all varieties (KWS-1, KWS-2, KWS-3, KWS-4, KWS-6 and KWS-7) in the LED study. Error bar shows least significant difference (LSD) at 5% significance between treatments (repeated measures analysis of variance,  $p=0.003$ ,  $n = 72$ ).

Consistent with the uninoculated treatment having the largest canopy area, the uninoculated plants also had the highest mean leaf dry weight at 3.327g compared to 3.249g for BMV-inoculated plants and 3.247g for BChV. However there was no statistical difference between any of the treatments (ANOVA,  $p=0.667$ ). Leaf dry weight did differ significantly between the varieties (Figure 4.26, ANOVA,  $p < 0.001$ ) but the response of the varieties to virus infection was equal (ANOVA,  $p = 0.165$  variety treatment interaction).

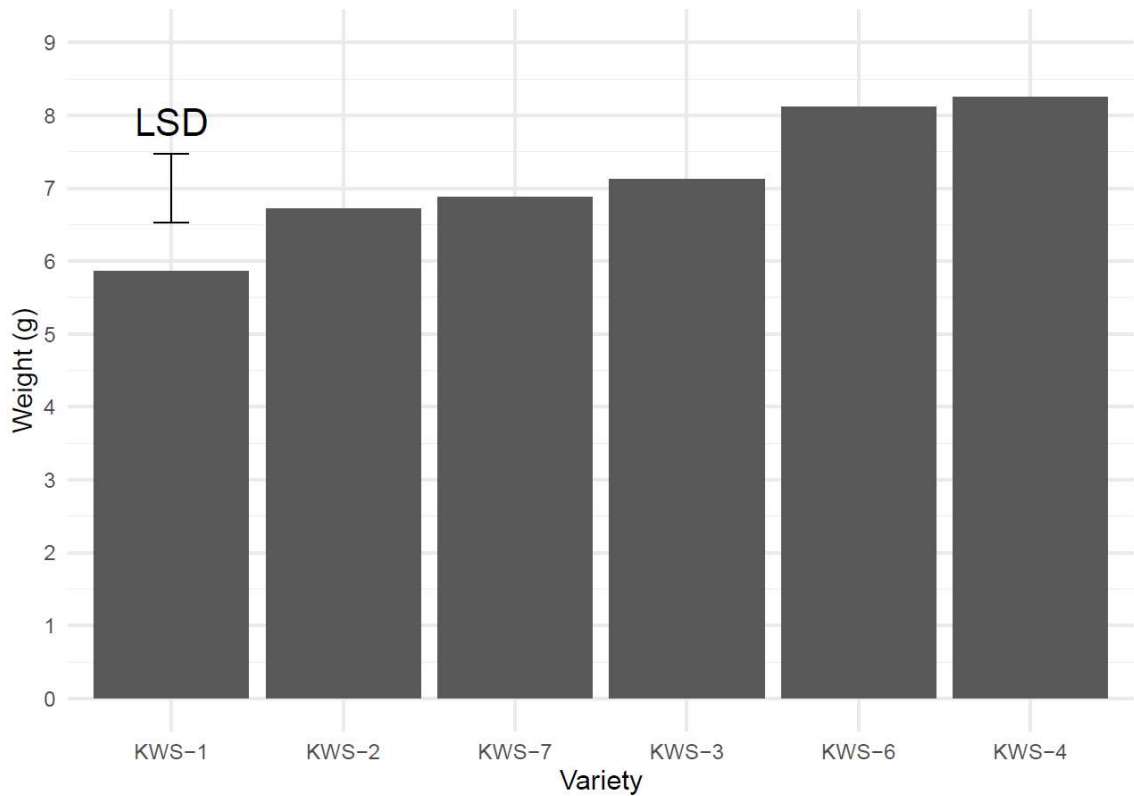


Figure 4.26: bar chart showing effect of variety on mean leaf dry weight across all treatments (uninoculated, Beet mild yellowing virus (BMV)-inoculated and Beet chlorosis virus (BChV)-inoculated) in the LED study. Error bar shows least significant difference (LSD) at 5% significance between varieties (analysis of variance (ANOVA),  $p < 0.001$ ,  $n = 72$ ).

#### 4.4.3.4 Root dry weight

Inoculation with either BMV or BChV had no significant impact on root dry weight. The mean root dry weight for the uninoculated plants was 6.81g compared to 6.96g for BChV and 7.35g for BMV. Significant differences were found between varieties (ANOVA,  $p < 0.001$ ), with variety KWS-1 producing the smallest roots (Figure 4.27) and KWS-4 the largest. No significant differences in how the varieties responded to virus infection was identified (ANOVA,  $p = 0.327$ ).

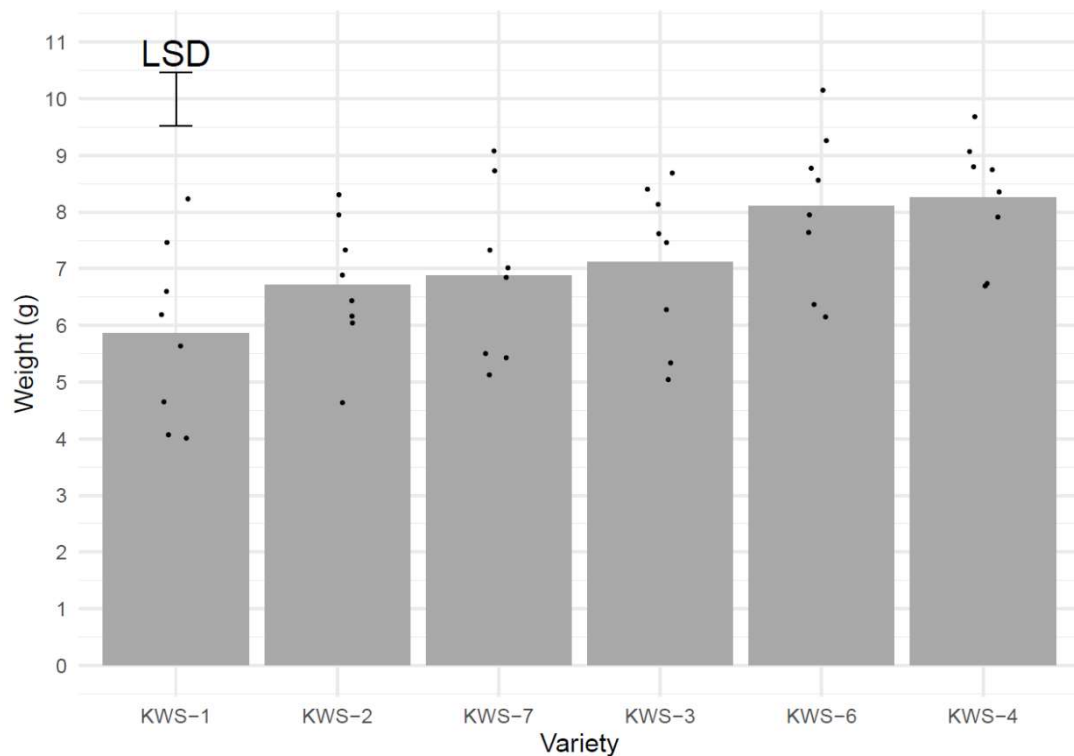


Figure 4.27: bar chart showing the effect of variety on mean dry root weight when inoculated with virus (Beet mild yellowing virus (BMV)-inoculated and Beet chlorosis virus (BChV)-inoculated treatments) in the LED study. Scatter points overlaid show individual data points. Error bar shows least significant difference (LSD) at 5% significance between varieties (analysis of variance (ANOVA),  $p < 0.001$ ,  $n = 54$ ).

## 4.5 Discussion

### 4.5.1 ELISA results

The adapted clip cage method using fine pins to secure the cages provided excellent aphid containment. ELISA results of the uninoculated treatments in all studies indicated no contamination with viruliferous aphids, with the ELISA results of the uninoculated plants falling below the positive threshold. Although the mean ELISA results for the inoculated treatments were above the positive threshold in all studies, some individual plants did not pass this threshold. When the pilot study was conducted variety KWS-5 was understood to be susceptible to virus infection, however following discussions with the seed breeder KWS, it was identified as having medium resistance to BMV and therefore was not the most suitable susceptible control variety to use. Nevertheless, KWS-5 is not fully resistant to BMV infection and therefore variety resistance was unlikely to be the only cause of these ELISA results. One explanation of these results is that the interval of only four weeks between inoculation and ELISA testing was not adequate time for virus infection to establish and any differences in titre be identified.

This hypothesis is supported by the ELISA results of both the glasshouse and LED studies, in which there was an eight-week interval between inoculation and ELISA testing. In both experiments significant differences were seen in the ELISA results of leaf ten between the inoculated and uninoculated plants, indicating the clip cage method of inoculation was successful. Notably, for the ELISA results of leaf five, although significant differences were found between inoculated and uninoculated plants in the glasshouse study, no differences were found in the LED study.

When sampling leaf five for ELISA testing, a higher level of leaf senescence was observed in the LED study compared to the glasshouse study. Senescing leaves are known to have lower virus titres (Peters, 1988) and is therefore likely to have caused the large variation seen in leaf five ELISA results in the LED study and the lower mean virus titres. In the glasshouse study, where lower levels of leaf senescence were observed, higher virus titres were found in leaf five than in leaf ten for the inoculated treatments. This could indicate differences in virus localisation within infected plants, consistent with the findings of Peters (1988), with a source-sink relationship arising between older and younger leaves. Virus is transported from older leaves with higher virus concentrations to younger leaves in a similar way to the translocation of photosynthates. However, as all the leaf five and all of the leaf ten samples were grouped together on the ELISA plates, these differences could be a consequence of variation between plates. In subsequent studies, randomising the samples across the ELISA plates would permit more reliable comparisons of results.

Focussing on the ELISA results of leaf five, the glasshouse study identified varieties KWS-3 and KWS-4 as having significantly higher ELISA results for the inoculated treatments than KWS-1 and KWS-2. Interestingly no significant differences were identified between KWS-1 and KWS-2, and KWS-6 and KWS-7 despite KWS-6 and KWS-7 being described as susceptible by the seed breeder. Only in the glasshouse study were significant differences in ELISA result found between varieties, indicating the glasshouse environment may be the better growth environment for ELISA based phenotyping. Nevertheless, both the glasshouse and the LED study identified KWS-1 as the variety with the lowest ELISA absorbance when inoculated. This indicates KWS-1 may have some resistance to beet polerovirus infection.

In all three experiments some inoculated plants did not pass the virus-positive ELISA threshold. This may be evidence for varietal resistance to virus infection, but equally could indicate that infection was not achieved via the clip cage method. Certainly, in the case of the susceptible varieties, namely KWS-6 and KWS-7, the latter explanation is more likely given no varietal resistance is described by the seed breeder (KWS).

One alternative to a clip cage method of inoculation would have been to move the plants into separate virus cultures to be infected, before returning them to the trialled growth conditions after the aphids had been removed. This methodology would have avoided any stress to the aphids resulting from transferring them into, and containing them within, the clip cage. Instead, the viruliferous aphids would have been able to feed on the test plants, and transmit the virus, unimpeded by the clip cage. This method would have significantly less time consuming, and more suited to large scale studies. However, the clip cage method utilised in this study has other advantages, such as allowing control over exactly how many viruliferous aphids are permitted to feed on each plant. Importantly, the clip cage approach also allows multiple virus treatments to be tested within the same controlled environment chamber, reducing the number of chambers required and the associated costs. Given the facilities available, conducting the trials described in this chapter would not have been possible if separate chambers were needed for each virus treatment. There is also no need to move plants between environments, minimising the stress incurred by the plants and reducing the risk of contamination.

As previously discussed, the use of fine pins to secure the aphid cages proved a successful method of containing aphids on sugar beet plants and had other practical advantages. Nevertheless, this approach was skilled and very time consuming (taking three to four minutes to affix one cage). Cages were positioned on one of the first true leaves, however on some plants it was not possible to situate the cage such that the pins did not go through the leaf tissue. This left holes in the leaves, which though very small would have placed additional stress on affected plants. Inoculating the first true leaves was reflective of the field situation, where aphids are attracted to younger sugar beet plants (Watson et al., 1946). However, the shape of the cotyledon leaves may be more conducive for clip cage attachment, avoiding the need to pin through the leaf tissue.

One change that was made between the pilot study and the subsequent glasshouse and LED studies regarding the clip cage methodology was the decision to not inoculate the non-virus treatments with non-viruliferous aphids in the latter experiments. With the increase in replicates from the 16 plants tested in the pilot study to the 72 and 96 plants tested in the second LED and glasshouse study respectively, it became impossible to clip cage every plant in a sustainable time frame. This may have affected the reliability of the results, with the inoculated plants suffering additional stress not only via virus inoculation but also the physical impacts of the clip cages. Best scientific practice would have been to continue to use non-viruliferous aphids contained within clip cages as the non-infected control treatment, and future studies should do this. One way around this problem would be to have more than one person affixing the cages, ensuring all plants were inoculated within a reasonable time frame.

#### 4.5.2 Root weight

Dry root weight was used as a proxy of harvest yield across all three experiments. In the pilot study, the “high” BMV resistant variety KWS-1 tended to yield higher than the “medium” BMV resistant variety KWS-5, however these results were not significant. As previously discussed, KWS-5 although understood to be susceptible to BMV was later confirmed by the breeder to have medium resistance to BMV and therefore was not the best control to compare the high resistance variety KWS-1 phenotype to. However, a number of other factors may well have contributed to the lack of statistically significant differences in this pilot study.

Sugar beet plants produce a long tap root, known to reach depths of more than one metre under field conditions (Jaggard and Qi, 2006). The small 9cm diameter, 9cm tall pots used in the pilot study, would not have allowed for normal sugar beet root development and may have prevented differences in varietal phenotypes being observed. There is therefore a trade-off between the convenience and practicality of smaller pot sizes and the closer to ‘normal’ root development permitted by larger pots. The decision to switch to 11cm diameter, 13.5cm tall pots in the subsequent studies described here, reflects this trade-off.

Another factor which is likely to have prevented phenotypic differences between virus treatments and varieties being identified in the pilot study is time between inoculation and harvest. During the pilot study, the plants were harvested four weeks after inoculation. This is enough time for symptom expression to develop under field conditions (Hossain et al., 2019), but when combined with the small pot size may not have been long enough for any yield differences to be distinguished under controlled conditions. Despite this, growing plants for long periods after inoculation was not practical in terms of resource use and negates the potential time benefits of controlled environment phenotyping. As a result of this trade off, in subsequent studies, test plants were grown for eight weeks after inoculation.

Following the pilot study, as well as altering the pot size and experiment length, it was also decided to change growing medium. In the pilot study, standard compost was used. Compost is a very convenient medium for growing plants under controlled conditions. It is lightweight, easy to use (requiring no mixing of multiple component mediums), and normally consistent in composition and readily available. Compost is also known to be a suitable medium for growing sugar beet under controlled conditions, being used by the BBRO to grow sugar beet plants to maintain their virus yellows cultures (BBRO, private communication).

However, during the first two years of this project the quality and availability of compost was reduced. This was caused in part by supply issues arising from the Covid19 pandemic and due to

manufacturers altering their compost recipes in response to the upcoming UK ban on horticultural peat use (Department for Environment, Natural England and The Rt Hon Lord Benyon, 2022). One example of these quality issues was demonstrated when growing sugar beet plants to maintain the virus cultures described in Chapter 2 section 2.1. As shown in Figure 4.28 multiple fungi and their fruiting bodies emerged from the compost (Premium All Purpose, Miracle-Gro), and as a result the seedlings showed reduced vigour.



Figure 4.28: images of fungal fruiting bodies which emerged from commercial compost (Premium All Purpose, Miracle-Gro) used to maintain virus cultures. Mushrooms shown here were kindly identified by Dr Tony Leech (the Norfolk county fungi recorder) in consultation with Kew Gardens as *Leucocoprinus straminellus*, this is the first recording of this species in East Anglia.

Aside from the issues described above, the other key issue with using compost as a growing medium is it is difficult to accurately control the available nutrients. Commercial compost such as that used in the pilot study described here, often has nutrients added (Evergreen garden care, 2023).



However, it is not possible to quantify the nutrients available to each test plant. Soil, or a soil-sand mix, is available without pre-mixed nutrients and therefore enables greater control of nutrients within the experimental design. It was owing to these issues, that the decision to switch to a 50:50 soil-sand growing medium was made following the pilot study.

Using a soil-sand mix avoided many of the problems associated with the compost growing medium, however it was not a perfect solution. The soil-sand medium lacked body, with little if any structure. Future studies may benefit from adding a courser sand or some organic matter to the soil-sand mix to improve the overall soil structure and drainage.

Following these changes in methodology, promising results were obtained with the uninoculated treatments producing significantly higher root yields than the inoculated treatments in the glasshouse study. Although not significant, the trend seen between the different virus treatments in the glasshouse trial was also consistent with the literature; BChV infection causing a smaller yield loss than BMV infection (Stevens et al., 2004). When assessing the performance of each variety under virus infection, of the two high resistance varieties KWS-1 which was identified as having a lower virus titre in both the glasshouse and LED studies compared to KWS-2, yielded higher. However, overall, there was a high level of variation in root yields across the glasshouse trial.

Lower variation was seen in root yield results from the LED study, and overall larger root weights were achieved. As in the glasshouse study no significant differences in how the varieties responded to the different virus treatments was identified. This suggests that the high resistant varieties are able to overcome the effects of both BMV and BChV infection. Under virus infection the lowest mean root yields were seen in KWS-1 and KWS-2, despite these varieties having high resistance. Although initially these results seem contrary to expectation (resistant varieties suffer lower yield losses than susceptible), it may be explained by the yield drag seen between novel resistant varieties and susceptible varieties which may be elite yielding varieties. As such, although the susceptible varieties suffer a greater yield loss, they may still outperform the lower yielding resistant varieties. This is analysed further in Chapter 6.

#### 4.5.3 Symptom development

None of the growth environments trialled here produced the typical yellowing symptoms of beet polerovirus infection consistently. The glasshouse proved the most promising controlled environment for phenotyping based on foliar symptoms, however as all treatments including the uninoculated showed some degree of pale leaf yellowing it was not possible to quantify these results. This pale yellowing could have many causes, including too low nutrient levels, watering regime or a possible stress response due to fluctuating temperatures within the glasshouse.

The LED environment provided much more consistent temperatures and permits experiments to be repeated under identical growth conditions. However, in the LED study plants across all varieties and treatments showed an unusual growth habit with canopy cover decreasing sharply six weeks after inoculation, explained by the onset of severe leaf rolling. This issue would need addressing if the controlled environment was to become a viable alternative to field trials. One explanation for the leaf rolling could be light stress. The light levels recorded during this experiment averaged  $539\mu\text{mol m}^{-2}\text{s}^{-1}$ , significantly higher than that of the MLR chamber ( $258\mu\text{mol m}^{-2}\text{s}^{-1}$ ).

Despite the unusual phenotype, only in the LED study was the canopy stunting effect of BMV and BChV infection observed, with the inoculated treatments exhibiting smaller canopies (both in terms of area and dry weight) compared to the uninoculated. Although differences between the high resistance and susceptible varieties were not detected, adjusting the light intensity and achieving a closer to field-like growth habit may allow for such differences to be found.

# Chapter 5: Characterising genetic variation between virus yellows isolates

## 5.1 Chapter Contributions

The sequence data further analysed here, were produced from the leaf samples collected as part of the work described in Chapter 3 by a combination of the BBRO network, British Sugar and Belchim Crop Protection. The consensus sequences were produced by Rocky Payet. All other data analysis (including sequence alignment and identification of changes to encoded amino acids) I completed myself.

## 5.2 Introduction

Recombination has been identified as a key driver behind polerovirus evolution (LaTourrette et al., 2021; Pagán and Holmes, 2010). This process, whereby the genetic material of two separate virus genomes is exchanged and combined giving rise to a new distinct virus (Cann, 2012a), has occurred frequently in the evolution of the polerovirus family (Pagán and Holmes, 2010). Genetic variation also arises thanks to the error prone nature of the RNA-dependent RNA-polymerase (RdRp). These mutations can be in the form of insertions, deletions or substitutions and, as with all genetic changes, selection pressure will then act determining if the mutation is maintained or removed (LaTourrette et al., 2021).

In the more extreme cases, the genetic variation arising from these events can give rise to viruses which meet the International Committee on Taxonomy of Viruses classification for a new polerovirus species (see Chapter 1 section 1.4.5). Examples of this include *Pepo aphid-borne yellows virus* (Ibaba et al., 2017) and *Pepper yellow leaf curl virus* (Dombrovsky et al., 2013). However, even when the genetic changes are not great enough to result in a new virus species, they can still affect viral properties particularly because of the overlapping nature of polerovirus genomes. This means that a single nucleotide mutation could result in alterations to the encoded amino acid in multiple proteins (LaTourrette et al., 2021), with possible consequences in virus pathogenicity.

As discussed in the previous chapter, assessment of sugar beet varieties' response to virus infection is predominantly undertaken via inoculated trials. The BBRO BMV culture has been widely used by sugar beet seed breeders, such as KWS, to develop BMV-resistant varieties. This virus isolate was brought into culture in 2013 and has been maintained in continuous culture, predominantly

within controlled glasshouse conditions or MLR-352 (Panasonic) growth chambers, ever since. In May 2020 a BChV culture was established at the BBRO and maintained within a Panasonic-MLR chamber. This culture has been used to challenge novel virus yellows resistant varieties.

Crucially, neither the BMV nor BChV culture have been sequenced and their current similarity to 'wild' virus isolates is unknown. As both cultures are now being used to test sugar beet varieties, ensuring that they are representative of the virus isolates likely to infect commercial sugar beet crops in the field is vital. If the BBRO cultures were to exhibit genetic differences there is a risk that varieties developed to overcome the BBRO BMV isolate, for example, may not stand up against 'wild' BMV infection. The rate of genetic change within the BMV or BChV cultures is also unknown. This has potential consequences when comparing trial data conducted across multiple years, particularly if the pathogenicity of the virus is changing.

More generally, since the introduction of neonicotinoid seed treatments in the 1990s limited, if any, research has been conducted into the genetic variation within beet polerovirus species. It is therefore not known whether the virus populations are largely homogeneous, or if differing, dominant genotypes are present. This could have important effects on virus-resistant varieties, particularly if isolates are present with resistance-breaking characteristics. Intra-species variation also has consequences on the reliability of current diagnostics, especially those deploying molecular techniques. If the target regions of primer-based assays are variable within virus populations, infection may be going undetected with symptoms instead being attributed to another cause of sugar beet leaf yellowing (see Chapter 1 section 1.2.3.1).

In the research presented here, I have further interrogated the sequencing data generated in Chapter 3 to elucidate the genetic variation within BMV and BChV populations within this study. As part of the virus prevalence study outlined in Chapter 3, the BBRO BMV and BChV cultures were sequenced across multiple years to act as positive control small RNA (sRNA) libraries. This also allows us to compare the changes in these data between years and gain an understanding of the impact of continuous culture on these virus isolates.

## 5.3 Materials and Methods

### 5.3.1 Sample collection and sequencing

The sequencing data, generated from the symptomatic sugar, fodder and sea beet leaves collected in the virus prevalence survey (reported in Chapter 3), were analysed further to identify genetic

variation between isolates. The locations of sequenced samples are shown in Figure 5.1, whilst additional sampling details are listed in appendix B.

The percentage of target genome present in a sRNA library sample was calculated using the software Integrative Genomics Viewer (IGV) as described in Chapter 3 section 3.3.5.2. Samples were then filtered to include only those containing more than 80% of the target virus genome.

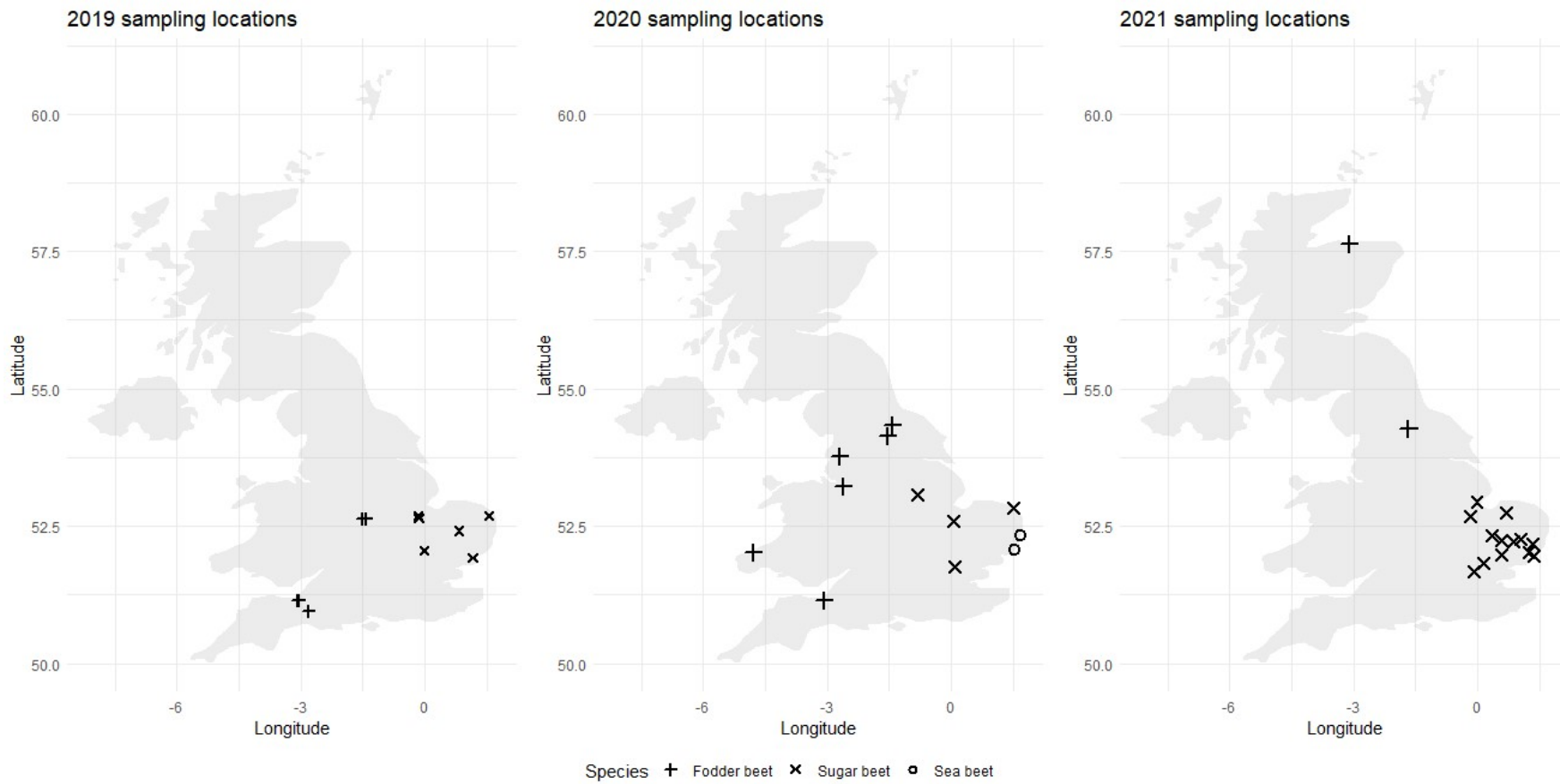


Figure 5.1: sampling locations of virus yellows symptomatic sugar, fodder and sea beet leaves collected between August and October 2019 – 2021, from which small RNA libraries were produced and next generation sequencing data analysed.

### 5.3.2 BCFtools consensus sequences

Consensus sequences for each sample were produced by Rocky Payet (UEA), using the variant-calling tool BCFtools (Li, 2011). The sample consensus sequences produced via BCFtools are based on a reference genome which is adjusted according to the input sequence data. When no mapping data is available the reference genome is defaulted to. Hence, using only samples known to contain more than 80% of the target genome reduced the risk of sample homogeneity arising because of the bioinformatic analysis methodology.

The NCBI reference sequences for each virus were used as the reference genomes in this analysis as detailed in Table 5.1.

*Table 5.1: National Center for Biotechnology Information (NCBI) reference genomes for Beet chlorosis virus, Beet mild yellowing virus and Beet yellows virus from which variation was called by BCFtools to the sample consensus sequences.*

<b>Virus</b>	<b>GenBank accession number</b>	<b>Origin location</b>	<b>Reference</b>
Beet chlorosis virus	NC_002766.1	East Anglia, England	Hauser et al., 2002
Beet mild yellowing virus	NC_003491.1	France	Guilley et al., 1995
Beet yellows virus	NC_001598.1	Ukraine	Agranovsky et al., 1994

### 5.3.3 Sequence alignment and percentage identity

The resultant sample consensus sequences were aligned, and the percentage identity determined, using ClustalOmega multiple sequence alignment tool (Sievers et al., 2011). When only two samples were compared, pairwise identity was determined using EMBOSS Needle (Needleman and Wunsch, 1970).

### 5.3.4 Locations of nucleotide differences and predictions of amino acid changes

Additional analysis to identify the open reading frames (ORFs) affected by the nucleotide variation was conducted on the samples which were brought into culture and included in the field trials described in Chapter 6. These samples were: BMV isolate 21-254, BChV isolates 21-024, 21-278 and 21-290, and the BBRO cultures of BMV and BChV as sequenced in 2021.

ORF locations were determined based on genome annotations of (Hauser et al., 2002) for BChV and (Guilley et al., 1995) for BMV. The effect of the nucleotide variation on the amino acid encoded, by the affected triplet codon, was then predicted for each ORF.

### 5.3.5 Sequence alignment to current diagnostics

Any genetic variability occurring in the target sites of the qPCR diagnostic assays used within this thesis was also determined, namely the DSMZ qPCR assay used on leaf tissue (detailed in Chapter 2, section 2.5) and the Rothamsted Research assay used to detect viruliferous aphids (detailed in Chapter 2, section 2.6). Nucleotide variation in the primer/probe binding sites was determined via alignment of all generated sample consensus sequences with the ClustalOmega multiple sequence tool (Sievers et al., 2011).

## 5.4 Results

### 5.4.1 BMV

Overall genetic diversity between sequenced BMV genomes within this study was low. The nucleic acid pairwise identity between isolates ranged from 99.49 to 99.91% (Figure 5.2). The highest divergence was seen in the BBRO BMV isolate sequenced in 2021 (BBRO-BMV21), which had a pairwise identity of 99.49 to 99.60% when compared to the other BMV isolates. Notably this high divergence was not observed in the BBRO BMV isolate sequenced in 2019 or 2020, indicating the isolate may have become more distinct from the collected isolates over time. Isolate 21-254 also had a relatively high level of divergence, with pairwise identity ranging from 99.56 and 99.76%. The closest related isolates, based on nucleic acid sequence, were 20-009, 20-014 and 21-056 all sharing 99.91% sequence identity.

Geographic location and host plant had limited effect on genetic similarity. Comparing isolates collected in the same year, 20-009 and 20-010 originated from fodder beet fields approximately 22km apart and shared 99.79% nucleic acid identity. In comparison, isolates 21-056 (from sugar beet) and 21-294 (from fodder beet) were separated by approximately 317km yet shared 99.88% identity.



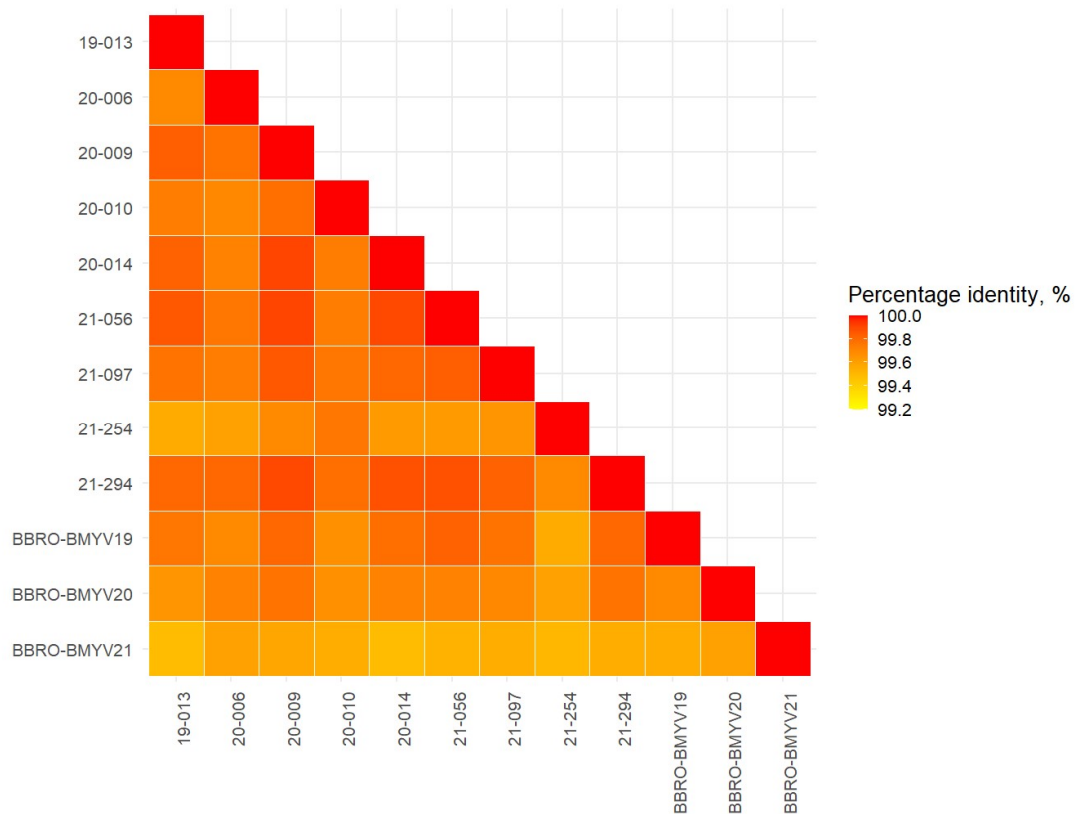


Figure 5.2: pairwise identity matrix depicting the whole genome nucleotide identity between Beet mild yellowing virus (BMV) isolates. Full descriptions of the isolate collection details are given in appendix B. The 2019, 2020 and 2021 sequences of the BBRO BMV isolate are included in the matrix (BBRO-BMYV19, BBRO-BMYV20 and BBRO-BMYV21 respectively).

As previously discussed, comparison of the BBRO BMV isolate sequences produced in 2019, 2020 and 2021 showed genetic variation over time. Between the 2019 and 2020 sequencing, eighteen nucleotide changes were identified. Seven of these changes were predicted to alter the encoded amino acid in at least one ORF (Figure 5.3). ORF0 and ORF4 each had two predicted amino acid changes, whilst ORF1, 3 and 5 each had one amino acid change. Greater variation was found between 2020 and 2021, with a total of 23 differing nucleotides identified. Nine of these nucleotide changes were predicted to alter the encoded amino acid; four changes to ORF2, two changes in ORF0 and ORF4 and one change in ORF1.

The majority of nucleotide changes which were predicted to be synonymous were identified in ORF1 and 2 (Figure 5.4). No nucleotide changes were identified in the intergenic region between ORF2 and 3.

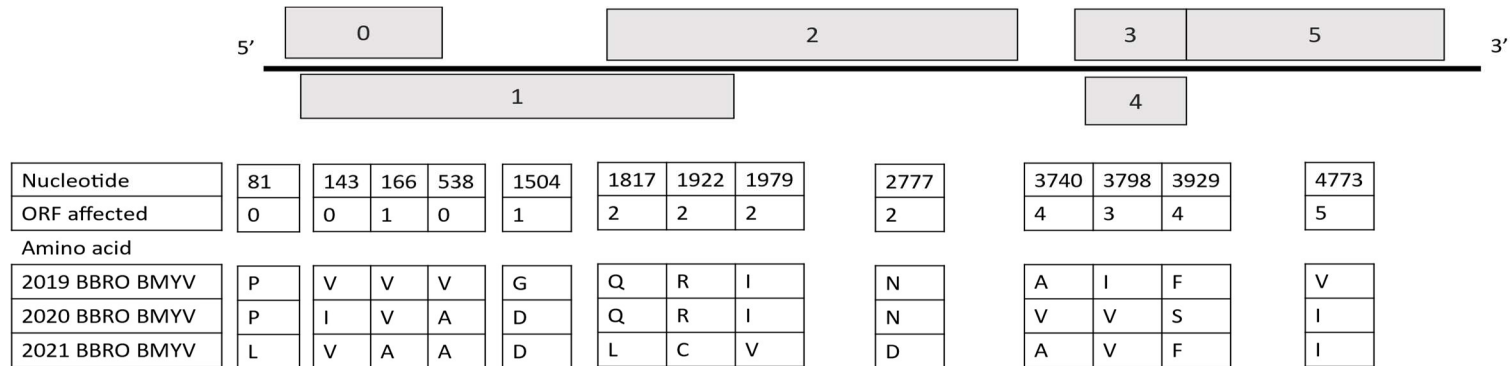


Figure 5.3: schematic diagram showing the organisation of the Beet mild yellowing virus (BMV) open reading frames (ORFs) and the predicted effect of nucleotide changes in the BBRO BMV isolate between 2019 and 2021 on the encoded amino acid.

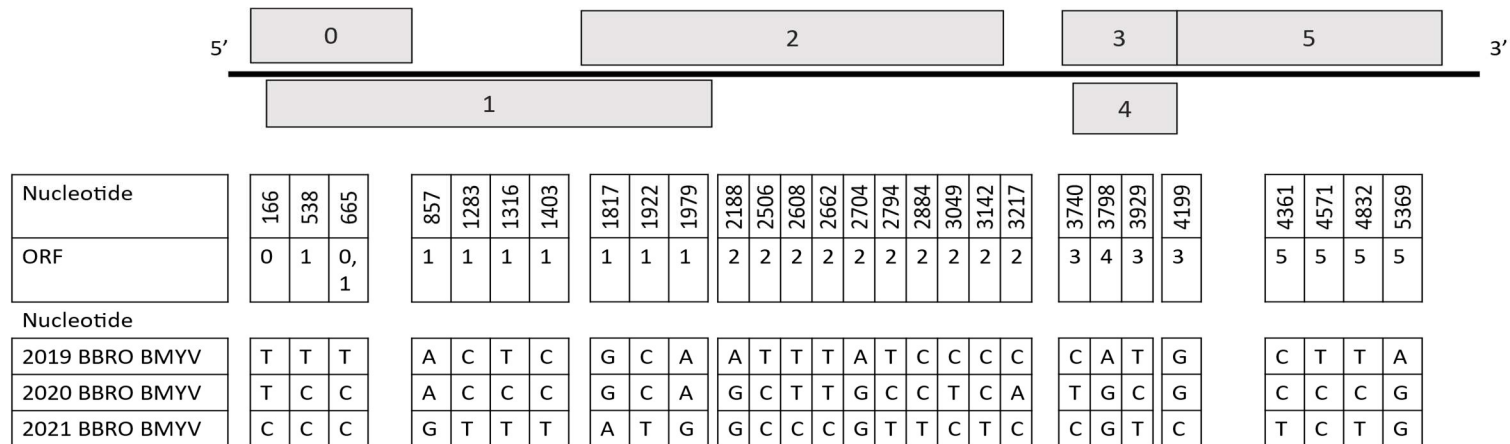


Figure 5.4: schematic diagram showing the organisation of the Beet mild yellowing virus (BMV) open reading frames (ORFs) and the nucleotide changes in the BBRO BMV isolate between 2019 and 2021 predicted to not alter the encoded amino acid.

#### 5.4.2 BMVYV included in field trial

The two cultured BMVYV isolates (BBRO BMVYV and 21-254), included in the field trial described in Chapter 6, differed at 28 nucleotide (nt) positions, with a pairwise identity of 99.51%. Further analysis of these nucleotide differences identified nine potential amino acid changes (Figure 5.5). Most of these changes affected the 5' coding region, with four amino acids differing in ORF 2. Three of these changes in ORF2 (nt1817, 1979 and 2777) along with the ORF0 change (nt81) were only seen in the BBRO BMVYV isolate and none of the other sequenced BMVYV isolates (data shown in appendix C). At the 3' end of the genome, only two amino acid differences were predicted between BBRO BMVYV and isolate 21-254; one change within ORF3 and one within ORF5. No amino acid changes were predicted within ORF4.

Nucleotide changes between isolate 21-254 and the BBRO BMVYV which were predicted not to result in an amino acid change (synonymous) were also identified (Figure 5.6). Again, these changes were more prevalent in the 5' coding region than the 3'. ORF1 contained the majority of these changes, with twelve differing nucleotides. Six synonymous changes were identified in ORF2, four in ORF3 and one change in each of ORF0, ORF3 and ORF4. No nucleotide changes were identified in the intergenic region between ORF2 and 3.

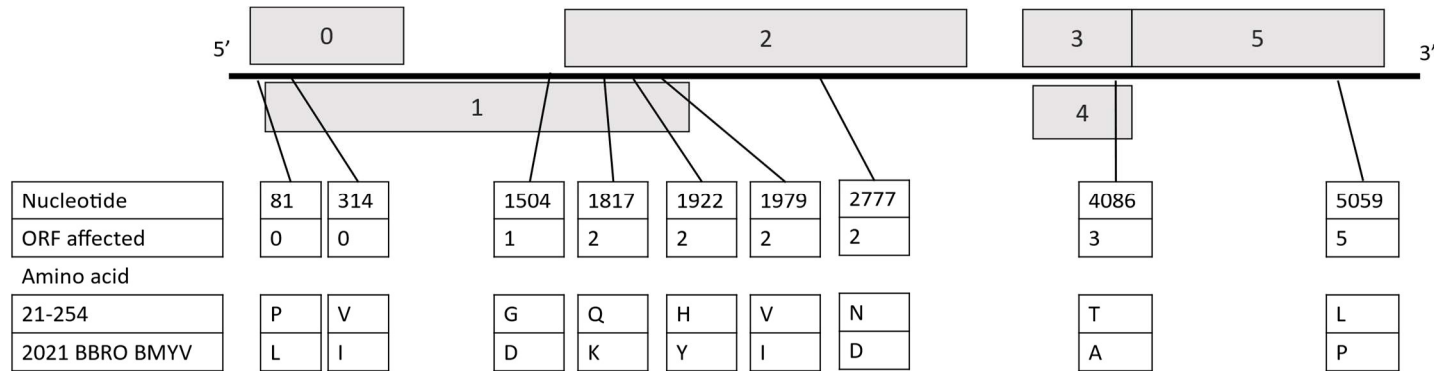


Figure 5.5: schematic diagram showing the organisation of the Beet mild yellowing virus (BMVYV) open reading frames (ORFs) and the predicted effect of nucleotide differences on the encoded amino acid, between the two BMVYV isolates included in the field trial discussed in Chapter 6 (isolate 21-254 and the BBRO BMVYV isolate as sequenced in 2021).

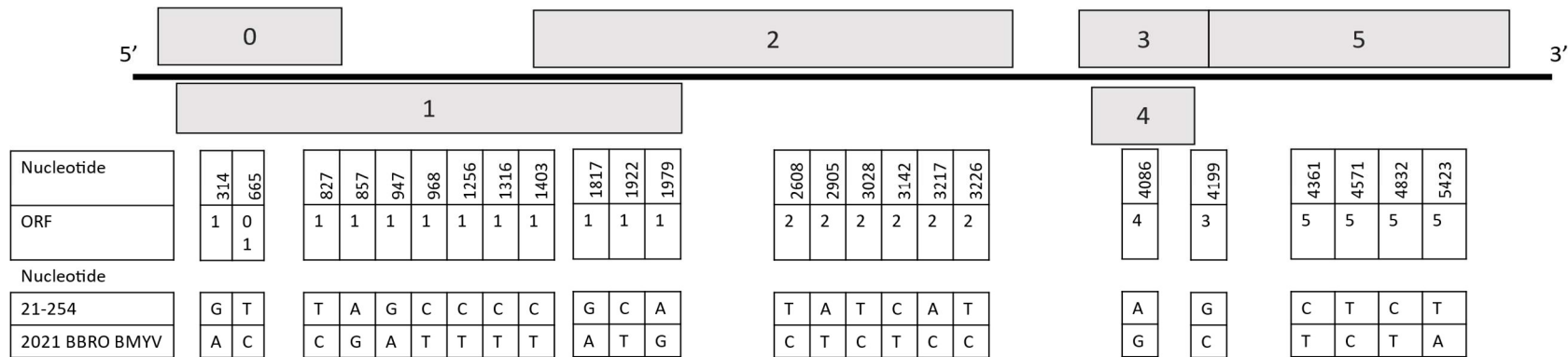


Figure 5.6: schematic diagram showing the organisation of the Beet mild yellowing virus (BMVYV) open reading frames (ORFs) and the nucleotide differences, predicted to not affect the encoded amino acid, between the two BMVYV isolates included in the field trial discussed in Chapter 6 (isolate 21-254 and the BBRO BMVYV isolate as sequenced in 2021).

### 5.4.3 BChV

Comparison of the BChV isolates showed a larger range of genetic diversity than seen between the BMV isolates, with the nucleic acid pairwise identity between isolates ranging from 99.27% to 100% (Figure 5.7). The highest divergence was seen in isolate 19-016 originating from fodder beet in Somerset, sharing only 99.53% identity with the most closely related isolate, BBRO BChV. Isolates 19-010, 20-017 and 20-018-1 shared identical nucleic acid sequences, however these sequences were also identical to the reference genome used by the variant-calling tool BCFtools.

Overall, based on the sequencing data from 2021, the BBRO BChV had a greater similarity to the collected BChV isolates than the BBRO BMV had to the BMV isolates, sharing between 99.53 and 99.84% sequence identity with the collected samples. Alignment of the 2020 and 2021 BBRO BChV sequences was broadly similar with no clear divergence of the BBRO BChV isolate away from the collected isolates.

As with the BMV sequences, geographic location and host plant had limited effect on genetic similarity. Isolate 19-016 shared only 99.48% identity with 19-015 despite both samples originating from neighbouring fodder beet fields. In comparison, despite being separated by approximately 265km and originating from different hosts, isolates 20-012 and 20-021 shared 99.84% identity. Unlike the BMV isolates, the BChV isolates did group loosely according to the year they were collected. However, this trend was not strong and was largely confined to the 2019 and 2020 isolates with the 2021 isolates showing more variation.

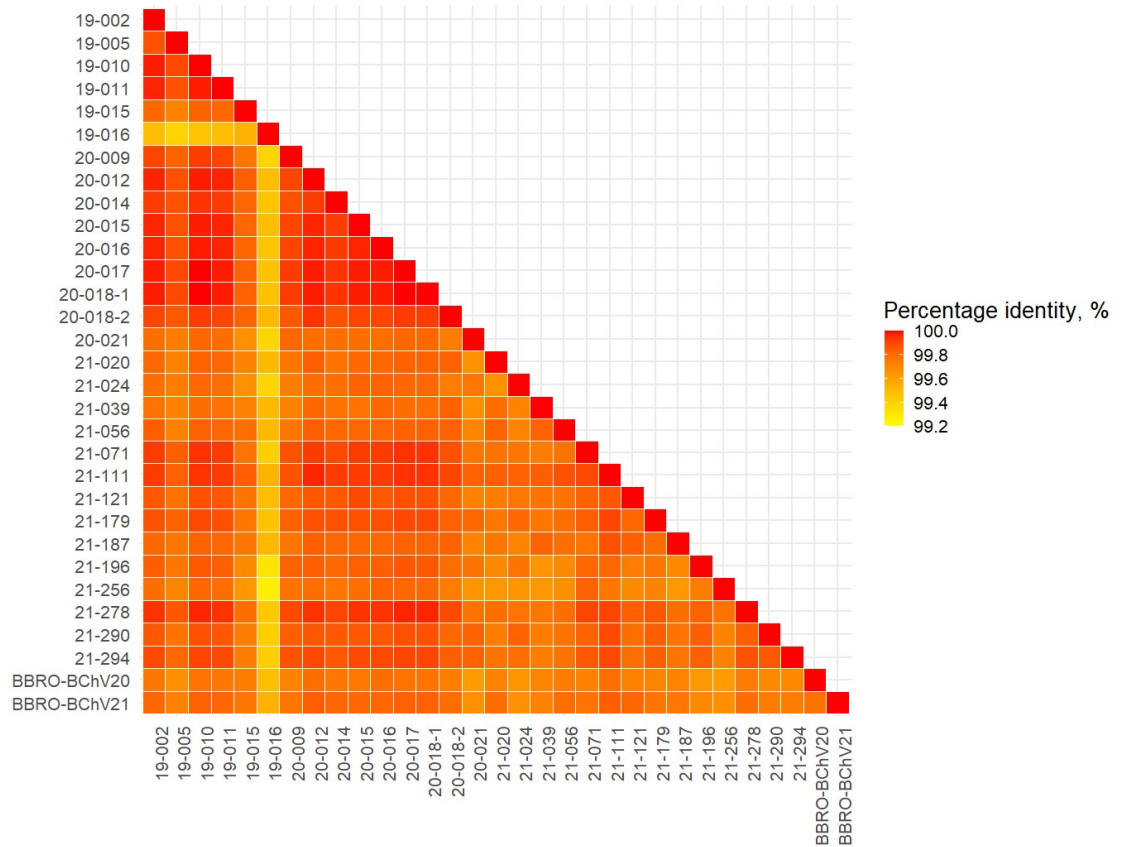


Figure 5.7: pairwise identity matrix depicting the whole genome nucleotide identity between Beet chlorosis virus (BChV) isolates. Full descriptions of the isolate collection details are given in appendix D. Both the 2020 and 2021 sequences of the BBRO BChV isolate are included in the matrix (BBRO-BChV20 and BBRO-BChV21 respectively).

The BBRO BChV isolate was sequenced in 2020 and 2021. Comparison of the 2020 and 2021 sequences showed 13 nucleotide changes, equating to a pairwise identity of 99.77%. All these nucleotides were located in the 5' proximal ORFs, with no changes detected in ORF3, 4, 5 or in the intergenic regions. Five nucleotide changes were predicted to alter the encoded amino acid in at least one ORF. Three of these changes occurred in ORF0, whilst two occurred in ORF2 (Figure 5.8). Six nucleotide changes which were predicted to be synonymous were identified in ORF1 and five in ORF2 (Figure 5.9).

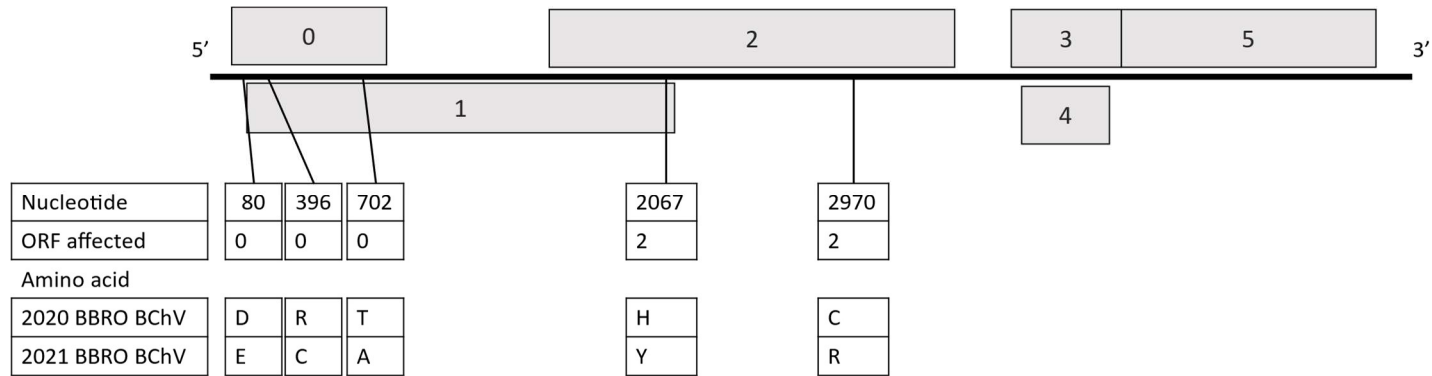


Figure 5.8: schematic diagram showing the organisation of the Beet chlorosis virus (BChV) open reading frames (ORFs) and the predicted effect of nucleotide changes in the BBRO BChV isolate between 2020 and 2021 on the encoded amino acid.

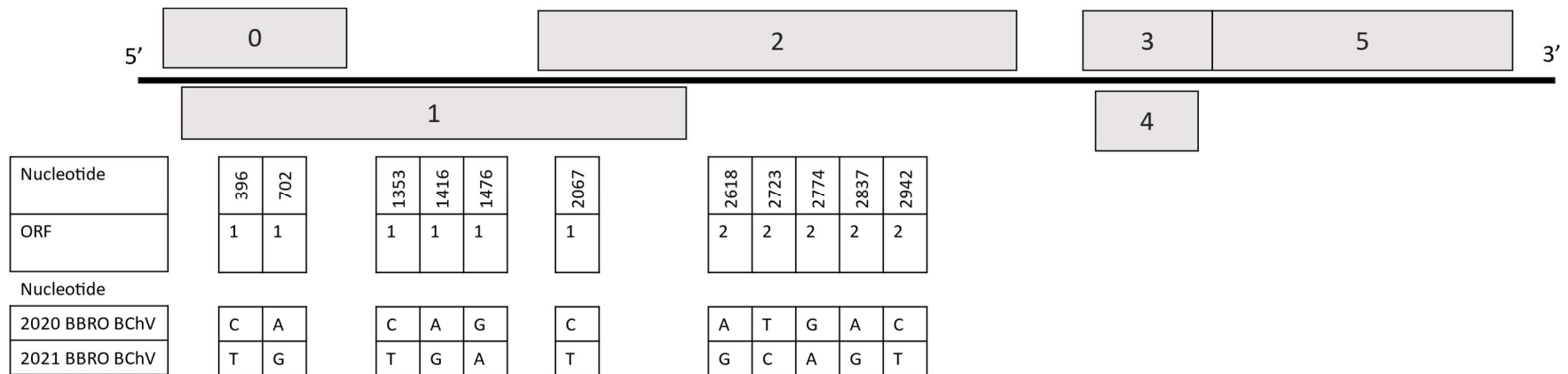


Figure 5.9: schematic diagram showing the organisation of the Beet chlorosis virus (BChV) open reading frames (ORFs) and the nucleotide changes in the BBRO BChV isolate between 2020 and 2021 predicted to not alter the encoded amino acid.

#### 5.4.4 BChV included in field trial

Of the three BChV isolates brought into culture, for the field trial described in Chapter 6, 21-278 was the most similar to BBRO BChV, with 99.79% pairwise identity. The other two BChV isolates, 21-024 and 21-290 each shared 99.67% and 99.74% identity with BBRO BChV respectively. Further analysis of these nucleotide differences identified potential amino acid differences between isolates (Figure 5.10). Three of these predicted changes were conserved across all three of the new BChV cultures but differed in BBRO BChV, suggesting a change in the BBRO BChV genome. These amino acid changes were located in ORF0, ORF2 and ORF5. These were the only amino acid changes predicted between isolate 21-278 and BBRO BChV, however additional amino acid changes were predicted between the other two new BChV cultures and BBRO BChV. Isolate 21-024 had the most potentially differing amino acids compared to BBRO BChV, with three additional changes affected ORF0, ORF1 and ORF2. Two additional amino acid changes were predicted between isolate 21-290 and BBRO BChV affecting ORF1 and ORF2.

Nucleotide changes between BBRO BChV and the newly cultured isolates which were predicted to be synonymous were also identified (Figure 5.11). Eight nucleotides were conserved across all three newly cultured BChV isolates but differed in BBRO BChV. Two of these changes were in ORF1, two in ORF2 and four in ORF5. In addition to these eight nucleotide changes, isolate 21-024 differed to BBRO BChV by a further nine nucleotides, isolate 21-290 by five nucleotides and isolate 21-278 by three nucleotides.

Like the BMV cultures, most nucleotide changes were found in the ORFs located at the 5' end of the genome. Genetic variation was also present in ORF5, but unlike the BMV cultures no nucleotide changes were identified in ORF3 or ORF4.



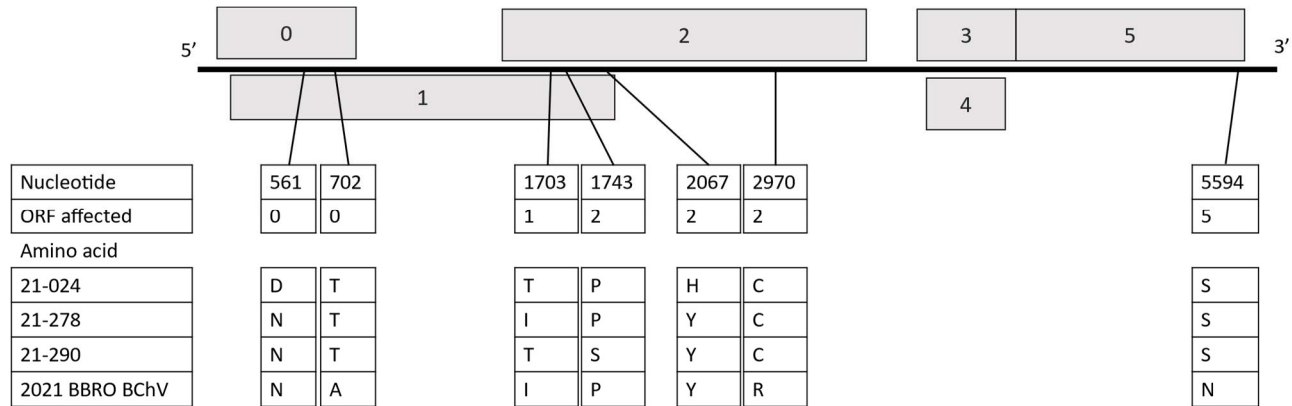


Figure 5.10: schematic diagram showing the organisation of the Beet chlorosis virus (BChV) open reading frames (ORFs) and the predicted effect of nucleotide differences on the encoded amino acid, between the four BChV isolates included in the field trial discussed in Chapter 6.

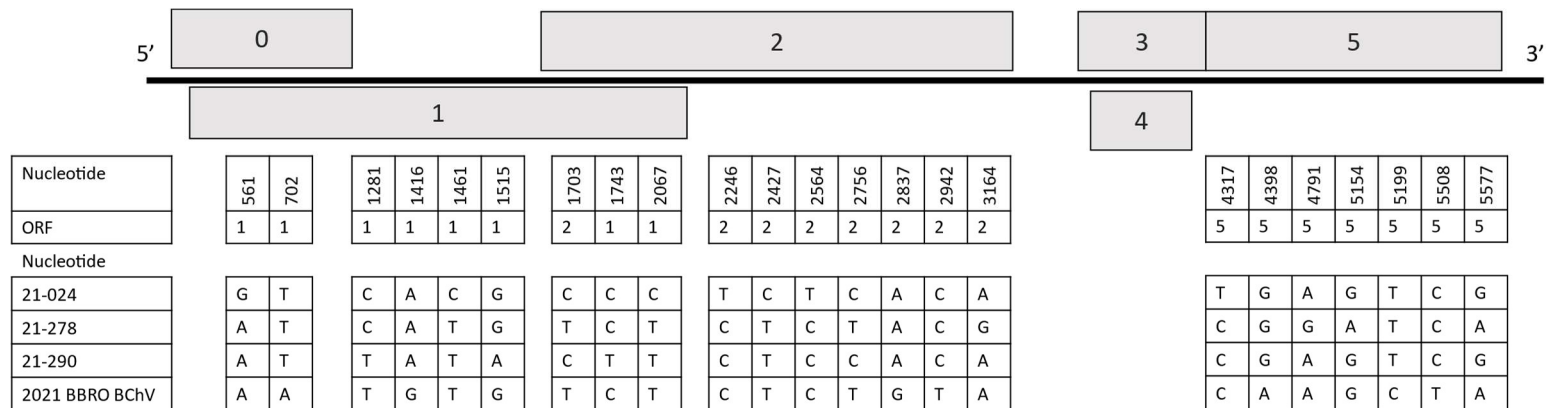


Figure 5.11: schematic diagram showing the organisation of the Beet chlorosis virus (BChV) open reading frames (ORFs) and the nucleotide differences (not predicted to alter the encoded amino acid) between the four BChV isolates included in the field trial discussed in Chapter 6.

#### 5.4.5 Influence of genetic diversity on reliability of current diagnostics

Sequence alignment of all the BMVYV isolates showed no nucleotide variation in the primer binding sites for the DSMZ BMVYV RT-qPCR assay (Figure 5.12). At the probe binding site, isolate 20-006 differed by one nucleotide whilst all other isolates showed 100% complementarity. The risk of cross-reactivity with the DSMZ BChV RT-qPCR assay was very low. Only mis-matching alignment of the primer sequences were identified and at sites too distant to produce a viable amplicon (forward primer at site 827-841nt, reverse primer at 5618-5636nt).

<b>a</b>			<b>b</b>		
BMVYV_F	---	GAGTGACGTACACATCTGAGAA---	BMVYV_R	---	TTGCTCTGTCGGATGGTGA---
19-013	TCAGAGT	GACGTACACATCTGAGAAAAG 1021	19-013	TCTTTGCT	CTGTCGGATGGTGAATG 1150
20-006	TCAGAGT	GACGTACACATCTGAGAAAAG 1021	20-006	TCTTTGCT	CTGTCGGATGGTGAATG 1150
20-009	TCAGAGT	GACGTACACATCTGAGAAAAG 1021	20-009	TCTTTGCT	CTGTCGGATGGTGAATG 1150
20-010	TCAGAGT	GACGTACACATCTGAGAAAAG 1021	20-010	TCTTTGCT	CTGTCGGATGGTGAATG 1150
20-014	TCAGAGT	GACGTACACATCTGAGAAAAG 1021	20-014	TCTTTGCT	CTGTCGGATGGTGAATG 1150
21-056	TCAGAGT	GACGTACACATCTGAGAAAAG 1021	21-056	TCTTTGCT	CTGTCGGATGGTGAATG 1150
21-097	TCAGAGT	GACGTACACATCTGAGAAAAG 1021	21-097	TCTTTGCT	CTGTCGGATGGTGAATG 1150
21-254	TCAGAGT	GACGTACACATCTGAGAAAAG 1021	21-254	TCTTTGCT	CTGTCGGATGGTGAATG 1150
21-294	TCAGAGT	GACGTACACATCTGAGAAAAG 1021	21-294	TCTTTGCT	CTGTCGGATGGTGAATG 1150
BBRO-BMYV21	TCAGAGT	GACGTACACATCTGAGAAAAG 1021	BBRO-BMYV21	TCTTTGCT	CTGTCGGATGGTGAATG 1150
		*****			*****
<b>c</b>					
BMVYV_probe	---	CCAAACTGGGAAGGAGTTCTTGC---			
19-013	CCCCCAA	ACTGGGAAGGAGTTCTTGCCTG 1072			
20-006	CCCCCAA	ACTGGGAAGGAGTTCTTGCCTG 1072			
20-009	CCCCCAA	ACTGGGAAGGAGTTCTTGCCTG 1072			
20-010	CCCCCAA	ACTGGGAAGGAGTTCTTGCCTG 1072			
20-014	CCCCCAA	ACTGGGAAGGAGTTCTTGCCTG 1072			
21-056	CCCCCAA	ACTGGGAAGGAGTTCTTGCCTG 1072			
21-097	CCCCCAA	ACTGGGAAGGAGTTCTTGCCTG 1072			
21-254	CCCCCAA	ACTGGGAAGGAGTTCTTGCCTG 1072			
21-294	CCCCCAA	ACTGGGAAGGAGTTCTTGCCTG 1072			
BBRO-BMYV21	CCCCCAA	ACTGGGAAGGAGTTCTTGCCTG 1072			
		*****			

Figure 5.12: DSMZ Beet mild yellowing virus (BMVYV) reverse transcription-qPCR assay alignment with the produced BMVYV isolate sequences. a – BMVYV forward primer, b – BMVYV reverse primer (shown in reverse compliment), c – BMVYV probe.

Similarly, no nucleotide variation was seen amongst the BChV isolate sequences at the DSMZ BChV primer binding site locations (Figure 5.13). All isolates showed exact complementarity to the BChV probe, except for isolate 19-016 which varied by one nucleotide. No viable binding sites for the DSMZ BMVYV primers or probe were identified in the BChV sequences.

<b>a</b>	<pre> BChV_F      ---AGTCATGACGACGGTTCACA--- 19_005      ATAAGTCATGACGACGGTTCACACGC 881 19_010      ATAAGTCATGACGACGGTTCACACGC 881 19_011      ATAAGTCATGACGACGGTTCACACGC 881 19_015      ATAAGTCATGACGACGGTTCACACGC 881 19_016      ATAAGTCATGACGACGGTTCACACGC 881 19-002      ATAAGTCATGACGACGGTTCACACGC 881 20-009      ATAAGTCATGACGACGGTTCACACGC 881 20-012      ATAAGTCATGACGACGGTTCACACGC 881 20-014      ATAAGTCATGACGACGGTTCACACGC 881 20-015      ATAAGTCATGACGACGGTTCACACGC 881 20-016      ATAAGTCATGACGACGGTTCACACGC 881 20-017      ATAAGTCATGACGACGGTTCACACGC 881 20-018-1    ATAAGTCATGACGACGGTTCACACGC 881 20-018-2    ATAAGTCATGACGACGGTTCACACGC 881 20-021      ATAAGTCATGACGACGGTTCACACGC 881 21_024      ATAAGTCATGACGACGGTTCACACGC 881 21_278      ATAAGTCATGACGACGGTTCACACGC 881 21_290      ATAAGTCATGACGACGGTTCACACGC 881 21-020      ATAAGTCATGACGACGGTTCACACGC 881 21-039      ATAAGTCATGACGACGGTTCACACGC 881 21-056      ATAAGTCATGACGACGGTTCACACGC 881 21-071      ATAAGTCATGACGACGGTTCACACGC 881 21-111      ATAAGTCATGACGACGGTTCACACGC 881 21-121      ATAAGTCATGACGACGGTTCACACGC 881 21-179      ATAAGTCATGACGACGGTTCACACGC 881 21-187      ATAAGTCATGACGACGGTTCACACGC 881 21-196      ATAAGTCATGACGACGGTTCACACGC 881 21-256      ATAAGTCATGACGACGGTTCACACGC 881 21-294      ATAAGTCATGACGACGGTTCACACGC 881 BBRO-BChV21 ATAAGTCATGACGACGGTTCACACGC 881 ***** </pre>	<b>b</b>	<pre> BChV_probe  ---TGATAACCTGCTCGCATGCTCC--- 19_005      GTTTGATAACCTGCTCGCATGCTCCCAT 959 19_010      GTTTGATAACCTGCTCGCATGCTCCCAT 959 19_011      GTTTGATAACCTGCTCGCATGCTCCCAT 959 19_015      GTTTGATAACCTGCTCGCATGCTCCCAT 959 19_016      GTTTGATAACCTGCTCGCATGCTCCCAT 959 19-002      GTTTGATAACCTGCTCGCATGCTCCCAT 959 20-009      GTTTGATAACCTGCTCGCATGCTCCCAT 959 20-012      GTTTGATAACCTGCTCGCATGCTCCCAT 959 20-014      GTTTGATAACCTGCTCGCATGCTCCCAT 959 20-015      GTTTGATAACCTGCTCGCATGCTCCCAT 959 20-016      GTTTGATAACCTGCTCGCATGCTCCCAT 959 20-017      GTTTGATAACCTGCTCGCATGCTCCCAT 959 20-018-1    GTTTGATAACCTGCTCGCATGCTCCCAT 959 20-018-2    GTTTGATAACCTGCTCGCATGCTCCCAT 959 20-021      GTTTGATAACCTGCTCGCATGCTCCCAT 959 21_024      GTTTGATAACCTGCTCGCATGCTCCCAT 959 21_278      GTTTGATAACCTGCTCGCATGCTCCCAT 959 21_290      GTTTGATAACCTGCTCGCATGCTCCCAT 959 21-020      GTTTGATAACCTGCTCGCATGCTCCCAT 959 21-039      GTTTGATAACCTGCTCGCATGCTCCCAT 959 21-056      GTTTGATAACCTGCTCGCATGCTCCCAT 959 21-071      GTTTGATAACCTGCTCGCATGCTCCCAT 959 21-111      GTTTGATAACCTGCTCGCATGCTCCCAT 959 21-121      GTTTGATAACCTGCTCGCATGCTCCCAT 959 21-179      GTTTGATAACCTGCTCGCATGCTCCCAT 959 21-187      GTTTGATAACCTGCTCGCATGCTCCCAT 959 21-196      GTTTGATAACCTGCTCGCATGCTCCCAT 959 21-256      GTTTGATAACCTGCTCGCATGCTCCCAT 959 21-294      GTTTGATAACCTGCTCGCATGCTCCCAT 959 BBRO-BChV21 GTTTGATAACCTGCTCGCATGCTCCCAT 959 ***** </pre>	<b>c</b>	<pre> BChV_R      ---TCTCAACCGTCACTGGCAA--- 1015 19_005      TTTTCTCAACCGTCACTGGCAATAA 1015 19_010      TTTTCTCAACCGTCACTGGCAATAA 1015 19_011      TTTTCTCAACCGTCACTGGCAATAA 1015 19_015      TTTTCTCAACCGTCACTGGCAATAA 1015 19_016      TTTTCTCAACCGTCACTGGCAATAA 1015 19-002      TTTTCTCAACCGTCACTGGCAATAA 1015 20-009      TTTTCTCAACCGTCACTGGCAATAA 1015 20-012      TTTTCTCAACCGTCACTGGCAATAA 1015 20-014      TTTTCTCAACCGTCACTGGCAATAA 1015 20-015      TTTTCTCAACCGTCACTGGCAATAA 1015 20-016      TTTTCTCAACCGTCACTGGCAATAA 1015 20-017      TTTTCTCAACCGTCACTGGCAATAA 1015 20-018-1    TTTTCTCAACCGTCACTGGCAATAA 1015 20-018-2    TTTTCTCAACCGTCACTGGCAATAA 1015 20-021      TTTTCTCAACCGTCACTGGCAATAA 1015 21_024      TTTTCTCAACCGTCACTGGCAATAA 1015 21_278      TTTTCTCAACCGTCACTGGCAATAA 1015 21_290      TTTTCTCAACCGTCACTGGCAATAA 1015 21-020      TTTTCTCAACCGTCACTGGCAATAA 1015 21-039      TTTTCTCAACCGTCACTGGCAATAA 1015 21-056      TTTTCTCAACCGTCACTGGCAATAA 1015 21-071      TTTTCTCAACCGTCACTGGCAATAA 1015 21-111      TTTTCTCAACCGTCACTGGCAATAA 1015 21-121      TTTTCTCAACCGTCACTGGCAATAA 1015 21-179      TTTTCTCAACCGTCACTGGCAATAA 1015 21-187      TTTTCTCAACCGTCACTGGCAATAA 1015 21-196      TTTTCTCAACCGTCACTGGCAATAA 1015 21-256      TTTTCTCAACCGTCACTGGCAATAA 1015 21-294      TTTTCTCAACCGTCACTGGCAATAA 1015 BBRO-BChV21 TTTTCTCAACCGTCACTGGCAATAA 1015 ***** </pre>
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Figure 5.13: DSMZ Beet chlorosis virus (BChV) reverse transcription-qPCR assay alignment with the produced BChV isolate sequences. a – BChV forward primer, b – BChV probe, c – BChV reverse primer (shown in reverse complement).

Aligning the BMV isolate sequences with the Rothamsted Research aphid RT-qPCR primers, identified one nucleotide mismatch in the reverse transcription primer and two nucleotide mismatches in the qPCR forward primer, (Figure 5.14). All three of these sites were conserved amongst the BMV sequences. The qPCR reverse primer and qPCR probe had exact complementarity to all BMV sequences with no sequence variation identified at these genome regions.

Alignment to the BChV sequences showed no variation in the qPCR primer or probe binding sites, with the primers and probes binding with exact complementarity. The same nucleotide mismatch that was identified in the reverse transcription primer binding site to the BMV sequences was also present in the BChV sequences (Figure 5.15).

<b>a</b>		<b>b</b>	
VR2	---CC <b>r</b> CTACAAAGGCAATGGTTC---	Pol_F	---CGTT <b>T</b> ACAGCGTCTTACATCAACG---
19-013	GATCCTCTACAAAGGCAATGGTTC 4086	19-013	GGGCATTTCAGCGTCTTACATCAACGGGG 4086
20-006	GATCCTCTACAAAGGCAATGGTTC 4086	20-006	GGGCATTTCAGCGTCTTACATCAACGGGG 4086
20-009	GATCCTCTACAAAGGCAATGGTTC 4086	20-009	GGGCATTTCAGCGTCTTACATCAACGGGG 4086
20-010	GATCCTCTACAAAGGCAATGGTTC 4086	20-010	GGGCATTTCAGCGTCTTACATCAACGGGG 4086
20-014	GATCCTCTACAAAGGCAATGGTTC 4086	20-014	GGGCATTTCAGCGTCTTACATCAACGGGG 4086
21-056	GATCCTCTACAAAGGCAATGGTTC 4086	21-056	GGGCATTTCAGCGTCTTACATCAACGGGG 4086
21-097	GATCCTCTACAAAGGCAATGGTTC 4086	21-097	GGGCATTTCAGCGTCTTACATCAACGGGG 4086
21-254	GATCCTCTACAAAGGCAATGGTTC 4086	21-254	GGGCATTTCAGCGTCTTACATCAACGGGA 4086
21-294	GATCCTCTACAAAGGCAATGGTTC 4086	21-294	GGGCATTTCAGCGTCTTACATCAACGGGG 4086
BBRO-BMYV21	GATCCTCTACAAAGGCAATGGTTC 4086	BBRO-BMYV21	GGGCATTTCAGCGTCTTACATCAACGGGG 4086
	*****		**** **
<b>c</b>		<b>d</b>	
Pol_R	---AGGATCCTCTACAAAGGCAATGG---	Pol_probe	---CGTTGCCaAGGACCA---
19-013	TTCAGGATCCTCTACAAAGGCAATGGTTC 4144	19-013	TGACGTTGCCAAGGACCAATT 4017
20-006	TTCAGGATCCTCTACAAAGGCAATGGTTC 4144	20-006	TGACGTTGCCAAGGACCAATT 4017
20-009	TTCAGGATCCTCTACAAAGGCAATGGTTC 4144	20-009	TGACGTTGCCAAGGACCAATT 4017
20-010	TTCAGGATCCTCTACAAAGGCAATGGTTC 4144	20-010	TGACGTTGCCAAGGACCAATT 4017
20-014	TTCAGGATCCTCTACAAAGGCAATGGTTC 4144	20-014	TGACGTTGCCAAGGACCAATT 4017
21-056	TTCAGGATCCTCTACAAAGGCAATGGTTC 4144	21-056	TGACGTTGCCAAGGACCAATT 4017
21-097	TTCAGGATCCTCTACAAAGGCAATGGTTC 4144	21-097	TGACGTTGCCAAGGACCAATT 4017
21-254	TTCAGGATCCTCTACAAAGGCAATGGTTC 4144	21-254	TGACGTTGCCAAGGACCAATT 4017
21-294	TTCAGGATCCTCTACAAAGGCAATGGTTC 4144	21-294	TGACGTTGCCAAGGACCAATT 4017
BBRO-BMYV21	TTCAGGATCCTCTACAAAGGCAATGGTTC 4144	BBRO-BMYV21	TGACGTTGCCAAGGACCAATT 4017
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Figure 5.14: Rothamsted Research aphid reverse transcription-qPCR assay (which detects both Beet mild yellowing virus (BMV) and Beet chlorosis virus (BChV) aligned with the produced BMV isolate sequences. Bold letters indicate mismatching sites in the primer/probe where the sequence is conserved amongst the sequenced isolates. a – reverse transcription primer, VR2 (shown in reverse compliment), b – qPCR forward primer, Pol\_F, c - qPCR reverse primer, Pol\_r (shown in reverse compliment), d – qPCR probe, Pol\_probe (shown in reverse compliment)

a		b		c		d	
VR2	---CCCTCTACAAAGGCAATGGTCTTC---	Pol_F	---CGTTTACAGCGTCTTACATCAACGGG---	Pol_R	---AGGATCCTCTACAAAGGCAATGG---	Pol_probe	---COTTGCCaAGGACCA--- 4127
19_005	GATCCTCTACAAAGGCAATGGTCTTC 4157	19_005	GGGCGTTTACAGCGTCTTACATCAACGGGA 4096	19_005	TTCAGGATCCTCTACAAAGGCAATGGTTC 4154	19_005	TGACGTTGCCAAGGACCAATT 4127
19_010	GATCCTCTACAAAGGCAATGGTCTTC 4157	19_010	GGGCGTTTACAGCGTCTTACATCAACGGGA 4096	19_010	TTCAGGATCCTCTACAAAGGCAATGGTTC 4154	19_010	TGACGTTGCCAAGGACCAATT 4127
19_011	GATCCTCTACAAAGGCAATGGTCTTC 4157	19_011	GGGCGTTTACAGCGTCTTACATCAACGGGA 4096	19_011	TTCAGGATCCTCTACAAAGGCAATGGTTC 4154	19_011	TGACGTTGCCAAGGACCAATT 4127
19_015	GATCCTCTACAAAGGCAATGGTCTTC 4157	19_015	GGGCGTTTACAGCGTCTTACATCAACGGGA 4096	19_015	TTCAGGATCCTCTACAAAGGCAATGGTTC 4154	19_015	TGACGTTGCCAAGGACCAATT 4127
19_016	GATCCTCTACAAAGGCAATGGTCTTC 4157	19_016	GGGCGTTTACAGCGTCTTACATCAACGGGA 4096	19_016	TTCAGGATCCTCTACAAAGGCAATGGTTC 4154	19_016	TGACGTTGCCAAGGACCAATT 4127
19-002	GATCCTCTACAAAGGCAATGGTCTTC 4157	19-002	GGGCGTTTACAGCGTCTTACATCAACGGGA 4096	19-002	TTCAGGATCCTCTACAAAGGCAATGGTTC 4154	19-002	TGACGTTGCCAAGGACCAATT 4127
20-009	GATCCTCTACAAAGGCAATGGTCTTC 4157	20-009	GGGCGTTTACAGCGTCTTACATCAACGGGA 4096	20-009	TTCAGGATCCTCTACAAAGGCAATGGTTC 4154	20-009	TGACGTTGCCAAGGACCAATT 4127
20-012	GATCCTCTACAAAGGCAATGGTCTTC 4157	20-012	GGGCGTTTACAGCGTCTTACATCAACGGGA 4096	20-012	TTCAGGATCCTCTACAAAGGCAATGGTTC 4154	20-012	TGACGTTGCCAAGGACCAATT 4127
20-014	GATCCTCTACAAAGGCAATGGTCTTC 4157	20-014	GGGCGTTTACAGCGTCTTACATCAACGGGA 4096	20-014	TTCAGGATCCTCTACAAAGGCAATGGTTC 4154	20-014	TGACGTTGCCAAGGACCAATT 4127
20-015	GATCCTCTACAAAGGCAATGGTCTTC 4157	20-015	GGGCGTTTACAGCGTCTTACATCAACGGGA 4096	20-015	TTCAGGATCCTCTACAAAGGCAATGGTTC 4154	20-015	TGACGTTGCCAAGGACCAATT 4127
20-016	GATCCTCTACAAAGGCAATGGTCTTC 4157	20-016	GGGCGTTTACAGCGTCTTACATCAACGGGA 4096	20-016	TTCAGGATCCTCTACAAAGGCAATGGTTC 4154	20-016	TGACGTTGCCAAGGACCAATT 4127
20-017	GATCCTCTACAAAGGCAATGGTCTTC 4157	20-017	GGGCGTTTACAGCGTCTTACATCAACGGGA 4096	20-017	TTCAGGATCCTCTACAAAGGCAATGGTTC 4154	20-017	TGACGTTGCCAAGGACCAATT 4127
20-018-1	GATCCTCTACAAAGGCAATGGTCTTC 4157	20-018-1	GGGCGTTTACAGCGTCTTACATCAACGGGA 4096	20-018-1	TTCAGGATCCTCTACAAAGGCAATGGTTC 4154	20-018-1	TGACGTTGCCAAGGACCAATT 4127
20-018-2	GATCCTCTACAAAGGCAATGGTCTTC 4157	20-018-2	GGGCGTTTACAGCGTCTTACATCAACGGGA 4096	20-018-2	TTCAGGATCCTCTACAAAGGCAATGGTTC 4154	20-018-2	TGACGTTGCCAAGGACCAATT 4127
20-021	GATCCTCTACAAAGGCAATGGTCTTC 4157	20-021	GGGCGTTTACAGCGTCTTACATCAACGGGA 4096	20-021	TTCAGGATCCTCTACAAAGGCAATGGTTC 4154	20-021	TGACGTTGCCAAGGACCAATT 4127
21_024	GATCCTCTACAAAGGCAATGGTCTTC 4157	21_024	GGGCGTTTACAGCGTCTTACATCAACGGGA 4096	21_024	TTCAGGATCCTCTACAAAGGCAATGGTTC 4154	21_024	TGACGTTGCCAAGGACCAATT 4127
21_278	GATCCTCTACAAAGGCAATGGTCTTC 4157	21_278	GGGCGTTTACAGCGTCTTACATCAACGGGA 4096	21_278	TTCAGGATCCTCTACAAAGGCAATGGTTC 4154	21_278	TGACGTTGCCAAGGACCAATT 4127
21_290	GATCCTCTACAAAGGCAATGGTCTTC 4157	21_290	GGGCGTTTACAGCGTCTTACATCAACGGGA 4096	21_290	TTCAGGATCCTCTACAAAGGCAATGGTTC 4154	21_290	TGACGTTGCCAAGGACCAATT 4127
21-020	GATCCTCTACAAAGGCAATGGTCTTC 4157	21-020	GGGCGTTTACAGCGTCTTACATCAACGGGA 4096	21-020	TTCAGGATCCTCTACAAAGGCAATGGTTC 4154	21-020	TGACGTTGCCAAGGACCAATT 4127
21-039	GATCCTCTACAAAGGCAATGGTCTTC 4157	21-039	GGGCGTTTACAGCGTCTTACATCAACGGGA 4096	21-039	TTCAGGATCCTCTACAAAGGCAATGGTTC 4154	21-039	TGACGTTGCCAAGGACCAATT 4127
21-056	GATCCTCTACAAAGGCAATGGTCTTC 4157	21-056	GGGCGTTTACAGCGTCTTACATCAACGGGA 4096	21-056	TTCAGGATCCTCTACAAAGGCAATGGTTC 4154	21-056	TGACGTTGCCAAGGACCAATT 4127
21-071	GATCCTCTACAAAGGCAATGGTCTTC 4157	21-071	GGGCGTTTACAGCGTCTTACATCAACGGGA 4096	21-071	TTCAGGATCCTCTACAAAGGCAATGGTTC 4154	21-071	TGACGTTGCCAAGGACCAATT 4127
21-111	GATCCTCTACAAAGGCAATGGTCTTC 4157	21-111	GGGCGTTTACAGCGTCTTACATCAACGGGA 4096	21-111	TTCAGGATCCTCTACAAAGGCAATGGTTC 4154	21-111	TGACGTTGCCAAGGACCAATT 4127
21-121	GATCCTCTACAAAGGCAATGGTCTTC 4157	21-121	GGGCGTTTACAGCGTCTTACATCAACGGGA 4096	21-121	TTCAGGATCCTCTACAAAGGCAATGGTTC 4154	21-121	TGACGTTGCCAAGGACCAATT 4127
21-179	GATCCTCTACAAAGGCAATGGTCTTC 4157	21-179	GGGCGTTTACAGCGTCTTACATCAACGGGA 4096	21-179	TTCAGGATCCTCTACAAAGGCAATGGTTC 4154	21-179	TGACGTTGCCAAGGACCAATT 4127
21-187	GATCCTCTACAAAGGCAATGGTCTTC 4157	21-187	GGGCGTTTACAGCGTCTTACATCAACGGGA 4096	21-187	TTCAGGATCCTCTACAAAGGCAATGGTTC 4154	21-187	TGACGTTGCCAAGGACCAATT 4127
21-196	GATCCTCTACAAAGGCAATGGTCTTC 4157	21-196	GGGCGTTTACAGCGTCTTACATCAACGGGA 4096	21-196	TTCAGGATCCTCTACAAAGGCAATGGTTC 4154	21-196	TGACGTTGCCAAGGACCAATT 4127
21-256	GATCCTCTACAAAGGCAATGGTCTTC 4157	21-256	GGGCGTTTACAGCGTCTTACATCAACGGGA 4096	21-256	TTCAGGATCCTCTACAAAGGCAATGGTTC 4154	21-256	TGACGTTGCCAAGGACCAATT 4127
21-294	GATCCTCTACAAAGGCAATGGTCTTC 4157	21-294	GGGCGTTTACAGCGTCTTACATCAACGGGA 4096	21-294	TTCAGGATCCTCTACAAAGGCAATGGTTC 4154	21-294	TGACGTTGCCAAGGACCAATT 4127
BBRO-BChV21	GATCCTCTACAAAGGCAATGGTCTTC 4157	BBRO-BChV21	GGGCGTTTACAGCGTCTTACATCAACGGGA 4096	BBRO-BChV21	TTCAGGATCCTCTACAAAGGCAATGGTTC 4154	BBRO-BChV21	TGACGTTGCCAAGGACCAATT 4127
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Figure 5.15: Rothamsted Research aphid reverse transcription-qPCR assay (which detects both Beet mild yellowing virus (BMV) and Beet chlorosis virus (BChV) aligned with the produced BChV isolate sequences. Bold letters indicate mismatches in the primer/probe where the sequence is conserved amongst the sequenced isolates. a – reverse transcription primer, VR2 (shown in reverse complement), b – qPCR forward primer, Pol\_F, c - qPCR reverse primer, Pol\_r (shown in reverse complement), d – qPCR probe, Pol\_probe (shown in reverse complement)

## 5.5 Discussion

Overall levels of intra-species genetic variation were similar for both BMV and BChV. No isolates showed greater than 10% difference in amino acid sequence identity for any gene product and therefore would be not classed as a separate species under the International Committee on Taxonomy of Viruses guidelines (Sömera et al., 2021). One fundamental limitation of using this approach, to understanding the genetic diversity within BMV and BChV isolates, is that it cannot be used to detect recombination. Recombination events result in the swapping of genetic material between genetically distinct viruses. The approach taken here was to map the aligned short reads to a reference genome and then 'correct' that reference to give a consensus sequence of the sample. Were recombination to have occurred giving rise to a virus which differed significantly from the reference sequence, the reads would not have aligned and therefore would not contribute to the consensus sequence. Consequently, the results discussed here reflect the smaller genetic changes most likely arising from errors in replication caused by the viral RdRp.

Importantly, from a diagnostic perspective, very little genetic variation was identified in the regions targeted by the DSMZ BMV or BChV assays. Both the primers and probes showed a high level of complementarity to the sequenced isolates indicating the assays are reliable at detecting multiple virus isolates. High sequence conservation was also seen in the genome region targeted by the Rothamsted Research diagnostics, however based on the sequence data generated here, there is the potential for slight adjustments to the reverse transcription primer and forward qPCR primer which may improve primer binding and diagnostic sensitivity.

When drawing conclusions from this data set it is important to remember the origins of the sequencing data. Each sequenced sRNA library was derived from RNA extracted from virus-infected leaves collected from the field. Crucially this means leaves may have been initially infected with a population of genetically differing virus particles, rather than one single virion. As such, the consensus sequences of the isolates may not represent a single virus genome but rather give the consensus of the most prevalent nucleotides at each genome position across the viral population contained within the isolate.

As previously discussed, BCFtools is a variant calling software which has been used here to create the sample consensus sequences from which viral genetic variation has been investigated. The stringency parameters of the tool mean that nucleotide variants occurring at a low rate within the sample sequencing data will be discounted. This reduces the risk of nucleotide variation arising from sequencing errors being mistakenly attributed to true genetic variation, however it increases the risk of low frequency mutations going undetected. This may explain why three of BChV isolates

(19-010, 21-017 and 20-018-1) all shared 100% identity with the BChV reference genome; with any genetic variation present not being consistent enough within each isolate to appear within the generated consensus sequence. Despite this potential draw back, the stringency parameters used in the analysis do provide assurance that where nucleotide differences are seen they are true reflections of differences observed within that virus population.

Alternative approaches of analysing this data set, such as *de novo* assembly of the viral sRNAs may permit the identification of low frequency mutations (Singh et al., 2020). However, adopting such a methodology is problematic when samples may be infected with multiple closely related viruses (such as both BMV and BChV). Such mixed infections could result in erroneous contigs, formed from reads originating from two separate viruses, giving the illusion of greater genetic diversity (Boonham et al., 2014).

It was hypothesised that greater genetic variation would be seen between virus isolates with further geographic separation, however the results presented here do not support this. This suggests that other factors have a larger influence on virus genetic diversity than geographic location. One such factor may be the host plant from which the virus was acquired by the aphid prior to infecting the beet plant, particularly the overwinter host. Isolates which originated from the same weed species, for example, may share similar mutations which proved advantageous to replication within that host. This would be consistent with the differences seen in the BBRO BMV culture which is maintained predominantly in *Capsella bursa-pastoris* rather than sugar beet to help prevent contamination.

Alternatively, the species of aphid which transmitted the virus to the beet plant may contribute to the genetic variation, with certain viral mutations favouring aphid transmission by *Macrosiphum euphorbiae*, for example, over *Myzus persicae*. Again, this could have contributed to the differences seen in the BBRO BMV culture which is only transmitted to new plants using the BBRO *M. persicae* culture and never *M. euphorbiae*.

The intra-species genetic variation which was identified was predominantly in the ORFs located at the 5' end of the viral genome. Variation within ORF0 and ORF1 is consistent with plant host and aphid vector adaptation. P0, encoded by ORF0, is a silencing suppression protein acting against the host plant gene silencing system. It has also been linked to viral pathogenicity and host symptom development, as well as affecting plant-aphid interactions through the control of phytohormones. ORF1, aside from its role in viral replication, encoding the viral genome-binding protein, also has a phytohormone regulation activity. Therefore, alterations to these proteins could affect both virus-host and virus-vector interactions, potentially providing resistance-breaking properties.

Nevertheless, the ORF most affected by nucleotide changes predicted to alter the encoded amino acid was ORF2. Translation of ORF2 arises through a ribosomal frame shift from ORF1 resulting in the P1P2 fusion protein. Previous studies have identified the ORF2 region as encoding the viral RdRp and therefore this region has a fundamental role in viral replication. Hence, it is surprising that the highest number of predicted amino acid changes were identified within this protein, a finding which goes against research conducted into variation seen between poleroviruses species (Huang et al., 2005; LaTourrette et al., 2021). This may be a consequence of the sRNA sequencing methodology and using a variant-calling tool to identify nucleotide differences between isolates. The shorter reads lengths of sRNA library sequencing may have permitted sample reads originating from other parts of the virus genome, or indeed different viruses present within the sample, to mis-align to the P2 region of the reference sequence. This would cause an artificially increased nucleotide diversity within the region. Future studies, utilising longer-read methodology, would help clarify if this is the case or if the result is true; indicating a greater intra-species variation in this genome region than previously understood.

In both the BMV and BChV isolates, synonymous nucleotide differences were also identified between isolates. As these differences were not predicted to alter the encoded amino acid, they are less likely to significantly alter the physical properties of the virus. Nevertheless, previous studies have shown that synonymous mutations in RNA viruses can affect RNA secondary structure and alter the codon use bias, in turn affecting the physical properties of the virus. As with the amino acid altering nucleotides, the synonymous changes in both the BMV and BChV isolates were most prevalent in the 5' proximal ORFs and so may further contribute to changes within these proteins.

The BChV isolate 19-016, originating from a fodder beet field in Somerset, had the lowest similarity of any of the virus isolates. The reasons for this are not clear, particularly as the same differences were not seen in isolate 19-015 despite it also originating from a fodder beet plant in a neighbouring field. Although these results may be a true reflection of a viral population, we cannot rule out a methodology cause; possibly contamination during the sRNA library preparation. Storage of the sample and resulting RNA degradation may also have contributed to these results, particularly given that the 2019 sample had to be freeze-dried and stored for longer than planned due to the laboratory lockdowns during the Covid-19 pandemic. However why only this isolate may have been affected is unclear.

Focusing on the virus isolates maintained in culture at the BBRO, both the BMV and BChV cultures showed genetic changes over time, becoming more genetically distinct from the 'wild' virus isolates. These findings have important consequences in both fundamental virus yellows research



and virus resistant variety development. Fundamental research conducted using BMV or BChV viruses maintained in continuous culture may result in findings which would not be consistent with other BMV or BChV isolates. From a breeding perspective, the genetic differences identified here may result in differences in pathogenicity and therefore varieties developed to overcome the BBRO BMV or BChV isolate may not respond as expected when infected with other virus isolates. In the future, to ensure the reliability and relevance of the BBRO virus cultures, the cultures should be 'refreshed' periodically with a mixture of field collected virus isolates. Routine sequencing would also be valuable providing data on how closely the culture genetically reflects 'wild' isolates.

# Chapter 6: The effect of different virus populations on susceptible and resistant sugar beet varieties under field conditions

## 6.1 Chapter Contributions

The agronomic work described in this chapter was conducted by the BBRO trials team. Inoculation of the trial (see section 6.3.2.3) in 2022 was conducted by me and Stuart Harder alongside Mark Stevens, Simon Bowen, Alistair Wright, Kate Orman, Georgina Barratt all from the BBRO. In 2023 the trial was inoculated by me alongside Simon Bowen, Eleanor Towler, Hannah Carthy, Alistair Wright and Stephen Aldis from the BBRO. The BBRO trials team harvested the trial. Subsequent root weight, sugar content and impurity analysis were completed by operatives of the British Sugar BBRO tarehouse, with the results being verified by the independent National Farmers Union tarehouse representatives. Drone flights were completed by Alistair Wright. Leaf samples for ELISA testing were collected by me with support from Eleanor Towler and Ollie Hammond. All other work including aphid monitoring and aphid species identification I completed myself.

## 6.2 Introduction

The results of the previous chapter (Chapter 5) show that genetic variation is present within the BChV and BMV species analysed as part of this study. However, as outlined in Chapter 1, for a virus isolate to be termed a 'strain' it must exhibit unique and recognisable phenotypic characteristics. Based on genetic data alone we cannot definitively say any of the virus isolates collected and cultured here are distinct enough to be called a "strain". As discussed in Chapter 5, we can begin to make predictions about the effects of genetic differences, based on the genomic locations of nucleotide differences, but to fully appreciate the consequences of these differences, assessing the effect of the virus isolates on sugar beet plants is necessary.

No recent studies (since this identification of BChV) have characterised the effect on sugar beet of differing isolates from the same beet polerovirus species. Were the genetic differences identified between isolates (Chapter 5) to result in different disease severities this could have important consequences for resistance breeding. Predominantly in the UK, resistant sugar beet varieties have been developed through challenging them with the BBRO BMV isolate. The results detailed in Chapter 5 show the BBRO BMV isolate genome has diverged from the 'wild' BMV isolates. It is therefore possible that the resistant sugar beet varieties that have been developed may be specific to this isolate and less effective against other BMV isolates.

Currently only one variety is included on the UK recommended list, and available for sugar beet growers, with virus yellows resistance. This variety is Maruscha KWS, with claimed partial tolerance against BMV. Given the findings outlined in Chapter 3, where BChV was identified as the most prevalent cause of virus yellows disease in the UK, it is important to determine whether resistant varieties which have been developed to overcome BMV infection can also combat BChV. As with BMV, confirming any resistance mechanism is functional against all isolates of BChV is also important for the effective deployment of these varieties in the field.

As demonstrated in Chapter 4 it is difficult to simulate the yield impact of virus infection under controlled conditions. Randomised field trials enable experimental plants to be treated more akin to a commercial crop, whilst maintaining scientific rigour. Previous studies have utilised field trials to assess the impact of different virus yellows species on sugar beet yield (Hossain et al., 2021; Stevens et al., 2004), as well as for identifying resistant varieties (Wintermantel, 2005). Although some trials are conducted exploiting natural aphid/virus infection alone (Dunning and Winder, 1965), trials aiming to assess variety performance typically involve inoculating experimental plants with viruliferous aphids. This latter method ensures uniform virus pressure across the trial and has proven a successful method of identifying resistant sugar beet varieties (Wright and Stevens, 2023).

To determine the effect of the collected virus isolates on resistant and susceptible sugar beet varieties I conducted virus inoculated field trials. Replicated across two years, these experiments helped clarify the consequences of the genetic variation identified within BMV and BChV isolates. I have also improved our understanding on the resilience and durability of resistant varieties, assessing their performance under mixed virus infection.

## 6.3 Materials and Methods

### 6.3.1 Creation of virus cultures

To establish varietal response under differing virus isolates, cultures of virus populations were established. This was achieved using the virus-infected beet leaves received in the 2021 larger scale virus survey described in Chapter 3. Based on the qPCR assay results of this survey, one BMVYV infected and three BChV infected leaves were selected. These specific leaves were chosen based on their origin location, high virus titre as determined via ELISA, (Chapter 2, section 2.4) and their suitability to support aphid feeding (i.e. how fresh the leaves had arrived in the lab).

Virus cultures were established prior to the sRNA sequencing, due to time constraints and the need to collect leaves before commercial fields were harvested. Genetic variation was presumed to exist between virus isolates, and hypothesised to be greater between isolates which were more geographically distinct. As discussed in Chapter 5, geographic location did not correlate to genetic variation, however genetic differences were seen between the isolates brought into culture.

To keep the leaf samples turgid and viable for aphid feeding, approximately 2cm was trimmed from the basal end of the leaf petiole and the leaf placed in a beaker of deionised water. Five virus-free *Myzus persicae* were transferred onto the leaf blade using a paintbrush. The leaf and beaker were then sealed within an aphid proof bag. After three days the aphids, including any progeny, were carefully transferred from the leaf to a healthy sugar beet seedling with two to four true leaves. A plastic cup was placed over the plant to contain the aphids. Aphids were allowed to feed and transfer the virus for one week, before being sprayed with insecticide (0.1g/L InSyst containing 20% w/w acetamiprid, CertisBelchim).

Subsequent ELISA and qPCR testing (using the assay detailed in Chapter 2 section 2.4-2.5), confirmed the presence of BMVYV in the 21-254 culture and BChV in cultures 21-278 and 21-024. The other BChV culture (21-290) was found to also contain BYV (Table 6.1). Unfortunately, no plant host suitable to filter out the BYV from the culture was available, however, the culture was still included in the field trial to assess the impact of combined BChV and BYV infection on the varieties. Whilst the cultures were being maintained between inoculation of the 2022 field trial and the 2023 field trial, the 21-024 BChV culture became contaminated with BYV. As such in the 2023 field trial, plots inoculated with this culture became infected with both BChV and BYV.

Following this protocol, a total of four virus cultures were established (Table 6.1). These were used alongside the BBRO BMVYV and BChV reference cultures. Once established, cultures were maintained as per the virus culture protocol detailed in Chapter 2 section 2.1.

Table 6.1: origin details of virus cultures established in 2021 and used to inoculate field trials in 2022 and 2023.

Sample ID	Original leaf location	Original crop	Virus
21-254	Crowland, Lincolnshire	Sugar beet	Beet mild yellowing virus
21-278	Rougham, Norfolk	Sugar beet	Beet chlorosis virus
21-290	Thirn, North Yorkshire	Fodder beet	Beet chlorosis virus and Beet yellows virus
21-024	Letheringham, Suffolk	Sugar beet	Beet chlorosis virus (Beet yellows virus present in culture in 2023)

## 6.3.2 Field Experiment

### 6.3.2.1 Layout

A field experiment was designed to assess the performance of sugar beet varieties under the different virus isolates. Four varieties were included in the trial, two with resistance to polerovirus infection (KWS-1, KWS-10) and two susceptible varieties (KWS-6, KWS-9). These varieties were challenged with six different virus isolates: the four established cultures (Table 6.1), and the BBRO cultures of BMVY and BChV. In addition, a control treatment, which was not inoculated with aphids, since this would pose an even greater risk of contamination, was also included to assess variety performance when uninoculated with virus.

The experiment was arranged in a split-split-split plot design with four replicates of virus inoculated treatments and eight replicates of the uninoculated control treatment, split across two main blocks and four blocks (Figures 6.1 and 6.2). The uninoculated treatment was fixed at each end of the trial blocks to reduce the risk of contamination from the virus inoculated plots. These replicates are described as “Uninoculated 1” and “Uninoculated 2” for clarity. Each individual plot measured 1.5 by 4.5m and consisted of three rows of the allocated sugar beet variety.

Virus treatments were randomly assigned within in Block 1 and 3, then the same randomisation applied to Block 2 and 4 to minimise virus contamination between treatments. To reduce the risk of secondary spread of virus outside of the inoculated plots, KWS-1, a sugar beet variety with breeder-claimed resistance to BMVY, was used as a ‘buffer’ to surround each treatment. Spring rye (*Secale cereale*) was sown around the experimental area, and between the two main blocks to act as a wind break, in line with standard BBRO protocol for virus experiments. This reduced the risk of viruliferous aphids released into the treated plots being blown between the main blocks and helped

to shield the plots from naturally occurring aphids. In 2023 a strip of oil radish (*Raphinus sativus*) was sown along the field margin of the experimental area to act as a trap crop for aphids migrating into the field and other invertebrate pests such as flea beetle.

#### 6.3.2.2 Agronomy

In both 2022 and 2023 the field experiments were hosted within commercial sugar beet fields at Morley Farms Ltd. In 2022 the field was located at (52.56 N, 1.029 E) and in 2023 at (52.57 N, 1.021 E) on a loam soil. In 2022 the field trial was sown on the 14<sup>th</sup> April by the BBRO field trials team using a John Deere 6130R (RTK Guidance) with a Wintersteiger automated plot drill (6 row x 50cm). In 2023 the experiment was sown on the 25<sup>th</sup> April. Plots were double sown at a 9cm seed-spacing, then gapped (excess plants removed) prior to inoculation to provide a uniform plant stand with a target intra-row plant spacing of 18cm. All seed was treated with proprietary priming and pelleting technology (EPD 2.0) by KWS, along with the plant protection products tefluthrin (Force ST, Syngenta) and hymexazol (Tachigaren).

A standard commercial sugar beet herbicide, fertiliser and fungicide programme was conducted by the BBRO field trials team to keep the plots in good agronomic condition and free from weeds. Aphid numbers were monitored within the experiment from seedling emergence until the 16-leaf stage, after which the risk posed from virus infection is negligible (BBRO, 2023a; Schop et al., 2022). In 2022, aphid numbers were high and an insecticide (0.1g/L InSyst containing 20% w/w acetamiprid, CertisBelchim) was applied on 9<sup>th</sup> May to control numbers. No aphicide was required in 2023.

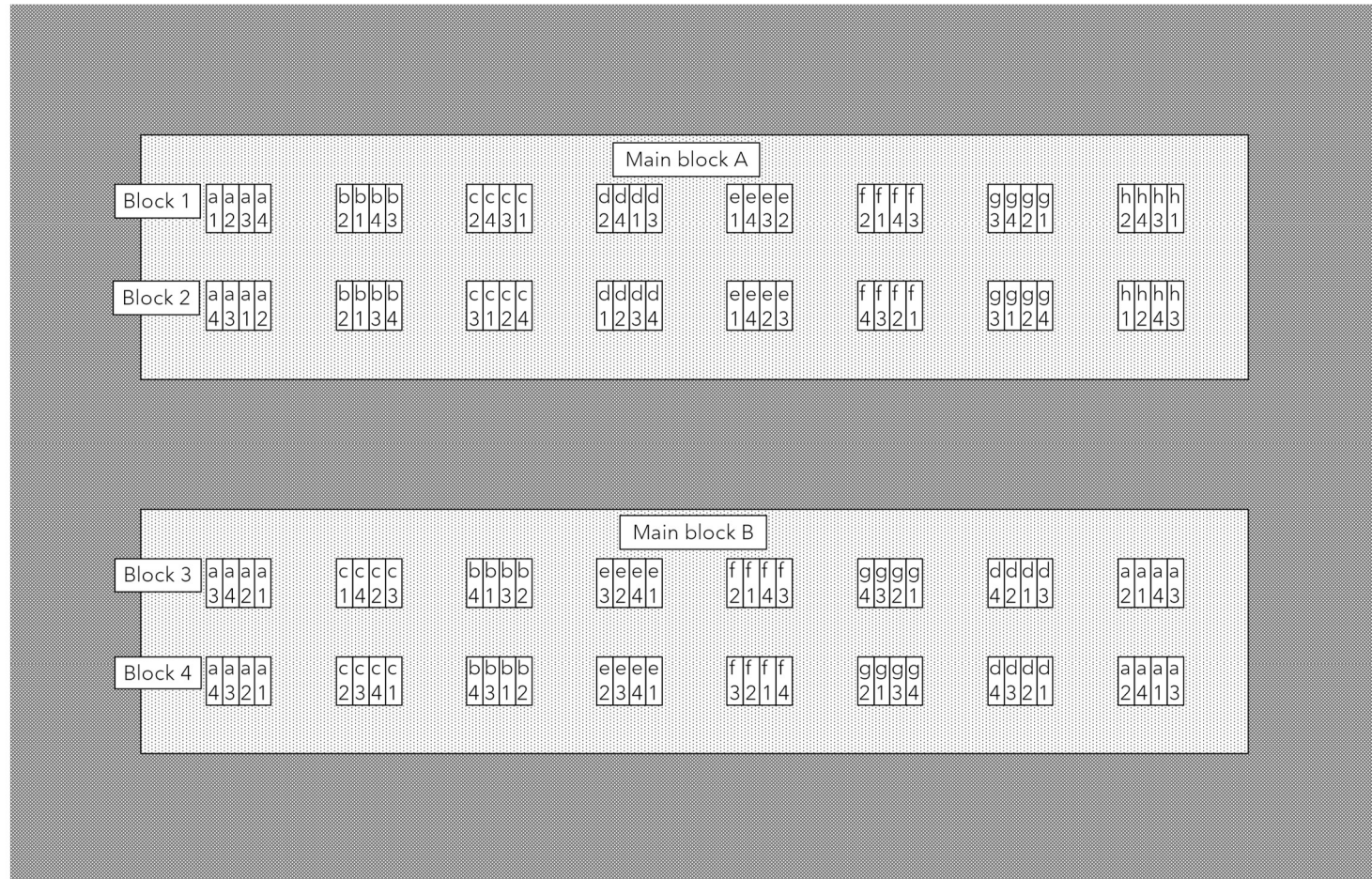
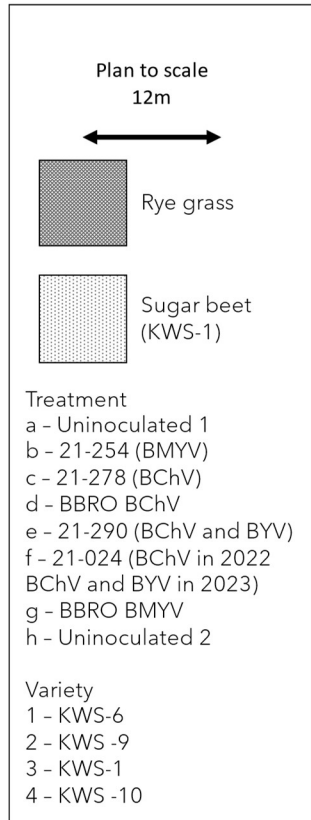


Figure 6.1: Experimental plan showing layout of sugar beet varieties and Beet mild yellowing virus (BMVY) and Beet chlorosis virus (BChV) virus treatments. Each plot (shown as white box) contained three rows of the specified sugar beet varieties, KWS-1 and KWS-10 had resistance to BMVY infection and KWS-6 and KWS-9 were susceptible. Plots were grouped together in treatments blocks, each receiving the same virus treatment. A buffer area of sugar beet was planted around each treatment block, then rye grass surrounding each main block.



Figure 6. 2: drone image of 2023 field trial, showing main blocks surrounded by rye taken on 23<sup>rd</sup> September 2023 taken by Alistair Wright (BBRO).

#### 6.3.2.3 Virus inoculation

Viruliferous aphids of each virus culture were reared in MLR-352 (Panasonic) reach-in growth chambers as described in Chapter 2 section 2.1. BMVY-viruliferous aphids were reared on *Capsella bursa-pastoris* to minimise the risk of contamination with BChV cultures. BChV-viruliferous aphids were reared on sugar beet as no reliable, discriminative host suitable for aphid rearing was available.

Inoculation of plots with viruliferous aphids was conducted on the 26<sup>th</sup> May in 2022 and 25<sup>th</sup> May in 2023 when plants were at the 2-4 true leaf stage. Migration of *M. persicae* into sugar beet fields typically peaks around the end of May beginning of June, hence inoculation was timed to mimic the likely timing of natural infection. Inoculation was achieved by placing a small piece of leaf tissue containing approximately 10 viruliferous aphids into the centre of every-other sugar beet plant within each plot.

Controlling the release of viruliferous aphids into the trial was vital to prevent contamination between treatments and minimise the risk to any neighbouring sugar beet crops. As such, multiple steps were taken to mitigate these risks. Inoculation was conducted by six different people, each responsible for one virus culture. Working entirely independently, each person prepared the inoculum containing the viruliferous aphids at the BBRO laboratory then travelled to the field trial site in separate vehicles. Once at the trial, 'inoculators' followed a pre-arranged specific route



walking through the rye to the plots they were to inoculate, avoiding walking near any other treatment plots or inoculator. Any viruliferous aphids which may have been present on clothing were brushed off into the plot before moving between blocks. After each inoculator had finished infecting every plot of their corresponding virus treatment, they exited the trial following the pre-arranged route, again walking through the rye and avoiding any other sugar beet plots.

In 2022 aphids were left to feed, and transfer the virus for 7 days, before being killed with an aphicide. In 2023 aphid numbers were low, with generally fewer than 10 viruliferous aphids per inoculated plant and aphid predator numbers were high. Consequently, 7 days after inoculation aphid numbers had not reached the agronomic threshold required to apply an aphicide (one green wingless aphid per four plants up to the twelve-leaf stage). Aphid numbers were continually closely monitored, however they did not reach the spray threshold as beneficial insects remained high throughout.

#### 6.3.2.4 Harvest

Plots were harvested into large bags by the BBRO trial team on the 31<sup>st</sup> October 2022 and the 4<sup>th</sup> October 2023 using a John Deere 6135R with modified Garford Victor sugar beet plot harvester (Figure 6.3). Bags were then transported to the BBRO plot facility tarehouse at the Wisington Beet Sugar factory (Norfolk, UK), where each plot was weighed dirty then washed and reweighed by the British Sugar BBRO tarehouse operatives. Samples from each plot were taken for sugar content and impurity analysis (ICUMSA, 2007) by the British Sugar BBRO tarehouse operatives and then verified by the independent National Farmers Union tarehouse representatives. Sucrose yield was calculated by multiplying the plot clean weight by the percentage sugar.



Figure 6.3: the BBRO trials team harvesting the 2023 field trial on the 4<sup>th</sup> October 2023, a) sugar beet plot harvester lifting roots from trial plots into large bags. b) harvested roots from one trial plot ready to be delivered to the BBRO tarehouse (photos by Suzannah Harder).

### 6.3.3 ELISA testing

ELISA testing of leaf samples was conducted to measure virus titre across the varieties and virus treatments. In 2022, ELISA testing was conducted four, eight, twelve and nineteen weeks after inoculation. The nineteen-week time point was not planned but added in response to the exceptionally high temperatures and dry conditions experienced at the trial site. Due to these conditions, at the twelve-week time point, all plants were severely droughted. By the nineteen-week time point plants were no longer showing symptoms of drought stress.

To avoid repeat sampling of the same plant, prior to any symptom development, five plants per experimental plot were randomly selected and marked using a cane. At the first sampling point, the plant nearest the cane was used. At the second sampling point, the plant one away from the cane was used and so on. At each time point, the largest leaf was taken from the selected plant as shown in Figure 6.4. In 2022, due to a beet moth (*Scrobipalpa ocellatella*) infestation, by the nineteen-week time point, only a very limited number of plants had an intact canopy (Figure 6.5), however the same protocol of sampling the largest leaf from the selected plant was followed. In 2023 ELISA testing was conducted at a single timepoint, fourteen weeks after inoculation. As in 2022, five plants were selected at random from each plot and the largest leaf sampled. In 2023 no beet moth damage was recorded.



Figure 6. 4: taking leaf samples from field trial plots for ELISA testing. a) sugar beet plant in trial plot marked with a cane prior to any symptom development, b) at first sampling time point, plant closest to the cane was selected. The largest leaf was identified by grouping the canopy together and determining the tallest leaf (photos by Suzannah Harder).



Figure 6.5: photos taken from field trial plots in 2022 showing damage caused by beet moth infestation (photos by Suzannah Harder).

All sampled leaves were placed in plastic sample bags and transported back to the laboratory where two 2.5cm diameter leaf discs were taken from each leaf. ELISA testing was conducted following the methodology described in Chapter 2 section 2.4. At every time point all leaves were tested using the Beet western yellows virus (BWYV) antibody set (RT-0049-0049/1, DSMZ). At the nineteen-

week post inoculation time points in 2022 and in 2023 the leaves were also tested using the BYV antibody set (RT-0185, DSMZ).

#### 6.3.4 Canopy cover and canopy chlorophyll estimation

Canopy cover and canopy chlorophyll estimation were determined based on aerial images captured by drone. Drone flights and subsequent image processing and analysis was undertaken by Alistair Wright (BBRO) following the methodology detailed by Wright et al. (2022). Drone flights were conducted on 22<sup>nd</sup> July and 15<sup>th</sup> September in 2022 and 9<sup>th</sup> July and 23<sup>rd</sup> September in 2023. Data was received in the form of RGB images, and the spectral reflectance index mNDblue (used to estimate canopy chlorophyll, (Jay et al., 2017)) of each experimental plot.

The percentage canopy cover per plot was calculated using a similar methodology to that described in Chapter 4 section 4.2.2.1. Plot images were loaded into the image analysis software ImageJ (version 1.53t). Then the 'colour threshold' tool used to remove any exposed ground by adjusting the hue settings to 40-145 (Figure 6.6) and a batch process macro run to calculate the percentage of image area selected.

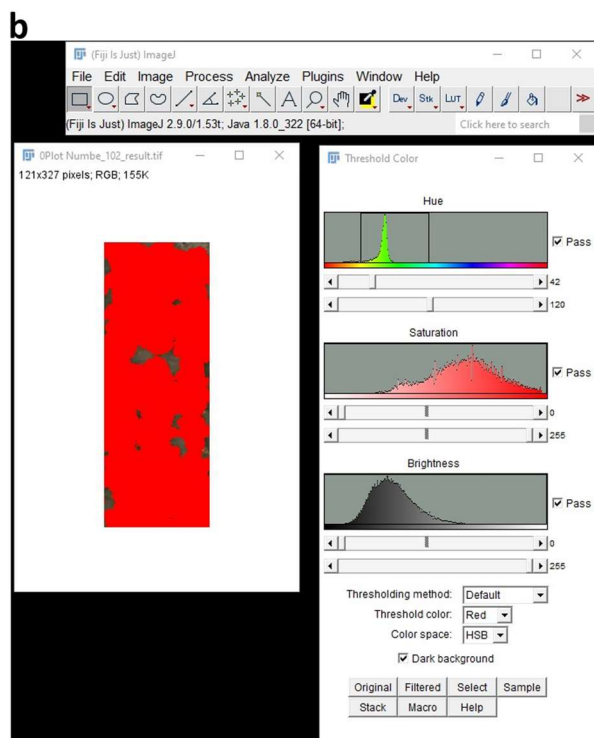
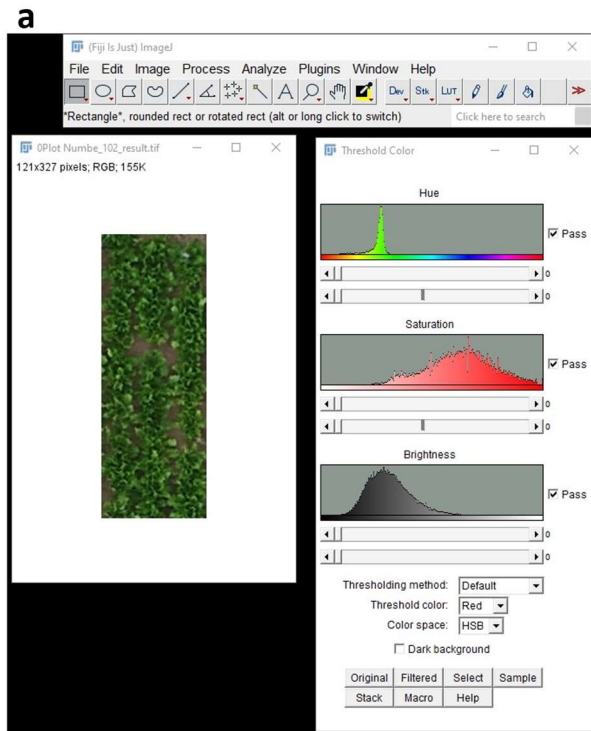


Figure 6.6: screen shots of user interface of ImageJ (version 1.53t) used to determine canopy cover, a) experimental plant image loaded into ImageJ, b) hue filter applied to select green/yellow image area.

### 6.3.5 Weather data

Air temperature and precipitation data was retrieved from the Morley Farm Weather Station (iMetos 3.3) via the Field Climate website (Pessl Instruments) and downloaded as an .xls file.

### 6.3.6 Monitoring natural aphid populations

Yellow water pan traps (YWP) were used to monitor the occurrence and abundance of naturally occurring *M. persicae* and *Macrosiphum euphorbiae* at the field trial site. Monitoring took place during the spring aphid migration from 25<sup>th</sup> April to 4<sup>th</sup> July (standard week 17 to 27) (Taylor, 2013). Three YWPs were installed as per the manufacturer's instructions (MN59930, Ringot France) in the discard sugar beet in the field experiment. YWPs were placed approximately 15m apart and the pans adjusted to be at sugar beet canopy height, as shown in Figure 6.7.

YWPs were filled with water containing approximately three drops of domestic washing up liquid. The detergent breaks the surface tension of the water to ensure any insects which landed in the pan were captured. The pans were emptied twice a week by pouring the contents into a funnel lined with a fine net cloth. The cloth and insects were then carefully transferred to a storage pot and taken for analysis in the lab.

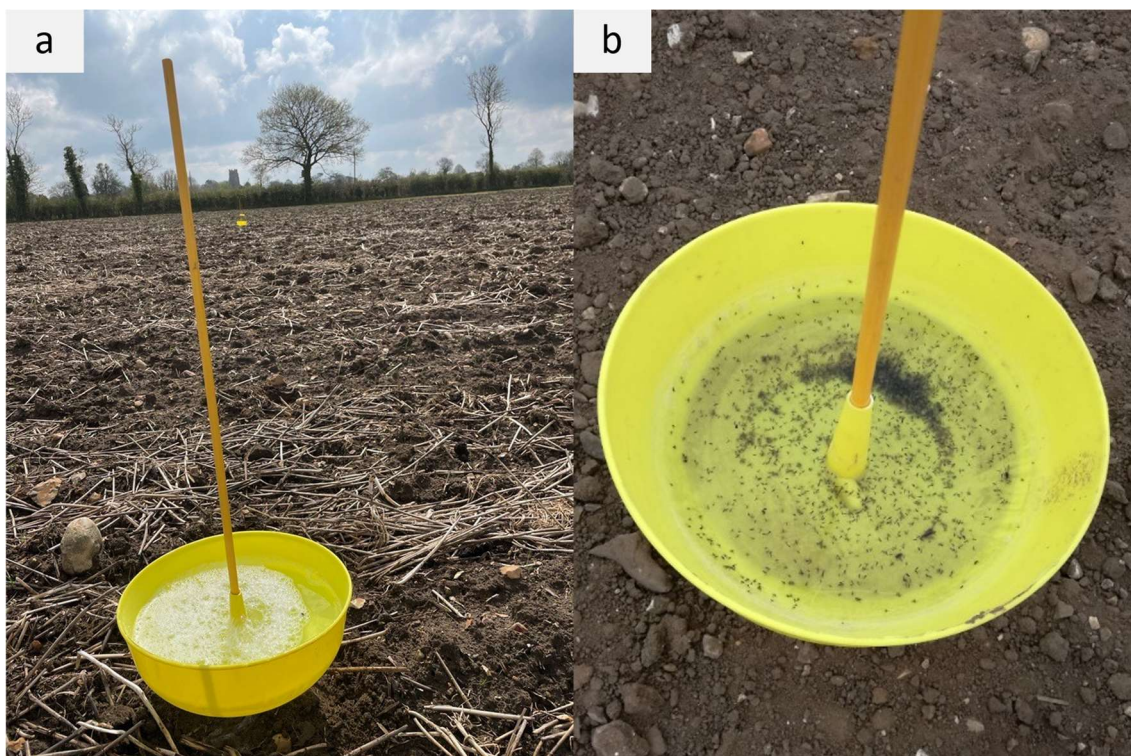


Figure 6.7: a) freshly filled yellow water pan (YWP) in discard beet of 2023 field trial, b) YWP on day of sample collection in 2022, (photos by Suzannah Harder).

In the laboratory, all insects were washed from the cloth into a white larval tray (such as A115, Watkins & Doncaster). Aphids of all species were identified and separated from the rest of the insect catch. A dissecting microscope (HN-3, Microtec) was used to identify the *M. persicae* and *M. euphorbiae* from all other aphid species (Figure 6.8). *M. persicae* and *M. euphorbiae* were identified based on their morphologically distinguishing features as described by Blackman (2010), and the total number of each species recorded. The total number of *Coccinellinae* sp (ladybirds) was also recorded, as an indication of natural aphid predator levels within the field.

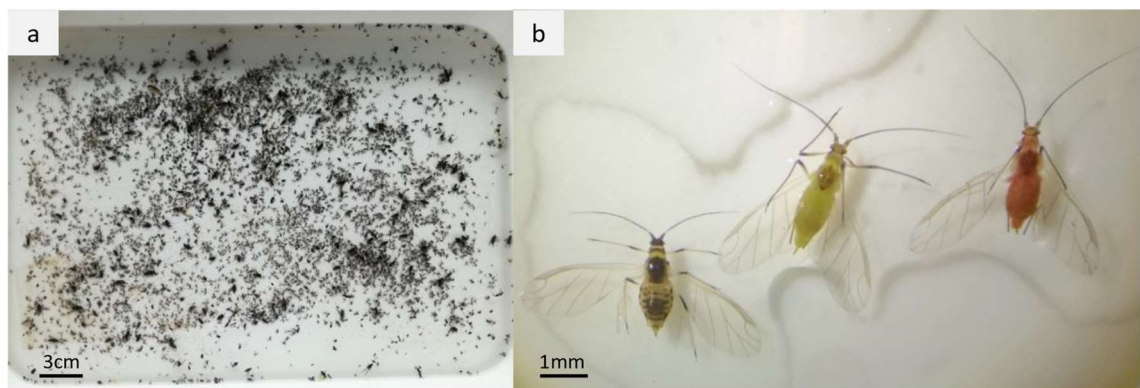


Figure 6.8: processing of yellow water pan (YWP) samples. a) contents of YWP sample emptied into white larval tray ready for aphid species to be separated, b) from left to right *M. persicae*, green form of *M. euphorbiae*, red form of *M. euphorbiae*, identified from YWP sample (photos by Suzannah Harder).

#### 6.3.6.1 Identification of polerovirus in YWP aphids

*M. persicae* and *M. euphorbiae* aphids caught in the YWPs were tested via two-step RT-qPCR to determine if they were carrying BMV and/or BChV using an unpublished protocol developed at Rothamsted Research (Martin Williamson, personal communication), as outlined in Chapter 2 section 2.6. The aphids were also tested for TuYV. TuYV is known to be carried by around 80% of the *M. persicae* population in the sugar beet growing region (BBRO, personal communication) and therefore acts as an inline positive control to confirm that poleroviruses were not lost through the capture process.

### 6.3.7 Statistical analysis

Results were analysed using GenSTAT 22<sup>nd</sup> edition (VSN International). All graphs were produced using RStudio version 2022.02.1+461 "Prairie Trillium" released for Windows (RStudio Team 2022).

#### 6.3.7.1 Analysis of canopy cover, canopy chlorophyll, sucrose yield and ELISA data

To analyse the canopy cover, canopy chlorophyll (estimated by mNDblue values) and sucrose yield data two-way analysis of variance (ANOVA) in randomised blocks were conducted using the GenSTAT 'General Analysis of Variance' statistical test. The experiment design was set to 'General analysis of variance', and the Y-variate to the relevant dependent variable (canopy cover, mNDblue value, sucrose yield). The treatment structure was defined using the fixed factors 'Variety x Treatment'. The block structure was set to 'Main block / Block / Treatment / Variety' where '/' indicates the nested blocking structure of the split-split-split plot trial design. The least significant difference (LSD) at 5% significance calculation was included in the ANOVA.

For analysis of ELISA data, prior to conducting the two-way ANOVAs as described above, the data were first summarised to prevent pseudo-replication. Data were loaded into GenSTAT and the 'Summary Statistics' function used to calculate the mean ELISA result of the five individual plants tested for each plot.

#### 6.3.7.1 Linear regression

Regression analysis was conducted on the percentage of the uninoculated sucrose yield achieved and the change in mNDblue value from the uninoculated using the GenSTAT 'Linear regression' statistical test. The regression type was set to 'simple linear regression' using the response variate (Y) 'percentage of uninoculated sucrose yield achieved' for the specified year (2022 or 2023). The explanatory variate (X) was set to 'change in mNDblue value from the uninoculated' for the relevant time point (July 2022, September 2022, July 2023 or September 2023).

Any improvement in the statistical significance of the model by the incorporation of the factors 'variety' and 'virus treatment' were explored using the GenSTAT 'Linear regression with groups' statistical test. This was conducted using the response variate and explanatory variate set as described above, with the addition of the 'Groups' structure set to 'Variety, Treatment'.



## 6.4 Results

### 6.4.1 Weather data

Weather conditions varied considerably between the two years the field experiments were conducted (Figure 6.9). In 2022, the field trial site experienced exceptionally hot and dry conditions resulting in plants being placed under considerable drought stress (Figure 6.10). In comparison, the 2023 experimental site experienced significantly more rainfall with 244.2mm recorded between the beginning of June and end of August compared to 39.6mm across the same period in 2022.

Air temperatures were also higher in 2022, with a total of 25 days having a mean air temperature above 20°C, compared to only 14 days in 2023. Exceptionally high maximum air temperatures were also recorded in 2022, with a maximum air temperature of 38.05°C being recorded (on the 19<sup>th</sup> July) compared to a maximum of 32.23°C in 2023 (on the 10<sup>th</sup> September).

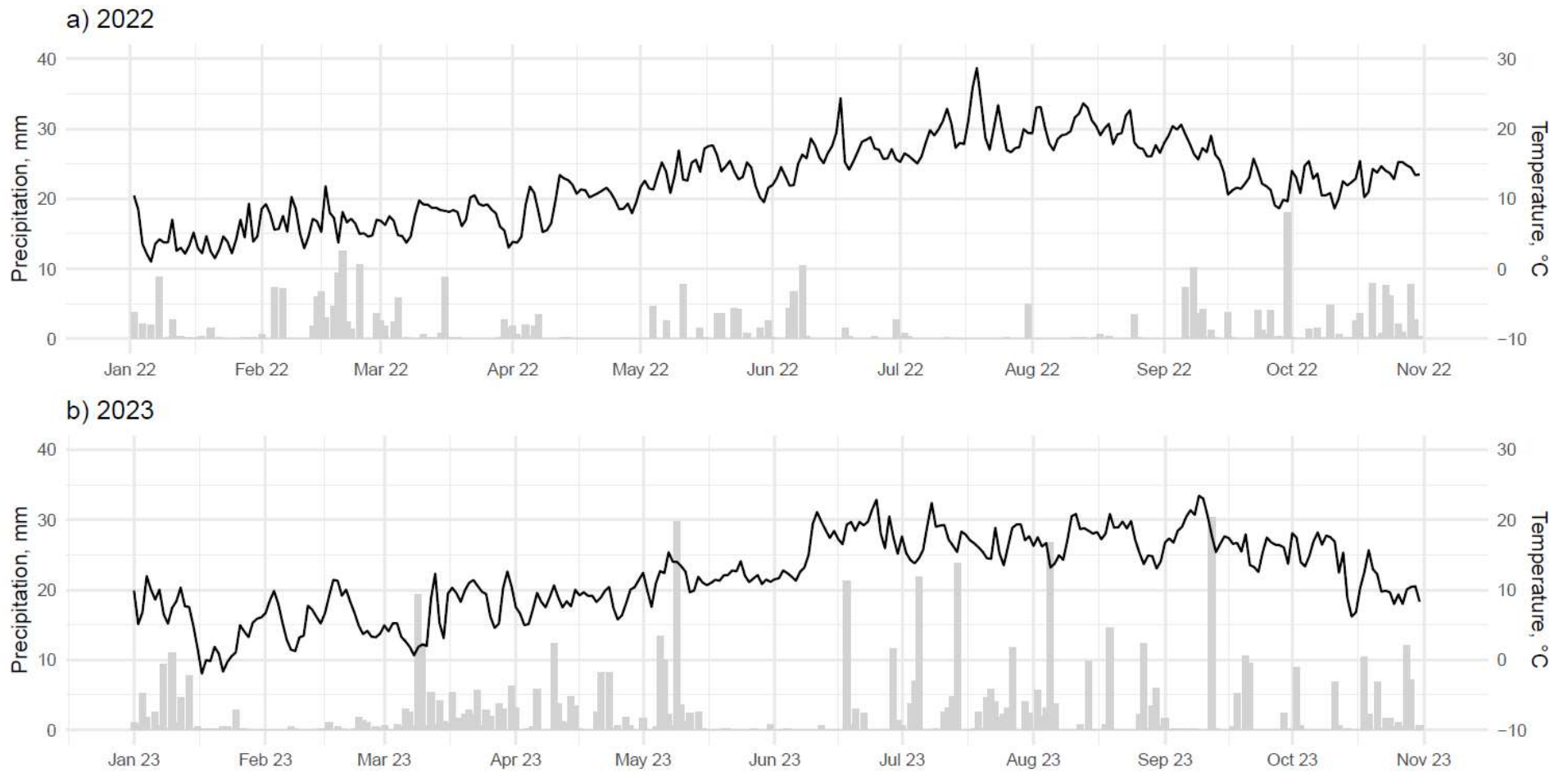


Figure 6.9: mean air temperature (shown as line) and daily total precipitation (shown as bar) recorded at the Morley weather station between 1<sup>st</sup> January and 31<sup>st</sup> October in a) 2022 b) 2023.



Figure 6.10: photo taken on 8<sup>th</sup> August 2022 of sugar beet plants in trial plot showing symptoms of severe drought stress (photo by Suzannah Harder).

#### 6.4.2 Aphid pressure – YWP22 and YWP23

Natural aphid pressure was significantly lower at the trial site location in 2023 than it was in 2022 (Figure 6.11). In 2022, 1002 *M. persicae* and 87 *M. euphorbiae* were captured and identified from the trial site YWPs, compared to only 128 *M. persicae* and 34 *M. euphorbiae* in 2023. Ladybird numbers were broadly similar between years, with a total of 59 and 46 ladybirds identified in the YWP catches in 2022 and 2023 respectively.

In 2022, seven *M. persicae* aphids captured in the YWPs were found to be positive for either BMVY and/or BChV. No BMVY and/or BChV positive aphids were identified from the 2023 YWP catches.

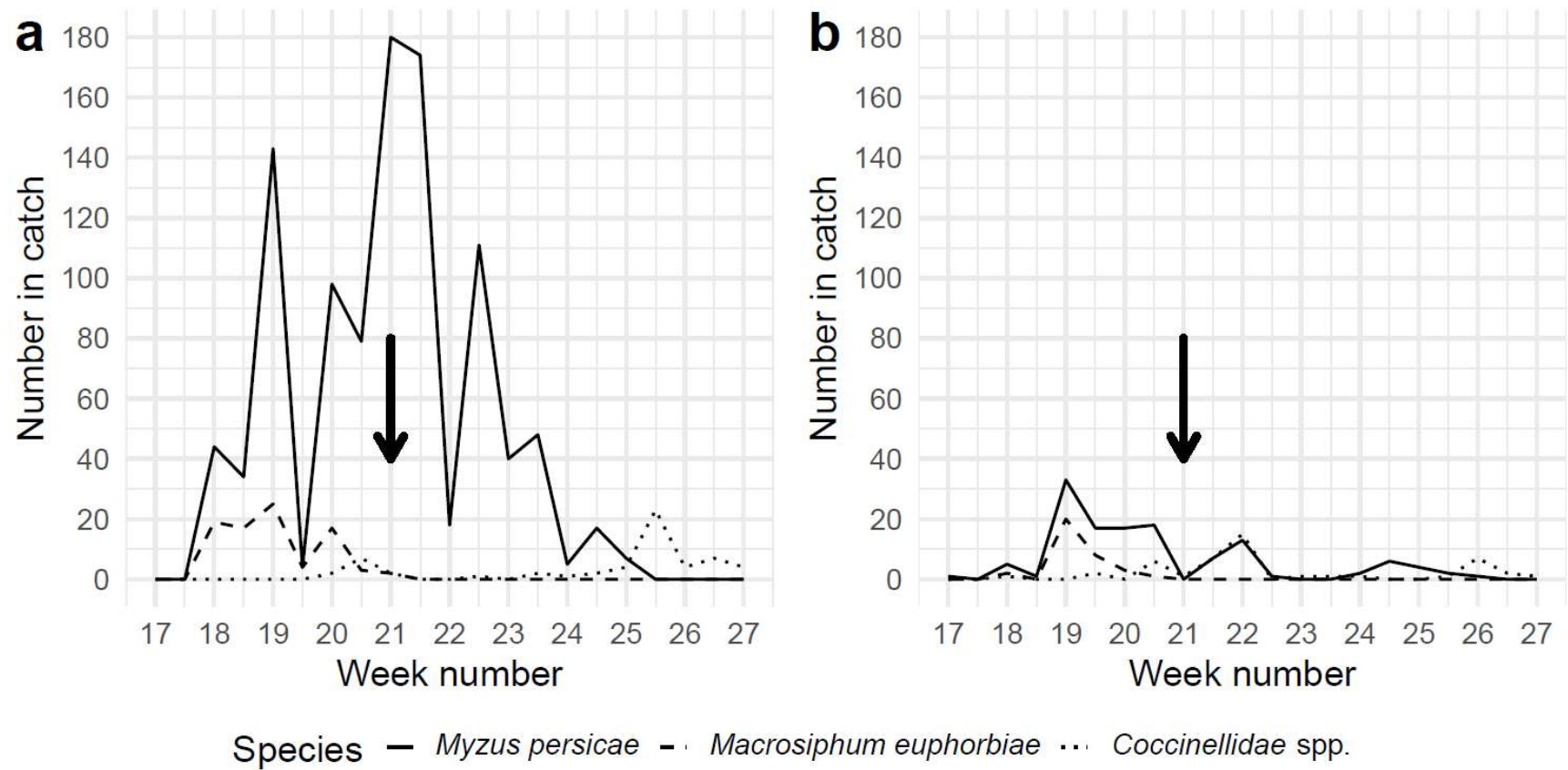


Figure 6.11: number of *Myzus persicae*, *Macrosiphum euphorbiae* and *Coccinellidae* species identified in the trial site yellow water pans (YWPs) in a) 2022, b) 2023. Arrow indicates when trial inoculation with *M. persicae* took place.

## 6.4.3 ELISA results

### 6.4.3.1 BYV

ELISA results from both 2022 and 2023 show BYV was present within the field trials (Figure 6.12). In 2022, the threshold above which a sample was deemed positive (as determined by the ELISA plate negative controls) was 0.159. The mean BYV ELISA result of every treatment exceeded this threshold, including both uninoculated treatments. However, the only treatment in which the ELISA result of every plot exceeded this threshold was 21-290 (known to contain both BChV and BYV) with individual plots ranging from 0.187 to 0.643. In 2022, Uninoculated 2 had a slightly higher mean BYV ELISA result than Uninoculated 1 at 0.2104 compared to 0.1725.

In 2023, only the two treatments known to contain BYV exceeded the negative control threshold determined to be 0.298. These were 21-290 which had a mean BYV ELISA result of 1.146, and 21-024 which had a mean ELISA result of 0.888. Both the uninoculated treatments fell below the positive threshold with mean ELISA results of 0.198 and 0.200 for Uninoculated 1 and 2 respectively.

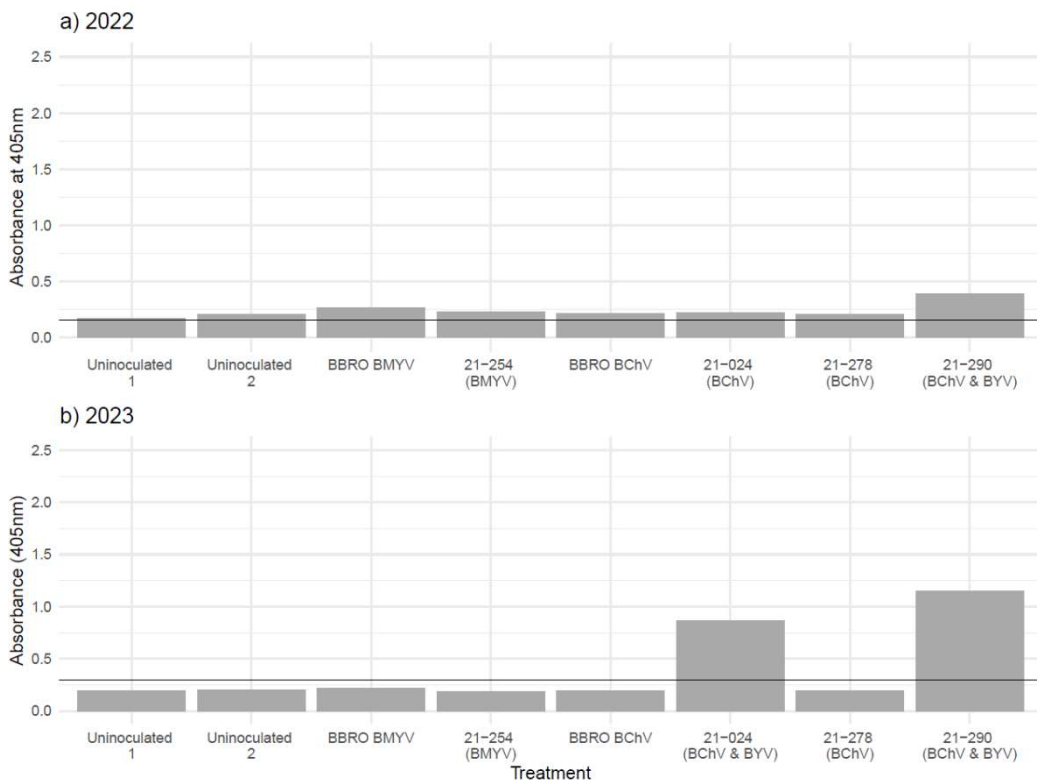


Figure 6.12: mean Beet yellow virus (BYV) ELISA results across all varieties (Polevirus resistant varieties KWS-1 and KWS-10 and susceptible varieties KWS-6 and KWS-9) for each treatment included in the field trial in a) 2022, b) 2023. Solid horizontal line indicates threshold set by negative controls above which a sample is deemed positive. BMYV – Beet mild yellowing virus, BChV – Beet chlorosis virus.

#### 6.4.3.2 Beet polerovirus - 2022

In 2022, significant differences in ELISA absorbance were identified between the different treatments at every sampling time point (Figure 6.13). Combining the ELISA results of all varieties, four weeks after inoculation, the BChV treatment 21-024 had the highest mean ELISA result at 2.41. This was significantly higher than both the BMV treatments (BBRO BMV and 21-254) which had ELISA results of 0.892 and 1.114 respectively (ANOVA,  $p < 0.001$ ). There was no significant difference between 21-024 and the BBRO BChV treatment, but 21-024 did have a significantly higher ELISA result than the other BChV cultures 21-278 and 21-290.

The lowest mean ELISA results for all virus inoculated treatments were seen eight weeks post inoculation when the site was under significant drought stress. As at the four-week time point, treatment 21-024 had the highest mean ELISA absorbance at 0.781, significantly higher than all other treatments (ANOVA,  $p < 0.001$ ). Of the virus inoculated treatments, 21-290 had the lowest ELISA absorbance with a mean of 0.292. Notably, the mean ELISA results of both treatments 21-290 and the BBRO BMV did not reach the threshold set by the negative controls above which a sample was determined positive (0.513).

ELISA absorbances recovered somewhat at the twelve-week post inoculation time point, but none of the virus inoculated treatments reached the ELISA absorbances seen four weeks after inoculation. Again 21-024 had the highest mean ELISA result, significantly higher than all other treatments at 1.732 (ANOVA,  $p < 0.001$ ). No significant difference was found in ELISA results of treatments 21-254, 21-278, BBRO BMV or BBRO BChV, however all these treatments did have significantly higher ELISA results than treatment 21-290.

Unlike at all other time points, where 21-290 had the lowest ELISA result, nineteen-weeks after inoculation BBRO BMV had the lowest ELISA result, significantly lower than all other inoculated treatments (ANOVA,  $p < 0.001$ ). No difference was seen between treatments 21-254, 21-278, 21-290 and BBRO BChV, however 21-024 again had a significantly higher mean ELISA absorbance than all other treatments.

The uninoculated treatments fell below the positive threshold as determined by the negative controls at both four- and eight-weeks post inoculation. However, at 12- and 19- weeks post inoculation, Uninoculated 2 had a mean ELISA result which exceeded this threshold and therefore was classed as positive for either BMV and/or BChV.

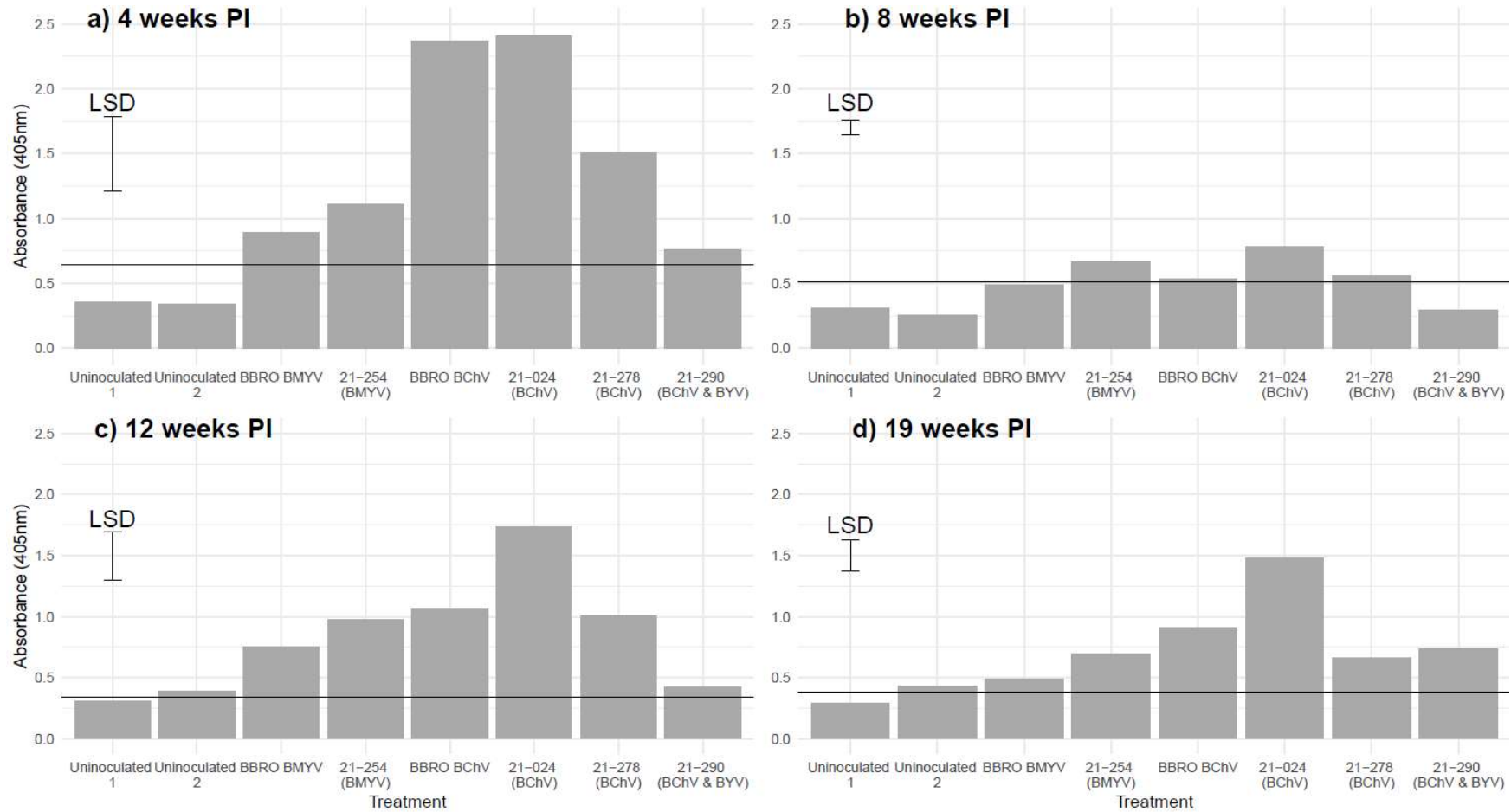


Figure 6.13: mean ELISA result of each treatment in the 2022 field trial across four time points. a) Four-weeks post inoculation (ANOVA  $p < 0.001$ ), b) eight-weeks post inoculation (ANOVA  $p < 0.001$ ) c) twelve-weeks post inoculation (ANOVA  $p < 0.001$ ), d) nineteen-weeks post inoculation (ANOVA  $p < 0.001$ ). Error bar shows least significant difference (LSD) at 5% significance. Solid horizontal line indicates threshold set by negative controls above which a sample is deemed positive. BMYV – Beet mild yellowing virus, BChV – Beet chlorosis virus, BYV – Beet yellows virus.

Focusing on the poliovirus treatments (removing the uninoculated and 21-290 BYV contaminated treatment from the analysis), significant differences were seen in mean ELISA result between the different varieties (Figure 6.14). Four weeks after inoculation, the resistant variety KWS-1 had the lowest mean ELISA result at 1.480, significantly lower than both susceptible varieties KWS-6 and KWS-9 (ANOVA,  $p < 0.001$ ). Although the other resistant variety, KWS-10, had a lower mean ELISA than both susceptible varieties, it was only significantly lower than KWS-6.

In comparison, at both the eight- and twelve-week time point, KWS-9 had a significantly higher mean ELISA result than any of the other varieties (eight-weeks; ANOVA,  $p = 0.005$ . twelve-weeks; ANOVA,  $p = 0.001$ ). Although no significant differences were identified between varieties at the nineteen-week time point, KWS-9 still had the highest mean ELISA result at 1.017. No significant differences between varieties KWS-1, KWS-10 and KWS-6 were identified at either eight- or twelve-weeks post inoculation.



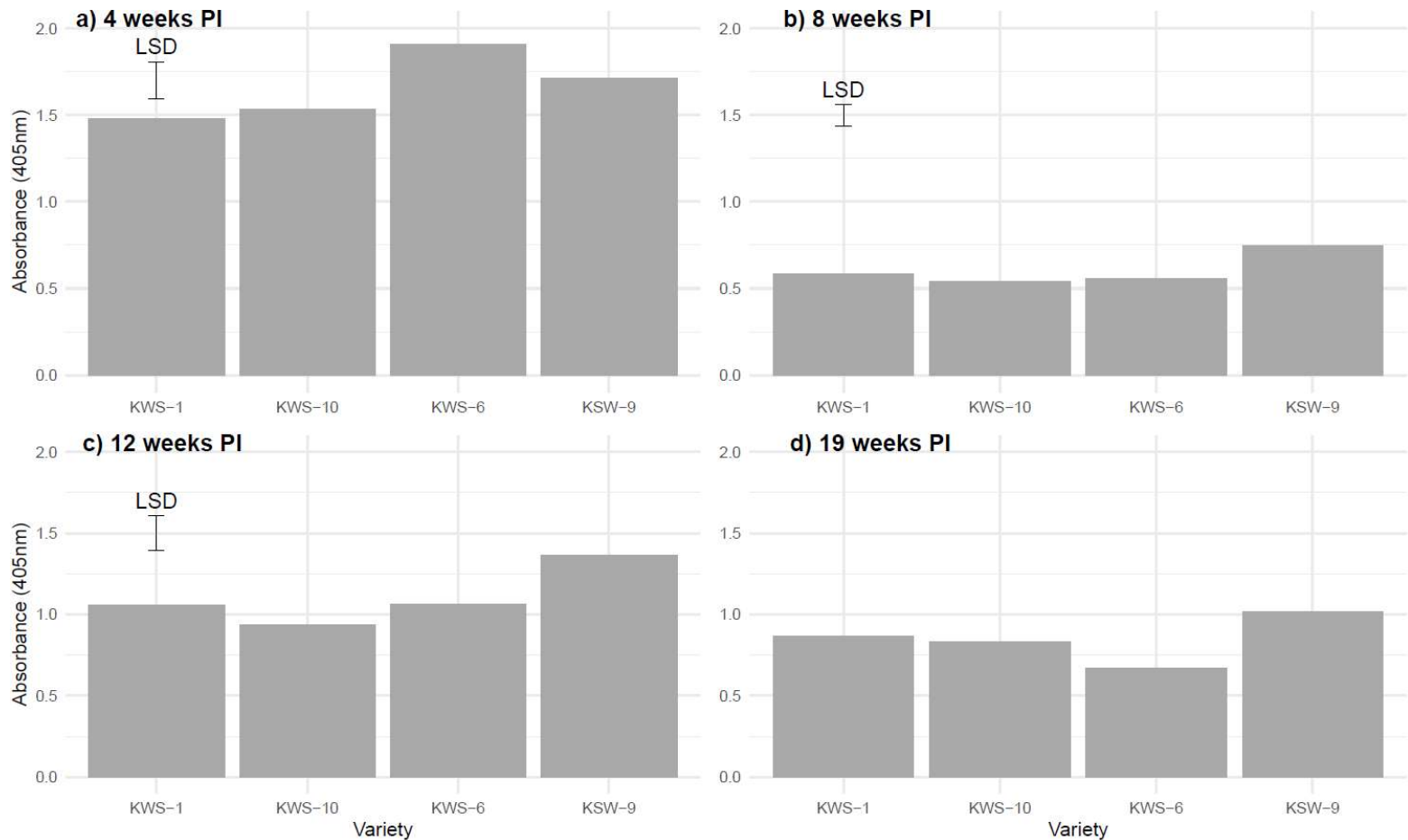


Figure 6.14: ELISA results from 2022 of varieties under Polerovirus infection (uninoculated and BYV inoculated treatments removed). KWS-1 and KWS-10 had resistance to Polerovirus infection and KWS-6 and KWS-9 were susceptible. a) 4 weeks post inoculation, ANOVA  $p < 0.001$ , b) 8 weeks post inoculation, ANOVA  $p = 0.005$ , c) 12 weeks post inoculation  $p = 0.001$ , d) 19 weeks post inoculation. Error bar shows least significant difference (LSD) at 5% significance.

At the twelve-week point a significant difference in varietal response to polerovirus treatment was detected (ANOVA treatment x variety,  $p = 0.019$ ). Under both the 21-254 and 21-278 virus treatments, KWS-9 had significantly higher mean ELISA results than the other varieties (Figure 6.15). In comparison, no differences were seen between the varieties when inoculated with 21-024, BBRO BMV or BBRO BChV.

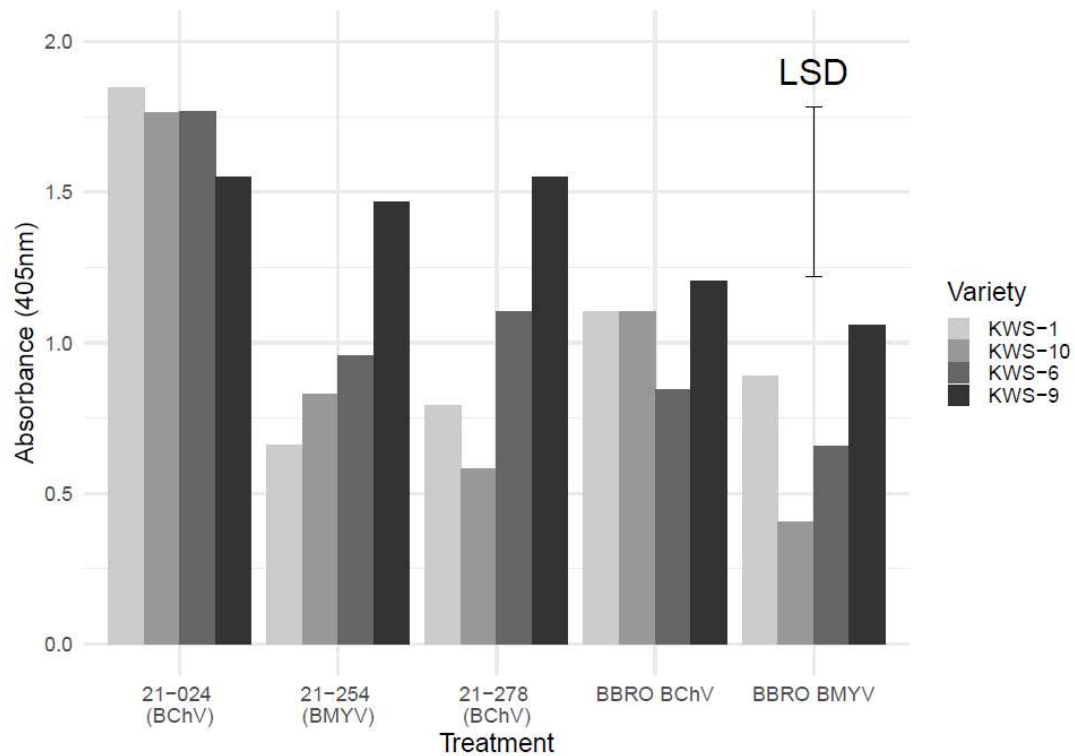


Figure 6.15: mean ELISA results of Beet mild yellowing virus (BMV) and Beet chlorosis virus (BChV) inoculated treatments from 2022, 12 weeks post inoculation for each variety (Polerovirus resistant varieties KWS-1 and KWS-10 and susceptible varieties KWS-6 and KWS-9). ANOVA (variety x treatment)  $p = 0.019$ , least significant difference (LSD) at 5% significance shown as error bar in figure. When comparing different varieties under the same treatment the LSD = 0.466.

#### 6.4.3.3 Beet polerovirus – 2023

In 2023 no significant difference in mean ELISA result was found between the virus inoculated treatments 14 weeks post inoculation (Figure 6.16). The mean ELISA result of both uninoculated treatments fell below the negative control threshold of 0.480 with ELISA results of 0.313 and 0.290 for Uninoculated 1 and Uninoculated 2 respectively. Both uninoculated treatments were also significantly lower than any of the virus treatments (ANOVA,  $p < 0.001$ ).

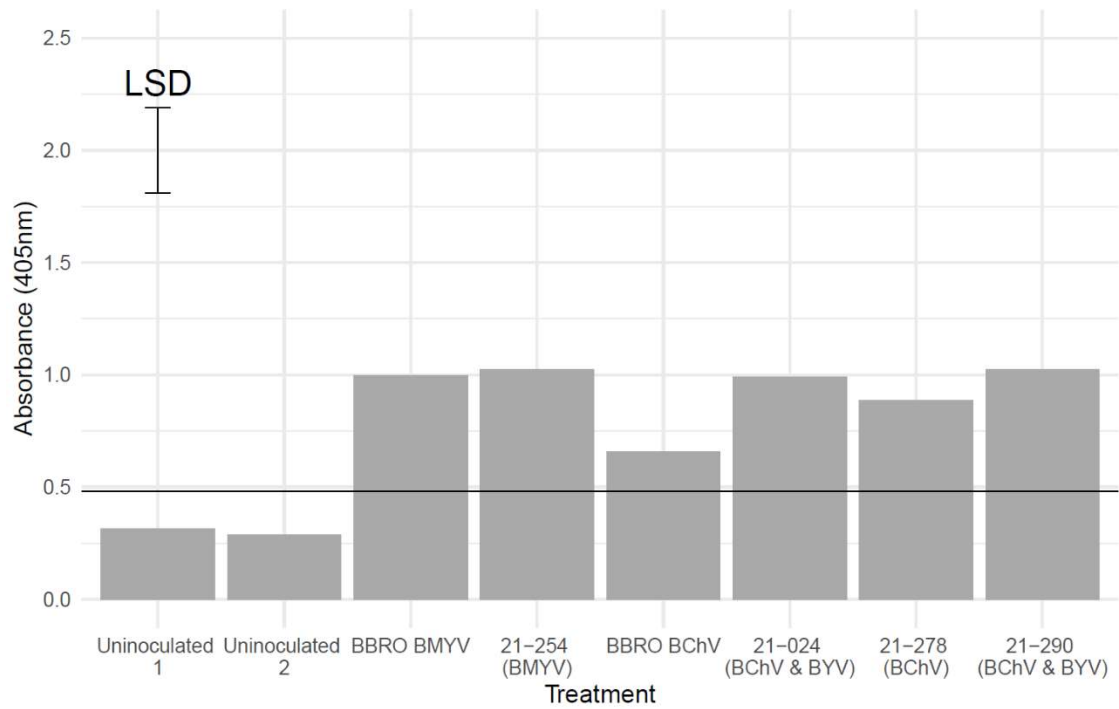


Figure 6.16: mean ELISA result for each treatment from 2023 field trial, 14 weeks post inoculation. Solid line indicates threshold set by negative controls above which an ELISA result is considered positive. ANOVA  $p < 0.001$ . Error bar shows least significant difference (LSD) at 5% significance. BMVYV – Beet mild yellowing virus, BChV – Beet chlorosis virus, BYV – Beet yellows virus.

Under polerovirus inoculation (excluding uninoculated and BYV contaminated treatments), KWS-9 had significantly higher mean ELISA result compared to the other three varieties (Figure 6.17, ANOVA  $p = 0.001$ ). The resistant variety KWS-10 had the lowest mean ELISA result, however it was not significantly lower than either the susceptible variety KWS-6 or the other resistant variety KWS-1.

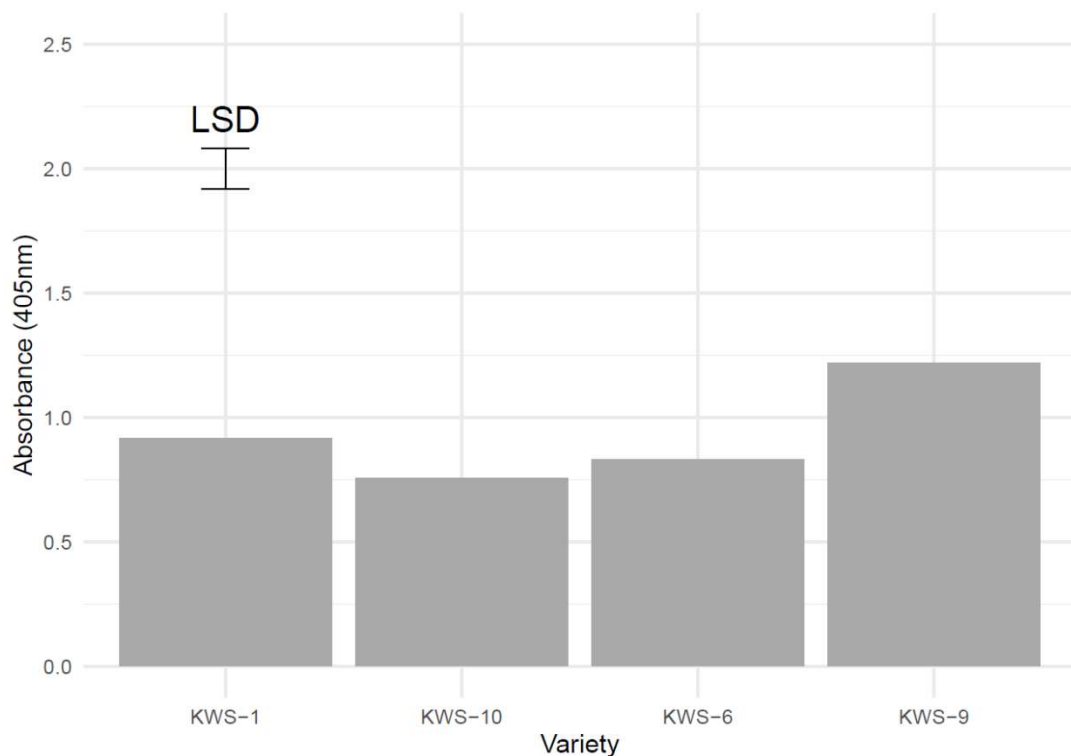


Figure 6. 17: mean ELISA result for each variety (Polerovirus resistant varieties KWS-1 and KWS-10 and susceptible varieties KWS-6 and KWS-9) under Polerovirus infection (excluding uninoculated and BYV infected treatments 21-290 and 21-024). Error bar shows least significant difference (LSD) at 5% significance. ANOVA  $p = 0.001$ .

#### 6.4.4 Canopy cover

In 2022, under virus inoculation, the resistant varieties KWS-1 and KWS-10 had significantly larger canopy cover than the susceptible varieties KWS-6 and KWS-9 (Figure 6.18, ANOVA,  $p < 0.001$  at both time points). There was no significant difference in canopy area between the two resistant varieties, however KWS-6 did have a significantly larger canopy area than KWS-9 in both July and September. Notably, the mean canopy areas of all varieties decreased between July and September most likely as result of the beet moth infestation (see Figure 6.5).

The mean canopy area of Uninoculated 1 was 74.21% in July 2022 and 69.47% in September 2022. This was higher than the mean canopy area of Uninoculated 2 which was 55.63% and 57.09% respectively. This corresponds to the ELISA data which identified a higher level of beet polerovirus and BYV in Uninoculated 2 plots compared to Uninoculated 1.

In July 2023, of the virus inoculated treatments, those infected only with a beet polerovirus (treatments BBRO BMYV, BBRO BChV, 211-254 and 21-278) had significantly larger canopy areas than those treatments which also contained BYV (21-024 and 21-290), (Figure 6.19a, ANOVA,  $p = 0.024$ ). Although significant differences were seen in canopy area between the varieties under virus

infection, they did not support the findings of 2022. In 2023, KWS-6 had the smallest mean canopy area under virus infection (Figure 6.19b), with no significant difference between the other three varieties. No significant differences in canopy area were identified in September 2023, with all but two plots (both in Uninoculated 2 treatments) having greater than 90% canopy cover.

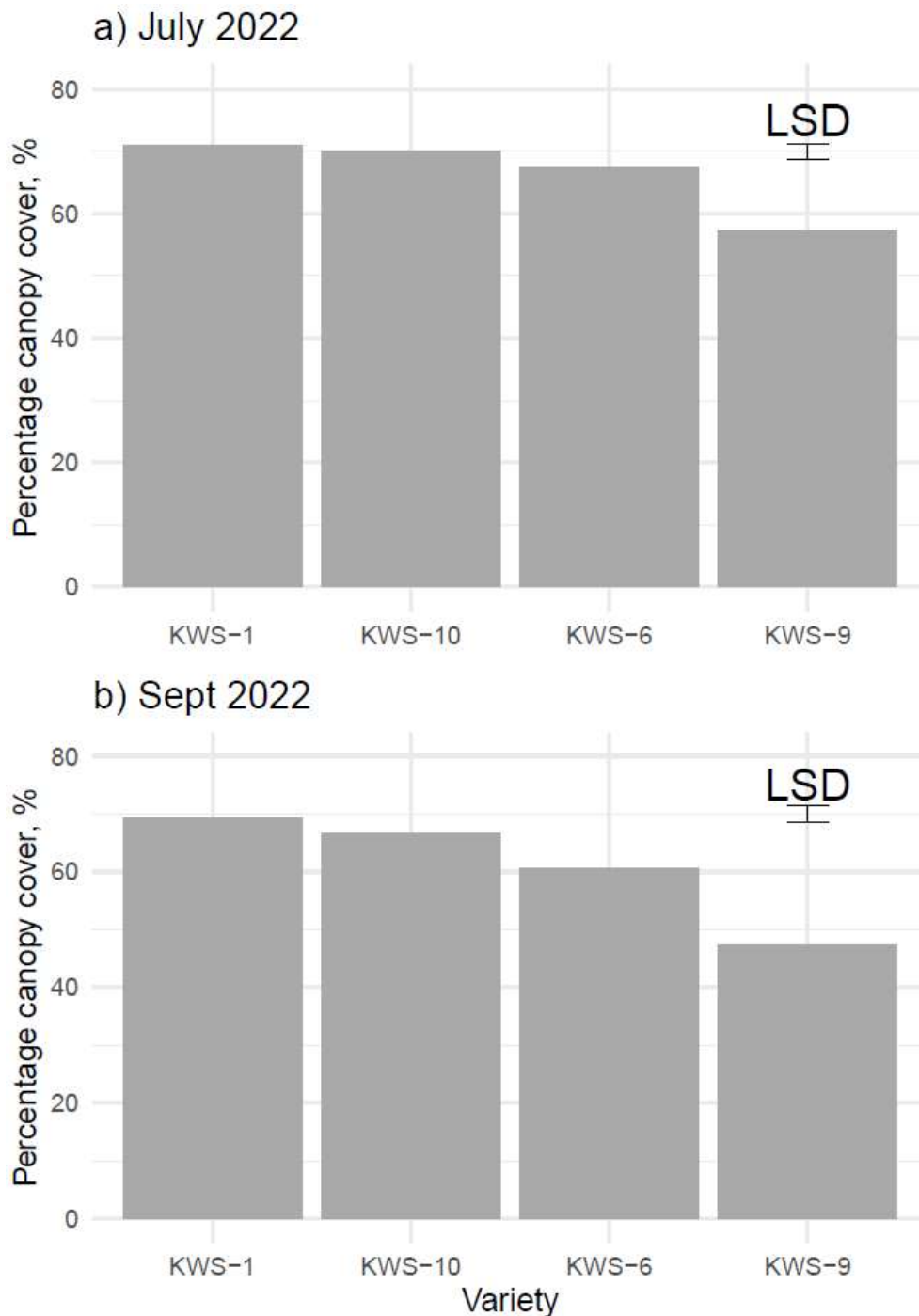


Figure 6.18: mean canopy cover (as a percentage of plot area) of each variety (KWS-1 and KWS-10 polerovirus resistant, KWS-6 and KWS-9 susceptible) in the 2022 field trial under virus inoculation (excluding both uninoculated treatments). a) on 22<sup>th</sup> July 2022, ANOVA  $p < 0.001$ , b) on 15<sup>th</sup> September 2022, ANOVA  $p < 0.001$ . Error bar shows least significant difference (LSD) at 5% significance.

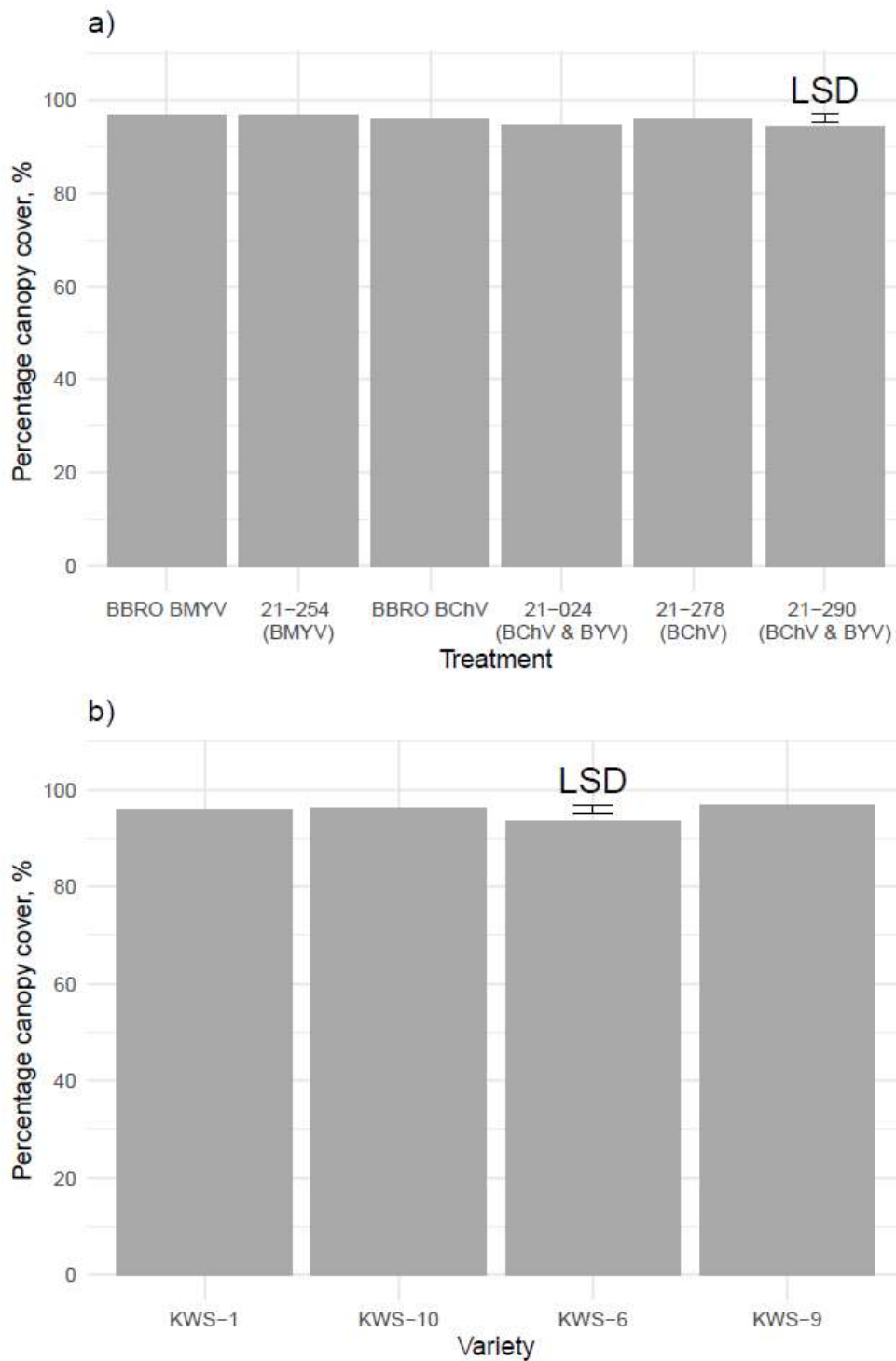


Figure 6.19: mean canopy cover (as percentage of plot area) for 2023 field trial a) mean of Beet mild yellowing virus (BMV), Beet chlorosis virus (BChV) and Beet yellows virus (BYV) inoculated treatments, ANOVA  $p = 0.024$ . b) mean of each variety (KWS-1 and KWS-10 polerovirus resistant, KWS-6 and KWS-9 susceptible) under virus infection, ANOVA  $p < 0.001$ . Error bars show least significant difference (LSD) at 5% significance.

#### 6.4.5 Canopy chlorophyll

In September 2022 canopy chlorophyll levels (as estimated by mNDblue value) were significantly higher in the resistant varieties than the susceptible varieties across all treatments (Figure 6.20, ANOVA,  $p < 0.001$ ). Under all virus treatments, KWS-10 had the highest mNDblue value though it was only significantly higher than KWS-1 under BBRO BChV, 21-278 and 21-290. Of the susceptible varieties, KWS-9 had higher canopy chlorophyll levels under virus infection than KWS-6. All varieties exhibited the lowest canopy chlorophyll levels under the 21-290 treatment (which contained both BChV and BYV). mNDblue results from July 2022 showed no significant differences, however the same trend of the susceptible varieties having lower values than the resistant varieties was observed.

Results from both July and September 2023 confirmed the findings of 2022, with higher canopy chlorophyll levels in the resistant varieties when under virus infection. However, of the two susceptible varieties, only KWS-6 had significantly lower mNDvalues than the resistant varieties across all virus treatments (ANOVA, July 2023  $p = 0.001$ , September 2023  $p < 0.001$ ). As in 2022, the lowest mNDblue values for all varieties were identified under the virus treatments that contained BYV (21-290 and 21-024 in 2023).

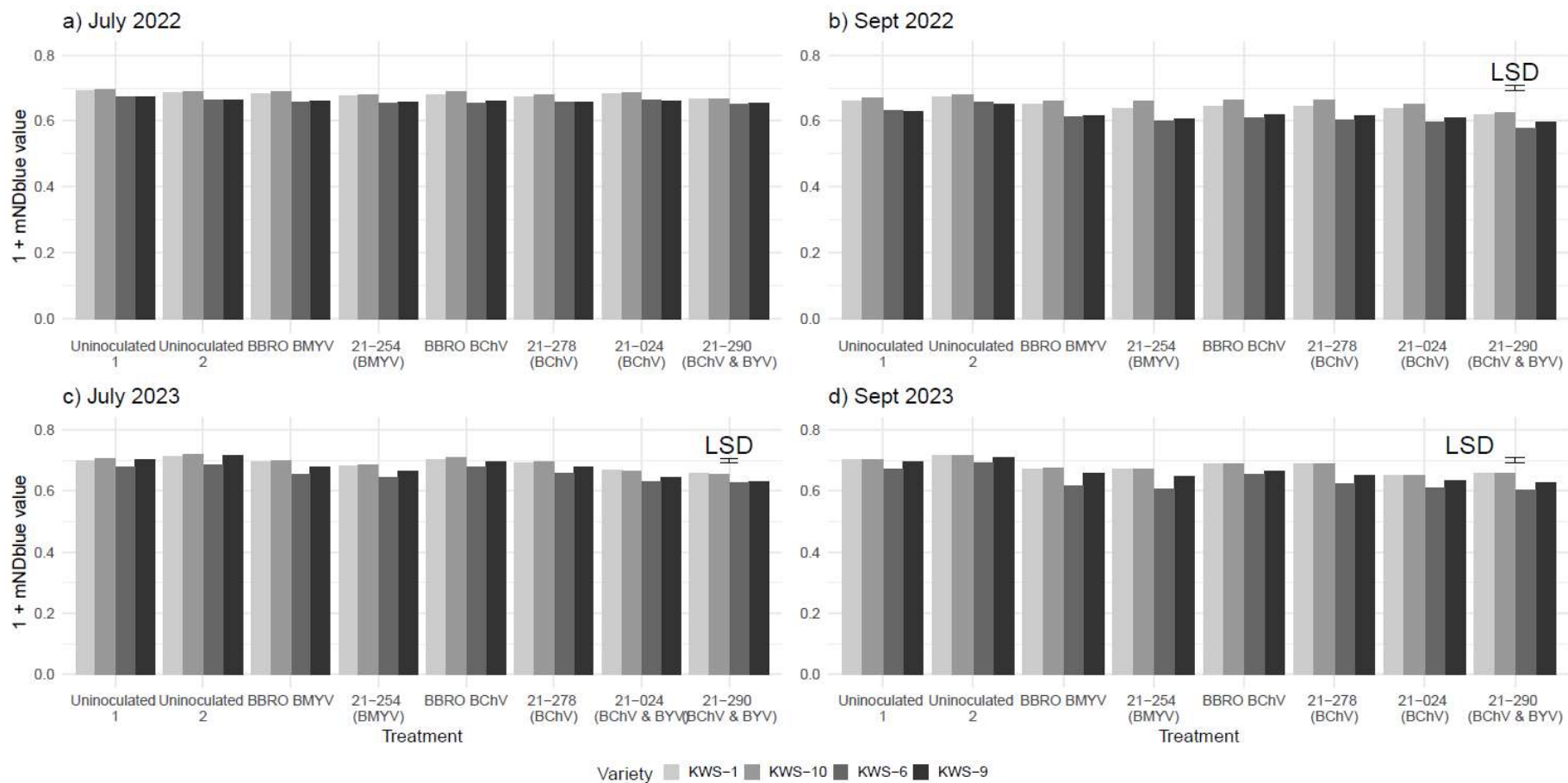


Figure 6.20: mean mNDblue value of varieties (KWS-1 and KWS-10 Polorovirus resistant, KWS-6 and KWS-9 susceptible) under different virus treatments. Results shown as 1 + mNDblue value to give a positive number and aid result interpretation, higher mNDblue values indicated plots with greater chlorophyll content, as measured on a) 22<sup>nd</sup> July 2022, b) 15<sup>th</sup> September 2022, ANOVA (variety x treatment)  $p < 0.001$ , c) 9<sup>th</sup> July 2023, ANOVA (variety x treatment)  $p = 0.001$ , d) 23<sup>rd</sup> September 2023, ANOVA (variety x treatment)  $p < 0.001$ . Error bars show least significant difference (LSD) at 5% significance. BMV – Beet mild yellowing virus, BChV – Beet chlorosis virus, BYV – Beet yellows virus.



#### 6.4.6 Sucrose yield

In both 2022 and 2023 the Uninoculated 2 treatment was removed from the harvest data analysis. In 2022 this was due to higher polerovirus ELISA result in the Uninoculated 2 treatment compared to the Uninoculated 1, indicating a higher level of virus contamination. In 2023 Uninoculated 2 was removed due to higher variation in the sucrose yield compared to Uninoculated 1. No other plots were removed from the analysis in 2023, however in 2022 three plots were removed due to low clean sample weights (each outside the range of the mean clean sample weight plus two times the standard deviation).

Overall, yields were higher in 2023 than in 2022 (Figure 6.21). In 2023, the mean sucrose yield across all varieties for the uninoculated treatment was 15.07 tonnes.ha<sup>-1</sup> compared to just 8.82 tonnes.ha<sup>-1</sup> in 2022. In both years, significant differences in sucrose yield were identified between treatments, however these differences were not consistent. In 2022 no significant difference was found between the sucrose yield of the uninoculated and the BBRO BMV treatments, although all other virus inoculated treatments did yield significantly lower than the uninoculated (ANOVA,  $p = 0.001$ ). Whereas in 2023, all virus treatments yielded significantly lower than the uninoculated including the BBRO BMV treatment (ANOVA,  $p < 0.001$ ). The treatments containing BYV yielded the lowest in both 2022 and 2023. In 2023 the two BYV containing treatments (21-290 and 21-024) had sucrose yields significantly lower than any other treatment (ANOVA,  $p < 0.001$ ).



Figure 6. 21: mean sucrose yield across all varieties (Polerovirus resistant varieties KWS-1 and KWS-10 and susceptible varieties KWS-6 and KWS-9) from a) 2022 field trial, ANOVA  $p = 0.001$ , b) 2023 field trial, ANOVA  $p < 0.001$ . Error bars show least significant difference (LSD) at 5% significance. BMYV – Beet mild yellowing virus, BChV – Beet chlorosis virus, BYV - Beet yellows virus.

Under virus infection, in 2022 the resistant varieties had higher mean sucrose yields than the susceptible varieties (Figure 6.22). However the only significant difference was between the resistant variety KWS-10, which had the highest yield at 7.60 tonnes.ha<sup>-1</sup>, and the susceptible variety KWS-9, which had the lowest yield at 6.63 tonnes.ha<sup>-1</sup> (ANOVA,  $p = 0.043$ ). These differences were not found in 2023, when KWS-1 (resistant variety) yielded significantly higher than any other variety at 11.58 tonnes.ha<sup>-1</sup> (ANOVA,  $p = 0.004$ ).

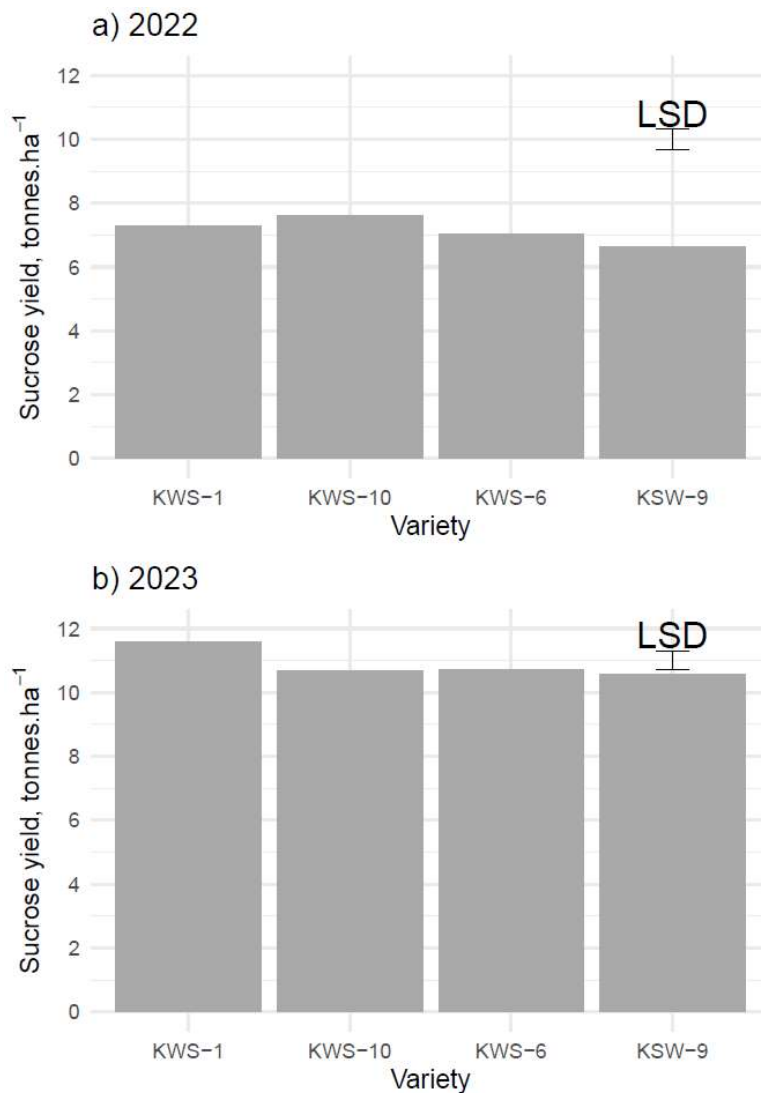


Figure 6.22: mean sucrose yield of each variety (Polerovirus resistant varieties KWS-1 and KWS-10 and susceptible varieties KWS-6 and KWS-9) when under virus infection (uninoculated treatments removed), a) from 2022 field trial, ANOVA  $p = 0.043$ . b) from 2023 field trial, ANOVA  $p=0.004$ . Error bars show least significant difference (LSD) at 5% significance.

Assessing the sucrose yield lost by each variety under virus infection (when compared to the uninoculated) in 2023 a clear trend was observed (Figure 6.23). Under all virus treatments, the resistant varieties (KWS-1 and KWS-10) maintained more of their uninoculated yield than the susceptible varieties. This trend was not seen in 2022, when the susceptible varieties had a lower yield loss under the 21-290 virus treatment than the resistant varieties.

Linear regression showed a significant relationship between mNDblue and sucrose yield achieved, in both 2022 and 2023 (Figure 6.24). This relationship shows that as plot mNDblue values deviate away from the controls the percentage yield achieved decreases. Overall, this relationship was more significant in 2023 than 2022, and in both years the July mNDblue data had higher  $R^2$  values

than the September data; Linear regression, July 2022  $p < 0.001$   $R^2 = 0.51$ , September 2022  $p = 0.009$   $R^2 = 0.27$ , July 2023  $p < 0.001$   $R^2 = 0.87$ , September 2023  $p < 0.001$   $R^2 = 0.84$ .

In both years, the lowest percentage of uninoculated yield achieved and the greatest change in mNDblue was seen in the BYV containing treatments. The BBRO BChV had the smallest mNDblue change however the model was not significantly improved by incorporating virus treatment or variety.

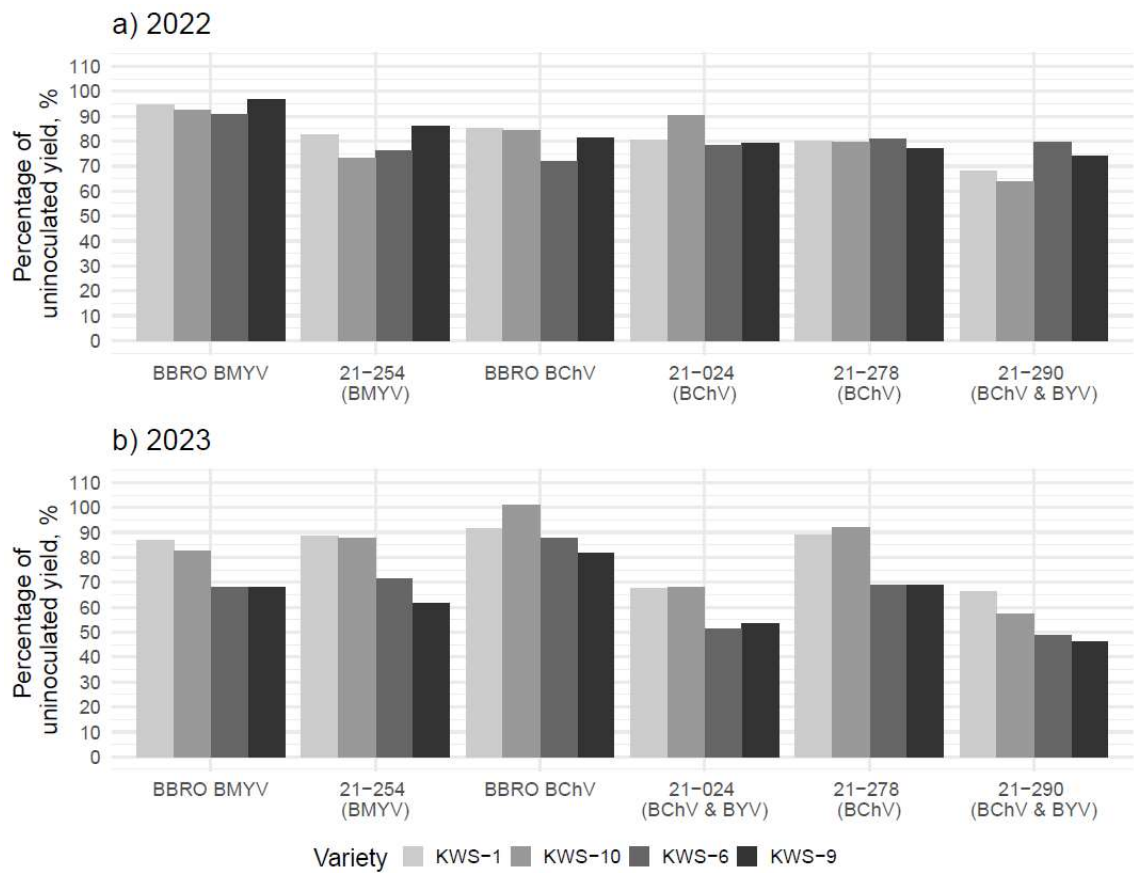


Figure 6.23: percentage of uninoculated yield achieved under virus infection (calculated as percentage of mean sucrose yield of the Uninoculated 1 treatment for each variety) a) from 2022 field trial b) from 2023 field trial. KWS-1 and KWS-10 had resistance to Polorovirus and KWS-6 and KWS-9 were susceptible. BMYV-Beet mild yellowing virus, BChV – Beet chlorosis virus, BYV – Beet yellows virus.

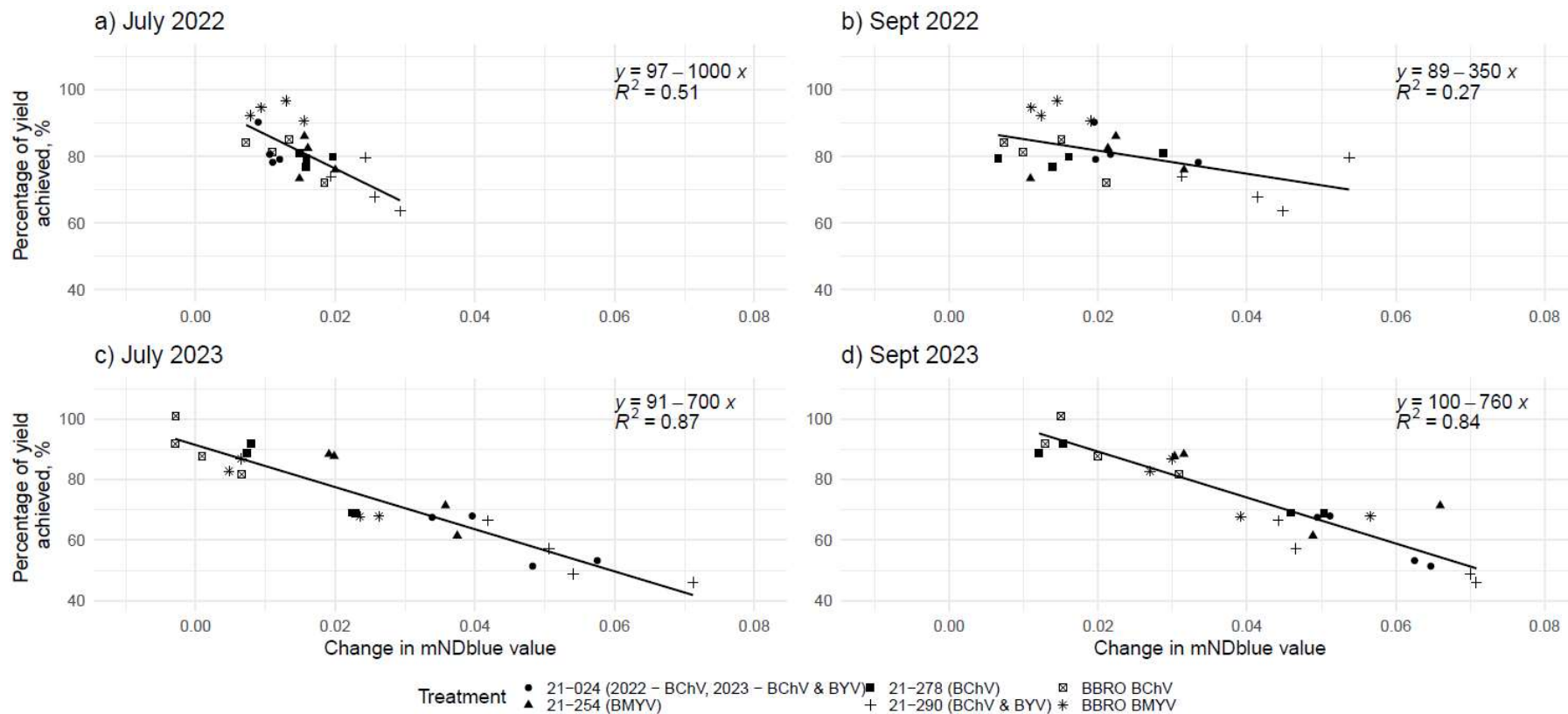


Figure 6.24: linear regression of percentage of uninoculated yield achieved and change in mNDblue value from the uninoculated across all varieties (Polerovirus resistant varieties KWS-1 and KWS-10 and susceptible varieties KWS-6 and KWS-9) under different virus treatments a) 2022 field trial using mNDblue value from 22<sup>nd</sup> July 2022, b) 2022 field trial using mNDblue value from 15<sup>th</sup> September 2022 c) 2023 field trial using mNDblue value from 9<sup>th</sup> July 2023, d) 2023 field trial using mNDblue value from 23<sup>rd</sup> September 2023. BMYV – Beet mild yellowing virus, BChV – Beet chlorosis virus, BYV – Beet yellows virus.

## 6.5 Discussion

### 6.5.1 Methodology

Ensuring the field experiments were kept free from natural aphid infection posed a significant challenge, particularly under the naturally high aphid pressure experienced in 2022. ELISA results from the uninoculated plots in 2022 showed that, unfortunately, contamination with naturally occurring viruliferous aphids had occurred. Some of this impact was mitigated by incorporating twice the number of uninoculated control plots into the experimental design, allowing for the less contaminated uninoculated controls to be used in the data analysis. However, it is likely that the virus treated plots were also contaminated with naturally occurring aphids and yellowing viruses. The lower natural aphid pressure in 2023 helped ensure a 'cleaner' experiment, with the uninoculated plots remaining free from natural virus infection and, presumably, therefore also the virus-treated plots.

Nevertheless, in both 2022 and 2023 the inoculation methodology was successful with susceptible varieties in all treatments becoming infected with virus. Importantly, good control of virus spread was also achieved, particularly in 2023 where BYV was only detected in those plots inoculated with a BYV containing virus culture. This was also evidenced by visual assessment of the trial showing a clear demarcation between trial plots and the non-inoculated discard sugar beet (Figure 6.25).

In 2022 visual assessment of yellowing symptoms also showed that, although only a 50% inoculation rate was used (infecting every other plant with viruliferous aphids), close to 100% infection was achieved. In 2023 aphid numbers in the laboratory virus cultures were lower than in 2022, particularly in the BBRO BChV culture. This meant that fewer aphids were released into the trial plots, and consequently yellowing symptoms were not visible in a small number of the susceptible variety plants. This may have contributed to the lower mean ELISA result and higher sucrose yield for this treatment compared to the other BChV treatment. Due to the scale of these field trials, counting an exact number of aphids on to each plant was not feasible, however future studies could do more to ensure aphid numbers were consistent between virus treatments. Nevertheless, the yield loss caused by the BBRO BChV treatment of around 20% for susceptible variety KWS-9 was consistent with published findings (Hossain et al., 2021; Stevens et al., 2004), and the overall effect of the few uninfected plants is likely to be minimal.



*Figure 6. 25: Image of inoculated trial plots on 30<sup>th</sup> June 2022, showing inoculated sugar beet within plots with yellowing symptoms whilst plants outside of treatment area remaining green (photo by Suzannah Harder).*

### 6.5.2 ELISA

Although the resistant varieties tended to have lower ELISA absorbances than the susceptible varieties (particularly KWS-9), their ELISA results still indicated virus infection under all virus treatments. This suggests these varieties have only partial, rather than complete, resistance to BMV and BChV infection. Interestingly, KWS-6, although being described by the seed breeder as susceptible, tended to align more closely to the resistant varieties than the other susceptible variety. This indicates it may have a low level of virus resistance, for example due to the involvement of a minor resistance gene not declared by the seed breeder. In 2022 the ELISA results from twelve-weeks after inoculation suggested that the resistant varieties resisted infection from some virus cultures better than others. However, this finding was not supported in 2023, or at any other time point in 2022 and is therefore more likely to be a consequence of the challenging growing conditions experienced in 2022.

Generally, ELISA results were not a good predictor of yield loss. In 2022 the 21-024 BChV treatment gave the highest mean ELISA results at every time point, however this treatment did not cause the

greatest yield loss. Virus titre also differed throughout the season, with the challenging growing conditions of 2022, namely the high air temperatures and drought conditions, clearly affecting virus titre within the inoculated plants. Reliably assessing variety performance under virus infection by ELISA is therefore difficult as weather conditions and time after inoculation has a large influence on results. From a diagnostic perspective, the ELISA results from 2022 show that virus testing via ELISA should not be conducted whilst test plants are under drought stress as false negative results are likely.

ELISA testing, as a tool for assessing variety performance, has the joint disadvantage of being time consuming whilst only considering a small number of plants per plot. The ELISA testing conducted here, assessed only five plants per plot yet took five days to complete at each time point. The methodology followed in 2023, where a single ELISA test was conducted shortly before harvest, would be more practical to adopt in future trials, in combination with remote sensing using multispectral and RGB imaging to monitor symptom development. This approach enables the resistance of test varieties to be established whilst minimising the time commitment and, in most years, avoiding the need to sample when the crop is under drought stress.

### 6.5.3 Canopy cover and chlorophyll estimation

The most severe foliar symptoms were observed under the combined BChV and BYV virus treatments. This result was expected and consistent with previous studies which identify BYV as the most damaging virus in the virus yellows complex (Smith and Hallsworth, 1990) and the findings of Hossain et al (Hossain et al., 2021) who found combined BYV and BChV infection caused more significant yield losses than either virus individually.

Unlike ELISA, drone assessments of canopy cover and chlorophyll levels considered whole trial plots, rather than a subset of plants from each plot. This made the measurements more reliable, with less chance of outliers skewing the data set. Drone analysis is also less time consuming, taking approximately one day to complete (including image analysis). This makes it an attractive phenotyping tool for this type of large-scale experiment. One drawback of these measurements, however, is that canopy health is affected by multiple factors not just virus infection. Nutrient deficiency, fungal infection and pest damage can all cause yellowing symptoms easily confused with virus infection (Stevens, 2019). Any measurement of yellowing symptoms, such as mNDblue measurements, are therefore not virus specific and should be used in conjunction with a diagnostic tool (such as ELISA) to ensure symptoms are due to virus infection.



Canopy cover measurements can also be affected by external factors. For example, in 2022 canopy cover was much smaller than in 2023 across all varieties due to the drought conditions experienced. Although differences between the varieties were identified in 2022, with the resistant varieties showing larger canopy areas than the susceptible, it is possible these differences were due to the varieties ability to tolerate the other stresses affecting the 2022 crop, particularly as these differences were not identified in the 2023 trial.

mNDblue measurements were used to estimate canopy chlorophyll levels, enabling quantification of the visible yellowing symptoms. As previously discussed, visual assessment of yellowing showed clear demarcation between inoculated and uninoculated plants. However, it was also possible to identify the susceptible and resistant varieties based on yellowing symptoms in both years (Figure 6.26). mNDblue measurements supported these observations, with the resistant varieties having higher mNDblue values than the susceptible under virus inoculation. Previously, yellowing symptoms have been scored through visual assessment (Hossain et al., 2021; Smith and Hallsworth, 1990), but this approach risks assessor bias, as well as inconsistency between assessors. Utilising drone based mNDblue measurements, which are captured within a short time period (approximately ten minute flight time to cover these experiments) and calibrated using a reflectance panel to correct the imagery, overcomes these risks whilst allowing significant differences in varietal performance under virus infection to be identified.



*Figure 6. 26: Image taken of 2022 field trial, showing clear differences in yellowing symptoms between BBRO Beet chlorosis virus inoculated plots. Plot on left is resistant variety KWS-10, plot on right is susceptible variety KWS-9. Image taken on 31<sup>st</sup> October 2022 (the same day the trial was harvested).*

#### 6.5.4 Yields

The confounding factors of drought and beet moth infestation clearly affected yields in 2022, with all treatments yielding lower than in 2023, including the uninoculated controls. Work by Clover et al (1999) showed no interaction between BYV infection and drought stress, with their effects on yield being additive. To date no studies have investigated the combined effect of BMV or BChV and drought, but they are assumed to exhibit the same additive relationship as BYV. Confirming this relationship will become more pertinent should drought conditions become more commonplace with future climate volatility.

Although beet moth is native to the UK, 2022 was the first year it caused significant damage to the UK sugar beet crop (Collier, 2023). It was unfortunate that beet moth occurred in the trial in 2022, but the infestation was at least consistent with visual assessment of the trial at the end of September confirming all plants within the trial field were infested. No differences in infestation

levels were observed between varieties or treatments and therefore the effect was presumed to be equal across the entire trial.

It was hypothesised that although the resistant varieties may not have higher sucrose yields than the susceptible varieties (due to yield drag (BBRO, 2023b)), they would achieve more of their potential yield when under virus infection. Given the challenges of the 2022 sugar beet growing season it is unsurprising that yield results were closer to this expectation in 2023 than they were in 2022. Nevertheless, the results of the 2022 experiment should not be assumed purely as consequence of the unusual growing season. One key finding from 2022, was the significantly lower yield loss caused by BBRO BMV culture compared to inoculation with the BMV culture 21-254. This result does fit with observations from BBRO virus trials, where the BBRO BMV culture appears to be having a lesser impact on yield year on year (personal communication, BBRO) and with the genetic divergence of the BBRO BMV isolate as outlined in Chapter 5.

The 2023 field trial results did not confirm the BBRO BMV culture as being 'weaker' than 21-254. There are many possible reasons for this. Firstly, although it is assumed that BMV will respond similarly to BYV when in combination with drought stress this may not be the case. The pathogenicity of the BBRO BMV culture may have been weakened by the drought conditions, whilst the 21-254 isolate was not. Alternatively, either isolate may have evolved between 2022 and 2023 with consequences on pathogenicity. Unfortunately, as the isolates were not re-sequenced ahead of the 2023 field trial it is not possible to confirm this. Future trials working with multiple virus strains should sequence all cultures at the point of trial inoculation to allow identification of genetic changes between years.

Conversely to the BMV results, although in 2022 no significant differences in yield loss were seen between the BChV cultures, differences were found in 2023 with the BBRO BChV causing lower yield loss than the 21-278 isolate. Importantly from a breeding perspective, however, under all virus treatments in 2023 the resistant varieties achieved more of their potential yield than the susceptible varieties. Therefore, although there do appear to be differences in pathogenicity between the BMV and BChV isolates tested here, none are considered "resistance breaking" strains.

In 2022 and 2023 the most severe yield losses were seen in the plots inoculated with BChV and BYV. As previously discussed, this is consistent with the findings of previous studies (Hossain et al., 2021; Smith and Hallsworth, 1990). Notably, although neither KWS-1 or KWS-10 have BYV resistance according to the seed breeder, both varieties achieved more of their potential yield under the combined BChV and BYV treatments in 2023 than the susceptible varieties. Nevertheless, these

varieties still suffered the greatest yield loss under these treatments and these results do not indicate the varieties have resistance to BYV. However, it does suggest that the resistance mechanism of both KWS-1 and KWS-10 to beet poleroviruses still holds whilst under BYV infection. Mixed infections are known to occur within the UK, a finding confirmed by the data outlined in Chapter 3. Therefore, to be of optimum benefit, resistant varieties will need to withstand combined infections and ideally also have BYV resistance.

#### 6.5.5 Predicting yield loss

One major drawback of field trials for assessment of variety performance is their significant time cost. Consequently, any way in which variety yield performance can be reliably predicted earlier in the growing season, rather than waiting for harvest in autumn/winter, is advantageous. The significant relationship identified here between mNDblue value and yield, enables such predictions and therefore has potentially large benefits to the sugar beet industry. The simplicity of the model is encouraging, accounting for all virus treatments and varieties tested here. The ability of the model to estimate yield even in challenging seasons like 2022, where confounding factors such as drought and beet moth infestation also affected the trial, is particularly promising. The model will, however, need further validation, for example, across different soil types and varieties from other seed houses.

# Chapter 7: General Discussion

## 7.1 Context of the project

It is ninety years since yellowing symptoms observed in sugar beet plants were suggested to arise from a viral infection (Quanjer, 1934). Since that time huge progress has been made in characterising the different viruses now known to cause virus yellows disease. From a disease control point of view, arguably the greatest breakthrough has been the use of neonicotinoid treated sugar beet seed since its introduction in 1994 until it was banned by the European Union in 2018. Although emergency authorisations, from the UK government, have resulted in the use of neonicotinoid treated sugar beet seed twice since 2018 (2022 and 2023), concerns over environmental safety and the need to adopt more sustainable farming practices mean neonicotinoid seed treatments are not a long-term solution (EASAC, 2023). Instead, the development of virus-resistant sugar beet varieties is now seen as the best solution to the problem of virus yellows disease, both in the UK and in Europe integrated alongside other aphid/virus management strategies.

Although progress was, and is, being made to develop novel tolerant and resistant sugar beet varieties, the need to renew our understanding of the profile of the disease and the likely durability of these varieties is vital. Through the survey work, genetic analysis, phenotyping assessments and field trials described in this thesis I have initiated an update to our knowledge base on this subject. Below the key findings of this thesis are discussed and areas of future work suggested.

## 7.2 Consequences of virus yellows species variation

To better understand the distribution and prevalence of the yellowing viruses in the UK a survey was undertaken between 2019 and 2022. The results of this survey (detailed in Chapter 2) indicated that BChV is now more prevalent in the UK than previous studies have found (Stevens et al., 2005b). This finding is important when considering the use of virus resistant or tolerant sugar beet varieties. Obviously, the ideal situation for growers and industry alike, would be to have sugar beet varieties with resistance to all three yellowing viruses. However, currently such a variety is not available.

The results of the survey conducted here indicate BMV is now the least likely cause of virus yellows disease in the UK, with BYV the most prevalent in epidemic years and BChV more common than

BMV. Therefore, there is a potentially high risk that if a variety such as Maruscha KWS (with partial tolerance against BMV) is used by a grower and becomes infected with BChV it may suffer significant yield loss. Fortunately, from an industry perspective, the results of the controlled environment phenotyping trials (Chapter 4) and the field trials (Chapter 6) conducted here, indicate resistance mechanisms may be shared against both BMV and BChV. However, all the sugar beet varieties tested as part of this research were from the same seed house, and it is plausible that different seed houses may not have this shared cross-protection.

Following the results of this survey, an in-house BChV culture was established by the BBRO and incorporated into their virus yellows variety trials. This means the BBRO now assess sugar beet variety performance under all three yellowing viruses, providing growers with more information on which to base their variety choice. Nevertheless, whilst work towards a variety with resistance to all three yellowing viruses is ongoing, it may be beneficial for growers to better understand their localised virus risk and utilise varieties with resistance to the virus most frequently detected in their fields.

To achieve this level of detailed epidemiological knowledge, reliable diagnostic methods are needed. Detecting and differentiating between the yellowing viruses, and particularly between the closely related beet poleroviruses, has proved challenging. Although the sRNA library sequencing technique used in Chapter 2, did permit this differentiation it does not present a realistic diagnostic approach for routine use owing to the time and economic cost involved. Previously, at the BBRO, routine virus testing has been conducted by ELISA however this approach does not differentiate BMV from BChV. Adopting a qPCR approach would allow BMV and/or BChV infections to be identified and provide growers with a better understanding of the viruses most prevalent in their area.

As has been previously found, BYV prevalence continues to vary significantly between years (Hossain et al., 2021). As discussed in Chapter 2 the reasons for this variation are not yet clear and warrant further investigation. One potential influencing factor is the availability and survival of overwinter host species. Increasingly, beet crops are being grown in the UK which are not destined for sugar production but instead for animal feed (fodder beet) or energy production via anaerobic digestion plants (energy beet). The survey conducted in this project (Chapter 2) included samples from fodder beet plants across the UK, finding virus yellows in regions across England and Wales. Future surveys would benefit from also including energy beet samples, as these crops are often not harvested until the spring and therefore provide an ideal source and green bridge of disease from which the next years sugar beet crop may become infected.

Late harvested sugar beet crops, and any root remnants which are left in the field after harvest and able to re-sprout in the spring, also provide potential sources of infection. With the impacts of climate change, and wetter winters favouring the survival of such material, the risk of virus infection arising from these sources is increasing. Therefore, sampling in the spring of such material would be beneficial.

It is also worth noting that the use of neonicotinoid seed treatments may still be having a legacy impact on the relative prevalence of the yellowing viruses. The use of neonicotinoid seed treatments does not prevent a sugar beet plant becoming infected with virus, because the aphid needs to feed on the plant to acquire the insecticide, but instead prevents the secondary spread of virus and hence controls the disease within a crop. During this feeding time, prior to aphid death, any virus present in the aphid may be transferred to the plant. The shorter transmission time of BYV compared to the beet poleroviruses (detailed in Chapter 1 section 1.2.4.3 and 1.2.5.3) means that it may be more likely to be transferred into a neonicotinoid treated plant than BMV or BChV. This in turn may alter the abundance of virus overwintering in beet crops and hence the risk posed in subsequent years.

If the reasons for the variation in BYV prevalence can be ascertained, the occurrence of the virus may be able to be better predicted, and more effective control measures put in place. Future work, sampling potential BYV overwinter hosts and mapping BYV presence to overwinter weather conditions, for example, would help to clarify the hypothesised impact of winter weather conditions on BYV prevalence. Such studies are particularly important given the results of the field trial (Chapter 6) which found the highest yield losses, of both resistant and susceptible varieties, occurred under combined BChV and BYV infection. This highlights the threat posed by BYV, and the importance of developing sugar beet varieties able to overcome infection to all yellowing viruses.

### 7.3 Consequences of beet polerovirus strain variation

Given the progress in the development of varieties able to overcome BMV infection, a more detailed investigation was undertaken to establish the genetic diversity of BMV and BChV isolates. Perhaps of most note, from the genetic analysis detailed in Chapter 5, was the divergence of the BBRO BMV isolate, which had been kept in continuous culture since 2013, from the 'wild' BMV isolates collected between 2019 and 2022. This finding is of particular importance because it is this BMV isolate which has been widely supplied to and used by sugar beet seed breeders to develop and trial resistant varieties. The consequences of this variation have not been completely

elucidated, with differing results between the 2022 and 2023 field trials (discussed in Chapter 6). Nonetheless, based on the genetic differences and potential changes in pathogenicity identified through this project, in 2023 the BBRO took the decision to replace their original BMV culture with isolate 21-254. It was also decided that the culture should be more frequently ‘refreshed’ with wild BMV isolates to help prevent any divergence arising through continued culture.

The field trials were conducted using both susceptible and “high resistance” varieties, as described by the seed breeder. Using the definitions outlined in Chapter 1 section 1.4.5, however, neither of these high resistance varieties were truly resistant to either BMV or BChV, as both tested positive for virus via ELISA. Based on the field trial results from 2023, and the findings from the controlled environment studies (Chapter 4), these varieties do, however, exhibit partial resistance having a lower virus titre than susceptible varieties. Crucially this partial resistance appears, based on the virus isolates tested here, to be robust with resistant varieties consistently suffering lower yield losses than susceptible varieties.

Based on these results, and the definition of a virus strain being an isolate which displays a unique and recognisable phenotypic characteristic, none of the virus isolates studied here should be described as distinct strains. Nevertheless, given the risk posed by ‘resistance-breaking’ virus strains, and the very limited number of isolates tested here, research in this area should continue. To date, the adoption of the BMV tolerant variety Maruscha KWS in commercial crops has been limited, likely due to its lower yield potential, and as such the effect of the use of tolerant varieties on the evolution of virus strains is not yet clear. In the future, it is feasible that partially resistant varieties may have a large impact on the development of resistance-breaking strains. Such varieties could act as a filter with only the more pathogenic virus strains able to infect. These infected plants may then act as a virus source from which other plants become infected. Therefore, specifically targeting partially resistant varieties would be beneficial when searching for resistance-breaking virus strains.

## 7.4 Assessment of variety performance under virus yellows infection

Characterising how a variety performs when infected with virus is vital in the development of resistant sugar beet. Predominantly such assessments are conducted via virus inoculated field trials. As discussed in Chapter 4, these trials have many drawbacks including their large time and resource cost. It was therefore hoped that a methodology for phenotyping variety performance under virus yellows inoculation could be developed utilising controlled environment facilities. Unfortunately no



viable alternative was identified, out of the three controlled environments tested here, which provided notable benefits over a field trial approach. Despite this, future efforts to identify such an environment should continue, particularly as technological advances in lighting and the control of other variables such as humidity and air circulation are made; an area both breeders and BBRO are investigating further.

As no controlled environment was found to be as good as the field (Chapter 4), the consequences of the genetic variation identified between polerovirus isolates (Chapter 5) was elucidated through a field trial (Chapter 6). In what is believed to be the first field trial of its kind, sugar beet varieties were assessed under inoculation with six differing virus isolates in a commercial field setting. Undertaking these trials was extremely challenging and the methodology developed, as well as the results achieved, are of significant benefit to the sugar beet industry. The most demanding aspect of these field trials was rearing enough inoculum, of the six differing virus isolates, to inoculate the field trials, whilst preventing any contamination between cultures. This was made more difficult as the cultures needed to be reared at the same time as the BBRO were rearing thousands of plants for their own field trials, and hence there was the added risk of contamination from neighbouring glasshouse facilities. Unfortunately, as shown in the results of Chapter 6, some contamination with BYV did occur. Paradoxically, this contamination event did give unintended but fascinating insights into the impact of 'mixed' infections on variety performance used in this study. Nevertheless, given the considerable pressures, maintaining these six cultures was a significant achievement.

The benefits of conducting research in controlled environment facilities was made clear during the 2022 field trial. The drought and beet moth issues experienced highlight the challenges and risks of conducting experimental trials outdoors where they are fundamentally at the mercy of the weather and pest challenges of that year. These conditions clearly affected both the final yield of the trial plots, as well as the in-season measurements such as canopy cover. The relationship between virus and plant also appears to have been affected, with lower virus titre (as determined via ELISA) when plants were under severe drought stress. It is important to note that, in the field trial study, ELISA testing was conducted on the largest non-senescent leaf. In the controlled environment studies (Chapter 4), differences were observed in virus titre between leaves of different ages, a finding which indicated a source-sink relationship of virus movement through the infected plant. It therefore suggests that drought stress may alter this relationship, even to the point where the older leaves of plants known to be infected with virus give a negative ELISA result.

This finding has potential consequences both for variety assessment and diagnostic testing of sugar beet more generally. To permit reliable comparisons to be made between variety-virus studies, the

industry would benefit from agreeing a standard virus titre assessment protocol with defined sampling times. A single sampling point shortly prior to harvest (such as that undertaken in the 2023 field trial) would likely provide a balance between the need to gather the necessary information on variety resistance and the significant time and resource cost of such testing. From a more general perspective, avoiding any assessment of virus titres whilst plants are under severe drought stress would help prevent false negative results as clearly highlighted by the differences shown between my 2022 and 2023 trials.

In spite of the difficulties experienced during these field trials, a significant relationship between canopy chlorophyll levels and subsequent yield loss was identified. If this model is upheld, following the necessary validation across different soil types and sugar beet varieties from other seed houses, it would provide a useful tool to both researchers and growers alike. For researchers and breeders, when used on inoculated field trials, it could provide a time benefit enabling high yielding varieties to be identified ahead of harvest. For growers, it may be possible to estimate yield losses on a field scale, better informing subsequent agronomic decisions such as when to harvest particular fields. However, as previously discussed (Chapter 1 section 1.2.3.1), multiple pathogens and deficiencies can give rise to sugar beet leaf yellowing and could interfere with this model. Future validation studies must, therefore, assess the impact of these factors on the model prior to any widespread adoption.

As well as achieving resistance to each virus individually, ultimately varieties will also need to overcome mixed infections. These mixed infections may include differing virus species (such as those identified in the survey in Chapter 3), and differing isolates or strains. Preliminary investigations into the effect of mixed infections on sugar beet varieties were conducted under glasshouse conditions (with BMV and BChV, Chapter 4) and field conditions (with BYV and BChV, Chapter 6). In both environments, these mixed infections resulted in the most severe symptoms. However further work is needed to validate these findings, and study in more detail the effect of these virus-virus interactions on resistance mechanisms.

## 7.5 Final conclusion

At the time this project was created, in 2019, there was growing concern in the sugar beet industry of the threat posed by virus yellows disease. The loss of neonicotinoid seed treatments, and the lack of alternative control strategies, raised fears of widespread virus yellows outbreaks, the like of which had not been seen since the mid-1970s. These fears were realised, in part, in 2020 when the

industry lost an estimated £67 million to the disease. Understandably, this increased threat led to a rapid acceleration in the development of a virus yellows strategy and research funding, which this PhD project forms part of (British Sugar, NFU Sugar and BBRO, 2023).

Arguably the most important finding of this project, for the sugar beet industry, is that the resistant varieties trialled here proved to be durable against differing isolates of both BMV and BChV. Given the genetic diversity amongst these isolates, this result is encouraging and possibly indicates a reliable and robust resistance mechanism that should be exploited by the industry. However, my findings also highlight the complexity of the virus yellows issue. Changing virus species dynamics, future strain variation and the ultimate need for varietal resistance to all three yellowing viruses must all be appreciated by the UK sugar beet industry to provide long term durability for disease management. The battle with virus yellows continues...

# Chapter 8: References

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## Appendix A – variety table

Additional information on the sugar beet varieties used in this project as provided by the seed breeder KWS.

<b>Level of resistance</b>	<b>Description</b>	<b>Code</b>	<b>Decoding</b>
High	res. source A	KWS-1	Maruscha KWS
High	res. source B	KWS-2	
Medium		KWS-3	
Medium		KWS-4	
Medium		KWS-5	
Low		KWS-6	
Low		KWS-7	
Low		KWS-9	
High	res. source A	KWS-10	

## Appendix B – sRNA library sample collection details

Collection details of samples used for sRNA library sequencing.

<b>Sample code</b>	<b>Collection year</b>	<b>Sample type</b>	<b>Collection location</b>
19-002	2019	Fodder beet	Field A, Market Bosworth, Leicestershire
19-003	2019	Fodder beet	Field B, Market Bosworth, Leicestershire
19-004	2019	Sugar beet	Field A, Brettenham, Norfolk
19-005	2019	Sugar beet	Field B, Brettenham, Norfolk
19-006	2019	Sugar beet	Field A, Crowland, Lincolnshire
19-007	2019	Sugar beet	Field B, Crowland, Lincolnshire
19-008	2019	Sugar beet	Wrabness, Suffolk
19-009	2019	Fodder beet	Charlinch, Somerset
19-010	2019	Fodder beet	Twycross, Leicestershire
19-011	2019	Fodder beet	Ashdown, Somerset
19-012	2019	Sugar beet	Field A, Royston, Hertfordshire
19-013	2019	Sugar beet	Field B, Royston, Hertfordshire
19-014	2019	Sugar beet	Thurne, Norfolk
19-015	2019	Fodder beet	Field A, South Petherton, Somerset
19-016	2019	Fodder beet	Field B, South Petherton, Somerset
20-006	2020	Sea beet	Southwold, Suffolk
20-008	2020	Sea beet	Havergate Island, Suffolk
20-009	2020	Fodder beet	Northallerton, North Yorkshire
20-010	2020	Fodder beet	Ripon, North Yorkshire
20-011	2020	Fodder beet	Field A, Cannington, Somerset
20-012	2020	Fodder beet	Field B, Cannington, Somerset
20-014	2020	Fodder beet	Field A, Cheshire
20-015	2020	Fodder beet	Field B, Lancashire
20-016	2020	Fodder beet	Nevern, Pembrokeshire
20-017	2020	Sugar beet	Walcott, Norfolk
20-018-1	2020	Sugar beet	Field A, Harlow, Essex
20-018-2	2020	Sugar beet	Field B, Harlow, Essex
20-020	2020	Sugar beet	Guyhirn, Cambridgeshire
20-021	2020	Sugar beet	Newark-on-Trent, Nottinghamshire
21-020	2021	Sugar beet	Barrow, Suffolk
21-024	2021	Sugar beet	Letheringham, Suffolk
21-039	2021	Sugar beet	Drinkstone, Suffolk
21-056	2021	Sugar beet	Nacton, Suffolk
21-071	2021	Sugar beet	Felixstowe, Suffolk
21-097	2021	Sugar beet	Spellbrook, Hertfordshire
21-111	2021	Sugar beet	Frampton, Lincolnshire
21-121	2021	Sugar beet	Cotton, Suffolk
21-179	2021	Sugar beet	Clay Hill, London
21-187	2021	Sugar beet	Soham, Cambridgeshire
21-196	2021	Sugar beet	Sible Hedingham, Essex
21-254	2021	Sugar beet	Field C, Crowland, Lincolnshire
21-256	2021	Sugar beet	Field D, Crowland, Lincolnshire
21-278	2021	Sugar beet	Rougham, Norfolk



21-290	2021	Fodder beet	Field A, Thirn, North Yorkshire
21-294	2021	Fodder beet	Field B, Thirn, North Yorkshire
21-303	2021	Fodder beet	Fochabers, Moray, Scotland
BBRO-BMYV19	2019	Sugar beet	BBRO BMYV culture
BBRO-BMYV20	2020	Sugar beet	BBRO BMYV culture
BBRO-BMYV21	2021	Sugar beet	BBRO BMYV culture
BBRO-BChV20	2020	Sugar beet	BBRO BChV culture
BBRO-BChV21	2021	Sugar beet	BBRO BChV culture

## Appendix C – BMVY consensus sequence alignment

BMVY consensus sequences produced using BCFtools (see Chapter 5 section 5.3.2), and aligned using ClustalOmega.

CLUSTAL O(1.2.4) multiple sequence alignment

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21-BMYV      ACAAAGAAACCAGCGAGGATCTAGCAGTCTATGCAATTTTCAGCTTAAACAAACAGTTT      60
21-254      ACAAAGAAACCAGCGAGGATCTAGCAGTCTATGCAATTTTCAGCTTAAACAAACAGTTT      60
20-BMYV      ACAAAGAAACCAGCGAGGATCTAGCAGTCTATGCAATTTTCAGCTTAAACAAACAGTTT      60
20-010      ACAAAGAAACCAGCGAGGATCTAGCAGTCTATGCAATTTTCAGCTTAAACAAACAGTTT      60
20-006      ACAAAGAAACCAGCGAGGATCTAGCAGTCTATGCAATTTTCAGCTTAAACAAACAGTTT      60
19-BMYV      ACAAAGAAACCAGCGAGGATCTAGCAGTCTATGCAATTTTCAGCTTAAACAAACAGTTT      60
21-097      ACAAAGAAACCAGCGAGGATCTAGCAGTCTATGCAATTTTCAGCTTAAACAAACAGTTT      60
19-013      ACAAAGAAACCAGCGAGGATCTAGCAGTCTATGCAATTTTCAGCTTAAACAAACAGTTT      60
21-294      ACAAAGAAACCAGCGAGGATCTAGCAGTCTATGCAATTTTCAGCTTAAACAAACAGTTT      60
21-056      ACAAAGAAACCAGCGAGGATCTAGCAGTCTATGCAATTTTCAGCTTAAACAAACAGTTT      60
20-009      ACAAAGAAACCAGCGAGGATCTAGCAGTCTATGCAATTTTCAGCTTAAACAAACAGTTT      60
20-014      ACAAAGAAACCAGCGAGGATCTAGCAGTCTATGCAATTTTCAGCTTAAACAAACAGTTT      60
*****

21-BMYV      CACTGTTCGTTGAACCGACTGCTAACAGCTACAGAGCGAGTTTTAAACACCGCGTATTT      120
21-254      CACTGTTCGTTGAACCGACTGCTAACAGCTACAGAGCGAGTTTTAAACACCGCGTATTT      120
20-BMYV      CACTGTTCGTTGAACCGACTGCTAACAGCTACAGAGCGAGTTTTAAACACCGCGTATTT      120
20-010      CACTGTTCGTTGAACCGACTGCTAACAGCTACAGAGCGAGTTTTAAACACCGCGTATTT      120
20-006      CACTGTTCGTTGAACCGACTGCTAACAGCTACAGAGCGAGTTTTAAACACCGCGTATTT      120
19-BMYV      CACTGTTCGTTGAACCGACTGCTAACAGCTACAGAGCGAGTTTTAAACACCGCGTATTT      120
21-097      CACTGTTCGTTGAACCGACTGCTAACAGCTACAGAGCGAGTTTTAAACACCGCGTATTT      120
19-013      CACTGTTCATTGAACCGACTGCTAACAGCTACAGAGCGAGTTTTAAACACCGCGTATTT      120
21-294      CACTGTTCGTTGAACCGACTGCTAACAGCTACAGAGCGAGTTTTAAACACCGCGTATTT      120
21-056      CACTGTTCGTTGAACCGACTGCTAACAGCTACAGAGCGAGTTTTAAACACCGCGTATTT      120
20-009      CACTGTTCGTTGAACCGACTGCTAACAGCTACAGAGCGAGTTTTAAACACCGCGTATTT      120
20-014      CACTGTTCGTTGAACCGACTGCTAACAGCTACAGAGCGAGTTTTAAACACCGCGTATTT      120
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21-BMYV      TCTTACGAATCATTTACCGCTCGTAACTTTTGAGAATGAAAACGTGATTCGTTCTCTTCT      180
21-254      TCTTACGAATCATTTACCGCTCGTAACTTTTGAGAATGAAAACGTGATTCGTTCTCTTCT      180
20-BMYV      TCTTACGAATCATTTACCGCTCATAACTTTTGAGAATGAAAACGTGATTCGTTCTCTTCT      180
20-010      TCTTACGAATCATTTACCGCTCGTAACTTTTGAGAATGAAAACGTGATTCGTTCTCTTCT      180
20-006      TCTTACGAATCATTTACCGCTCGTAACTTTTGAGAATGAAAACGTGATTCGTTCTCTTCT      180
19-BMYV      TCTTACGAATCATTTACCGCTCGTAACTTTTGAGAATGAAAACGTGATTCGTTCTCTTCT      180
21-097      TCTTACGAATCATTTACCGCTCGTAACTTTTGAGAATGAAAACGTGATTCGTTCTCTTCT      180
19-013      TCTTACGAATCATTTACCGCTCGTAACTTTTGAGAATGAAAACGTGATTCGTTCTCTTCT      180
21-294      TCTTACGAATCATTTACCGCTCGTAACTTTTGAGAATGAAAACGTGATTCGTTCTCTTCT      180
21-056      TCTTACGAATCATTTACCGCTCGTAACTTTTGAGAATGAAAACGTGATTCGTTCTCTTCT      180
20-009      TCTTACGAATCATTTACCGCTCGTAACTTTTGAGAATGAAAACGTGATTCGTTCTCTTCT      180
20-014      TCTTACGAATCATTTACCGCTCGTAACTTTTGAGAATGAAAACGTGATTCGTTCTCTTCT      180
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21-254      CGCTGCTCTGCCTTTGCTGCTCAGTAAGCAGCTCGACCCCGGGAGCTTCATTTACACTCC      240
20-BMYV      CGCTGCTCTGCCTTTGCTGCTCAGTAAGCAGCTCGACCCCGGGAGCTTCATTTACACTCC      240
20-010      CGCTGCTCTGCCTTTGCTGCTCAGTAAGCAGCTCGACCCCGGGAGCTTCATTTACACTCC      240
20-006      CGCTGCTCTGCCTTTGCTGCTCAGTAAGCAGCTCGACCCCGGGAGCTTCATTTACACTCC      240
19-BMYV      CGCTGCTCTGCCTTTGCTGCTCAGTAAGCAGCTCGACCCCGGGAGCTTCATTTACACTCC      240
21-097      CGCTGCTCTGCCTTTGCTGCTCAGTAAGCAGCTCGACCCCGGGAGCTTCGTTTACACTCC      240
19-013      CGCTGCTCTGCCTTTGCTGCTCAGTAAGCAGCTCGACCCCGGGAGCTTCATTTACACTCC      240
21-294      CGCTGCTCTGCCTTTGCTGCTCAGTAAGCAGCTCGACCCCGGGAGCTTCATTTACACTCC      240
21-056      CGCTGCTCTGCCTTTGCTGCTCAGTAAGCAGCTCGACCCCGGGAGCTTCATTTACACTCC      240
20-009      CGCTGCTCTGCCTTTGCTGCTCAGTAAGCAGCTCGACCCCGGGAGCTTCATTTACACTCC      240
20-014      CGCTGCTCTGCCTTTGCTGCTCAGTAAGCAGCTCGACCCCGGGAGCTTCATTTACACTCC      240
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21-254	CGGGAAACGCCAGTCTTTACGACTGGCCAGGTTCTACAATTACTGCGGAGCCGTGTTACC	300
20-BMYV	CGGGAAACGCCAGTCTTTACGACTGGCCAGGTTCTACAATTACTGCGGAGCCGTGTTACC	300
20-010	CGGGAAACGCCAGTCTTTACGACTGGCCAGGTTCTACAATTACTGCGGAGCCGTGTTACC	300
20-006	CGGGAAACGCCAGTCTTTACGACTGGCCAGGTTCTACAATTACTGCGGAGCCGTGTTACC	300
19-BMYV	CGGGAAACGCCAGTCTTTACGACTGGCCAGGTTCTACAATTACTGCGGAGCCGTGTTACC	300
21-097	CGGGAAACGCCAGTCTTTACGACTGGCCAGGTTCTACAATTACTGCGGAGCCGTGTTACC	300
19-013	CGGGAAACGCCAGTCTTTACGACTGGCCAGGTTCTACAATTACTGCGGAGCCGTGTTACC	300
21-294	CGGGAAACGCCAGTCTTTACGACTGGCCAGGTTCTACAATTACTGCGGAGCCGTGTTACC	300
21-056	CGGGAAACGCCAGTCTTTACGACTGGCCAGGTTCTACAATTACTGCGGAGCCGTGTTACC	300
20-009	CGGGAAACGCCAGTCTTTACGACTGGCCAGGTTCTACAATTACTGCGGAGCCGTGTTACC	300
20-014	CGGGAAACGCCAGTCTTTACGACTGGCCAGGTTCTACAATTACTGCGGAGCCGTGTTACC	300
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21-254	CAGCACTCGCAACATTGACTTACGAGTGCCCCCAGAAAAGACGTTAAAAGATTTTACCT	360
20-BMYV	CAGCACTCGCAACATTGACTTACGAGTGCCCCCAGAAAAGACGTTAAAAGATTTTACCT	360
20-010	CAGCACTCGCAACATTGACTTACGAGTGCCCCCAGAAAAGACGTTAAAAGATTTTACCT	360
20-006	CAGCACTCGCAACATTGACTTACGAGTGCCCCCAGAAAAGACGTTAAAAGATTTTACCT	360
19-BMYV	CAGCACTCGCAACATTGACTTACGAGTGCCCCCAGAAAAGACGTTAAAAGATTTTACCT	360
21-097	CAGCACTCGCAACATTGACTTACGAGTGCCCCCAGAAAAGACGTTAAAAGATTTTACCT	360
19-013	CAGCACTCGCAACATTGACTTACGAGTGCCCCCAGAAAAGACGTTAAAAGATTTTACCT	360
21-294	CAGCACTCGCAACATTGACTTACGAGTGCCCCCAGAAAAGACGTTAAAAGATTTTACCT	360
21-056	CAGCACTCGCAACATTGACTTACGAGTGCCCCCAGAAAAGACGTTAAAAGATTTTACCT	360
20-009	CAGCACTCGCAACATTGACTTACGAGTGCCCCCAGAAAAGACGTTAAAAGATTTTACCT	360
20-014	CAGCACTCGCAACATTGACTTACGAGTGCCCCCAGAAAAGACGTTAAAAGATTTTACCT	360
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21-BMYV	TGCCCGAAATTCAGGCAGAGATCTGGGGGAGAGGCTACAACGCCGCAGAGAAATTTTCTC	420
21-254	TGCCCGAAATTCAGGCAGAGATCTGGGGGAGAGGCTACAACGCCGCAGAGAAATTTTCTC	420
20-BMYV	TGCCCGAAATTCAGGCAGAGATCTGGGGGAGAGGCTACAACGCCGCAGAGAAATTTTCTC	420
20-010	TGCCCGAAATTCAGGCAGAAATCTGGGGGAGAGGCTACAACGCCGCAGAGAAATTTTCTC	420
20-006	TGCCCGAAATTCAGGCAGAGATCTGGGGGAGAGGCTACAACGCCGCAGAGAAATTTTCTC	420
19-BMYV	TGCCCGAAATTCAGGCAGAGATCTGGGGGAGAGGCTACAACGCCGCAGAGAAATTTTCTC	420
21-097	TGCCCGAAATTCAGGCAGAGATCTGGGGGAGAGGCTACAACGCCGCAGAGAAATTTTCTC	420
19-013	TGCCCGAAATTCAGGCAGAAATCTGGGGGAGAGGCTACAACGCCGCAGAGAAATTTTCTC	420
21-294	TGCCCGAAATTCAGGCAGAGATCTGGGGGAGAGGCTACAACGCCGCAGAGAAATTTTCTC	420
21-056	TGCCCGAAATTCAGGCAGAGATCTGGGGGAGAGGCTACAACGCCGCAGAGAAATTTTCTC	420
20-009	TGCCCGAAATTCAGGCAGAGATCTGGGGGAGAGGCTACAACGCCGCAGAGAAATTTTCTC	420
20-014	TGCCCGAAATTCAGGCAGAGATCTGGGGGAGAGGCTACAACGCCGCAGAGAAATTTTCTC	420
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21-BMYV	TCGCGGTGAAGCAGAGTTTAAAAAGTTCCCTTTCAGTATGGTGTGCTGAAAGCGAGAGAAA	480
21-254	TCGCGGTGAAGCAGAGTTTAAAAAGTTCCCTTTCAGTATGGTGTGCTGAAAGCGAGAGAAA	480
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20-010	TCGCGGTGAAGCAGAGTTTAAAAAGTTCCCTTTCAGTATGGTGTGCTGAAAGCGAGAGAAA	480
20-006	TCGCGGTGAAGCAGAGTTTAAAAAGTTCCCTTTCAGTATGGTGTGCTGAAAGCGAGAGAAA	480
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19-013	TCGCGGTGAAGCAGAGTTTAAAAAGTTCCCTTTCAGTATGGTGTGCTGAAAGCGAGAGAAA	480
21-294	TCGCGGTGAAGCAGAGTTTAAAAAGTTCCCTTTCAGTATGGTGTGCTGAAAGCGAGAGAAA	480
21-056	TCACGGTGAAGCAGAGTTTAAAAAGTTCCCTTTCAGTATGGTGTGCTGAAAGCGAGAGAAA	480
20-009	TCGCGGTGAAGCAGAGTTTAAAAAGTTCCCTTTCAGTATGGTGTGCTGAAAGCGAGAGAAA	480
20-014	TCGCGGTGAAGCAGAGTTTAAAAAGTTCCCTTTCAGTATGGTGTGCTGAAAGCGAGAGAAA	480
	** *****	
21-BMYV	GCTACGGGAGAGTCCATAAATCGATATTAGAATGGACCATATTATTATGGTCTTACGCGA	540
21-254	GCTACGGGAGAGTCCATAAATCGATATTAGAATGGACCATATTATTATGGTCTTACGCGA	540
20-BMYV	GCTACGGGAGAGTCCATAAATCGATATTAGAATGGACCATATTATTATGGTCTTACGCGA	540
20-010	GCTACGGGAGAGTCCATAAATCGATATTAGAATGGACCATATTATTATGGTCTTACGCGA	540
20-006	GCTACGGGAGAGTCCATAAATCGATATTAGAATGGACCATATTATTATGGTCTTACGCGA	540
19-BMYV	GCTACGGGAGAGTCCATAAATCGATATTAGAATGGACCATATTATTATGGTCTTACGCGA	540
21-097	GCTACGGGAGAGTCCATAAATCGATATTAGAATGGACCATATTATTATGGTCTTACGCGA	540
19-013	GCTACGGGAGAGTCCATAAATCGATATTAGAATGGACCATATTATTATGGTCTTACGCGA	540
21-294	GCTACGGGAGAGTCCATAAATCGATATTAGAATGGACCATATTATTATGGTCTTACGCGA	540
21-056	GCTACGGGAGAGTCCATAAATCGATATTAGAATGGACCATATTATTATGGTCTTACGCGA	540
20-009	GCTACGGGAGAGTCCATAAATCGATATTAGAATGGACCATATTATTATGGTCTTACGCGA	540
20-014	GCTACGGGAGAGTCCATAAATCGATATTAGAATGGACCATATTATTATGGTCTTACGCGA	540

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21-BMYV TATGGGCACTCTCTTGCACCGTCTGGTACTTGTGGAAGAACTATAACCATAGAAATACTTA 600  
21-254 TATGGGCACTCTCTTGCACCGTCTGGTACTTGTGGAAGAACTATAACCATAGAAATACTTA 600  
20-BMYV TATGGGCACTCTCTTGCACCGTCTGGTACTTGTGGAAGAACTATAACCATAGAAATACTTA 600  
20-010 TATGGGCACTCTCTTGCACCGTCTGGTACTTGTGGAAGAACTATAACCATAGAAATACTTA 600  
20-006 TATGGGCACTCTCTTGCACCGTCTGGTACTTGTGGAAGAACTATAACCATAGAAATACTTA 600  
19-BMYV TATGGGCACTCTCTTGCACCGTCTGGTACTTGTGGAAGAACTATAACCATAGAAATACTTA 600  
21-097 TATGGGCACTCTCTTGCACCGTCTGGTACTTGTGGAAGAACTATAACCATAGAAATACTTA 600  
19-013 TATGGGCACTCTCTTGCACCGTCTGGTACTTGTGGAAGAACTATAACCATAGAAATACTTA 600  
21-294 TATGGGCACTCTCTTGCACCGTCTGGTACTTGTGGAAGAACTATAACCATAGAAATACTTA 600  
21-056 TATGGGCACTCTCTTGCACCGTCTGGTACTTGTGGAAGAACTATAACCATAGAAATACTTA 600  
20-009 TATGGGCACTCTCTTGCACCGTCTGGTACTTGTGGAAGAACTATAACCATAGAAATACTTA 600  
20-014 TATGGGCACTCTCTTGCACCGTCTGGTACTTGTGGAAGAACTATAACCATAGAAATACTTA 600  
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21-BMYV TGCTGAGCTCGCTTTTTGCGTTCCACCACCTTTTTGGTGAAGCTCGTGGTATGGATTTTTG 660  
21-254 TGCTGAGCTCGCTTTTTGCGTTCCACCACCTTTTTGGTGAAGCTCGTGGTATGGATTTTTG 660  
20-BMYV TGCTGAGCTCGCTTTTTGCGTTCCACCACCTTTTTGGTGAAGCTCGTGGTATGGATTTTTG 660  
20-010 TGCTGAGCTCGCTTTTTGCGTTCCACCACCTTTTTGGTGAAGCTCGTGGTATGGATTTTTG 660  
20-006 TGCTGAGCTCGCTTTTTGCGTTCCACCACCTTTTTGGTGAAGCTCGTGGTATGGATTTTTG 660  
19-BMYV TGCTGAGCTCGCTTTTTGCGTTCCACCACCTTTTTGGTGAAGCTCGTGGTATGGATTTTTG 660  
21-097 TGCTGAGCTCGCTTTTTGCGTTCCACCACCTTTTTGGTGAAGCTCGTGGTATGGATTTTTG 660  
19-013 TGCTGAGCTCGCTTTTTGCGTTCCACCACCTTTTTGGTGAAGCTCGTGGTATGGATTTTTG 660  
21-294 TGCTGAGCTCGCTTTTTGCGTTCCACCACCTTTTTGGTGAAGCTCGTGGTATGGATTTTTG 660  
21-056 TGCTGAGCTCGCTTTTTGCGTTCCACCACCTTTTTGGTGAAGCTCGTGGTATGGATTTTTG 660  
20-009 TGCTGAGCTCGCTTTTTGCGTTCCACCACCTTTTTGGTGAAGCTCGTGGTATGGATTTTTG 660  
20-014 TGCTGAGCTCGCTTTTTGCGTTCCACCACCTTTTTGGTGAAGCTCGTGGTATGGATTTTTG 660  
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21-BMYV GCGGCTGGCTAACTCCCTGGTAAATGGTTTATTTGCTCTCACGAAATGTATTTTGAAAA 720  
21-254 GCGGCTGGCTAACTCCCTGGTAAATGGTTTATTTGCTCTCACGAAATGTATTTTGAAAA 720  
20-BMYV GCGGCTGGCTAACTCCCTGGTAAATGGTTTATTTGCTCTCACGAAATGTATTTTGAAAA 720  
20-010 GCGGCTGGCTAACTCCCTGGTAAATGGTTTATTTGCTCTCACGAAATGTATTTTGAAAA 720  
20-006 GCGGCTGGCTAACTCCCTGGTAAATGGTTTATTTGCTCTCACGAAATGTATTTTGAAAA 720  
19-BMYV GCGGCTGGCTAACTCCCTGGTAAATGGTTTATTTGCTCTCACGAAATGTATTTTGAAAA 720  
21-097 GCGGCTGGCTAACTCCCTGGTAAATGGTTTATTTGCTCTCACGAAATGTATTTTGAAAA 720  
19-013 GCGGCTGGCTAACTCCCTGGTAAATGGTTTATTTGCTCTCACGAAATGTATTTTGAAAA 720  
21-294 GCGGCTGGCTAACTCCCTGGTAAATGGTTTATTTGCTCTCACGAAATGTATTTTGAAAA 720  
21-056 GCGGCTGGCTAACTCCCTGGTAAATGGTTTATTTGCTCTCACGAAATGTATTTTGAAAA 720  
20-009 GCGGCTGGCTAACTCCCTGGTAAATGGTTTATTTGCTCTCACGAAATGTATTTTGAAAA 720  
20-014 GCGGCTGGCTAACTCCCTGGTAAATGGTTTATTTGCTCTCACGAAATGTATTTTGAAAA 720  
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21-BMYV CTCTTTCATCCAGAAAGAGCTACGTTTGTGAGCGATCTGTAAGGTTTTCTCACCTTTA 780  
21-254 CTCTTTCATCCAGAAAGAGCTACGTTTGTGAGCGATCTGTAAGGTTTTCTCACCTTTA 780  
20-BMYV CTCTTTCATCCAGAAAGAGCTACGTTTGTGAGCGATCTGTAAGGTTTTCTCACCTTTA 780  
20-010 CTCTTTCATCCAGAAAGAGCTACGTTTGTGAGCGATCTGTAAGGTTTTCTCACCTTTA 780  
20-006 CTCTTTCATCCAGAAAGAGCTACGTTTGTGAGCGATCTGTAAGGTTTTCTCACCTTTA 780  
19-BMYV CTCTTTCATCCAGAAAGAGCTACGTTTGTGAGCGATCTGTAAGGTTTTCTCACCTTTA 780  
21-097 CTCTTTCATCCAGAAAGAGCTACGTTTGTGAGCGATCTGTAAGGTTTTCTCACCTTTA 780  
19-013 CTCTTTCATCCAGAAAGAGCTACGTTTGTGAGCGATCTGTAAGGTTTTCTCACCTTTA 780  
21-294 CTCTTTCATCCAGAAAGAGCTACGTTTGTGAGCGATCTGTAAGGTTTTCTCACCTTTA 780  
21-056 CTCTTTCATCCAGAAAGAGCTACGTTTGTGAGCGATCTGTAAGGTTTTCTCACCTTTA 780  
20-009 CTCTTTCATCCAGAAAGAGCTACGTTTGTGAGCGATCTGTAAGGTTTTCTCACCTTTA 780  
20-014 CTCTTTCATCCAGAAAGAGCTACGTTTGTGAGCGATCTGTAAGGTTTTCTCACCTTTA 780  
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21-BMYV CCATCAAACAAAGCCCGCCGCGTAATTCGATTCTTCAAATACAACACGCAGACGGTTCCC 840  
21-254 CCATCAAACAAAGCCCGCCGCGTAATTCGATTCTTCAAATACAACACGCAGACGGTTCCC 840  
20-BMYV CCATCAAACAAAGCCCGCCGCGTAATTCGATTCTTCAAATACAACACGCAGACGGTTCCC 840  
20-010 CCATCAAACAAAGCCCGCCGCGTAATTCGATTCTTCAAATACAACACGCAGACGGTTCCC 840  
20-006 CCATCAAACAAAGCCCGCCGCGTAATTCGATTCTTCAAATACAACACGCAGACGGTTCCC 840  
19-BMYV CCATCAAACAAAGCCCGCCGCGTAATTCGATTCTTCAAATACAACACGCAGACGGTTCCC 840  
21-097 CCATCAAACAAAGCCCGCCGCGTAATTCGATTCTTCAAATACAACACGCAGACGGTTCCC 840  
19-013 CCATCAAACAAAGCCCGCCGCGTAATTCGATTCTTCAAATACAACACGCAGACGGTTCCC 840  
21-294 CCATCAAACAAAGCCCGCCGCGTAATTCGATTCTTCAAATACAACACGCAGACGGTTCCC 840  
21-056 CCATCAAACAAAGCCCGCCGCGTAATTCGATTCTTCAAATACAACACGCAGACGGTTCCC 840

20-009	CCATCAAACAAAGCCCGCCGCGTAATTGCATTCTTCAAATACAACACGCAGACGGTTCCC	840
20-014	CCATCAAACAAAGCCCGCCGCGTAATTGCATTCTTCAAATACAACACGCAGACGGTTCCC *****	840
21-BMYV	ATGCCGGTTATGCAACGTGCGTAACCTTATTCGACGGGACAAACGGATTGTTGACTGCGC	900
21-254	ATGCCGGTTATGCAACATGCGTAACCTTATTCGACGGGACAAACGGATTGTTGACTGCGC	900
20-BMYV	ATGCCGGTTATGCAACATGCGTAACCTTATTCGACGGGACAAACGGATTGTTGACTGCGC	900
20-010	ATGCCGGTTATGCAACATGCGTAACCTTATTCGACGGGACAAACGGATTGTTGACTGCGC	900
20-006	ATGCCGGTTATGCAACATGCGTAACCTTATTCGACGGGACAAACGGATTGTTGACTGCGC	900
19-BMYV	ATGCCGGTTATGCAACATGCGTAACCTTATTCGACGGGACAAACGGATTGTTGACTGCGC	900
21-097	ATGCCGGTTATGCAACATGCGTAACCTTATTCGACGGGACAAACGGATTGTTGACTGCGC	900
19-013	ATGCCGGTTATGCAACATGCGTAACCTTATTCGACGGGACAAACGGATTGTTGACTGCGC	900
21-294	ATGCCGGTTATGCAACATGCGTAACCTTATTCGACGGGACAAACGGATTGTTGACTGCGC	900
21-056	ATGCCGGTTATGCAACATGCGTAACCTTATTCGACGGGACAAACGGATTGTTGACTGCGC	900
20-009	ATGCCGGTTATGCAACATGCGTAACCTTATTCGACGGGACAAACGGATTGTTGACTGCGC	900
20-014	ATGCCGGTTATGCAACATGCGTAACCTTATTCGACGGGACAAACGGATTGTTGACTGCGC *****	900
21-BMYV	AACATGTAGTTGACGATTTTTACGAAGGAGACCCGAGAAAGACTCTAAAAGTCGTCTCCA	960
21-254	AACATGTAGTTGACGATTTTTACGAAGGAGACCCGAGAAAGACTCTAAAAGTCGTCTCCA	960
20-BMYV	AACATGTAGTTGACGATTTTTACGAAGGAGACCCGAGAAAGACTCTAAAAGTCGTCTCCA	960
20-010	AACATGTAGTTGACGATTTTTACGAAGGAGACCCGAGAAAGACTCTAAAAGTCGTCTCCA	960
20-006	AACATGTAGTTGACGATTTTTACGAAGGAGACCCGAGAAAGACTCTAAAAGTCGTCTCCA	960
19-BMYV	AACATGTAGTTGACGATTTTTACGAAGGAGACCCGAGAAAGACTCTAAAAGTCGTCTCCA	960
21-097	AACATGTAGTTGACGATTTTTACGAAGGAGACCCGAGAAAGACTCTAAAAGTCGTCTCCA	960
19-013	AACATGTAGTTGACGATTTTTACGAAGGAGACCCGAGAAAGACTCTAAAAGTCGTCTCCA	960
21-294	AACATGTAGTTGACGATTTTTACGAAGGAGACCCGAGAAAGACTCTAAAAGTCGTCTCCA	960
21-056	AACATGTAGTTGACGATTTTTACGAAGGAGACCCGAGAAAGACTCTAAAAGTCGTCTCCA	960
20-009	AACATGTAGTTGACGATTTTTACGAAGGAGACCCGAGAAAGACTCTAAAAGTCGTCTCCA	960
20-014	AACATGTAGTTGACGATTTTTACGAAGGAGACCCGAGAAAGACTCTAAAAGTCGTCTCCA *****	960
21-BMYV	CCCGCAATGGAACAAAATCCCCCTTGATGAATTCAGAGTGACGTACACATCTGAGAAAA	1020
21-254	CCCGCAACGGAACAAAATCCCCCTTGATGAATTCAGAGTGACGTACACATCTGAGAAAA	1020
20-BMYV	CCCGCAATGGAACAAAATCCCCCTTGATGAATTCAGAGTGACGTACACATCTGAGAAAA	1020
20-010	CCCGCAATGGAACAAAATCCCCCTTGATGAATTCAGAGTGACGTACACATCTGAGAAAA	1020
20-006	CCCGCAATGGAACAAAATCCCCCTTGATGAATTCAGAGTGACGTACACATCTGAGAAAA	1020
19-BMYV	CCCGCAATGGAACAAAATCCCCCTTGATGAATTCAGAGTGACGTACACATCTGAGAAAA	1020
21-097	CCCGCAATGGAACAAAATCCCCCTTGATGAATTCAGAGTGACGTACACATCTGAGAAAA	1020
19-013	CCCGCAATGGAACAAAATCCCCCTTGATGAATTCAGAGTGACGTACACATCTGAGAAAA	1020
21-294	CCCGCAATGGAACAAAATCCCCCTTGATGAATTCAGAGTGACGTACACATCTGAGAAAA	1020
21-056	CCCGCAATGGAACAAAATCCCCCTTGATGAATTCAGAGTGACGTACACATCTGAGAAAA	1020
20-009	CCCGCAATGGAACAAAATCCCCCTTGATGAATTCAGAGTGACGTACACATCTGAGAAAA	1020
20-014	CCCGCAATGGAACAAAATCCCCCTTGATGAATTCAGAGTGACGTACACATCTGAGAAAA *****	1020
21-BMYV	GGGATCAGTTGTTGATGCATGGGCCCCCAAACCTGGGAAGGAGTTCTTGCCGTGCAAGGCAG	1080
21-254	GGGATCAGTTGTTGATGCATGGGCCCCCAAACCTGGGAAGGAGTTCTTGCCGTGCAAGGCAG	1080
20-BMYV	GGGATCAGTTGTTGATGCATGGGCCCCCAAACCTGGGAAGGAGTTCTTGCCGTGCAAGGCAG	1080
20-010	GGGATCAGTTGTTGATGCATGGGCCCCCAAACCTGGGAAGGAGTTCTTGCCGTGCAAGGCAG	1080
20-006	GGGATCAGTTGTTGATGCATGGGCCCCCAAACCTGGGAAGGAGTTCTTGCCGTGCAAGGCAG	1080
19-BMYV	GGGATCAGTTGTTGATGCATGGGCCCCCAAACCTGGGAAGGAGTTCTTGCCGTGCAAGGCAG	1080
21-097	GGGATCAGTTGTTGATGCATGGGCCCCCAAACCTGGGAAGGAGTTCTTGCCGTGCAAGGCAG	1080
19-013	GGGATCAGTTGTTGATGCATGGGCCCCCAAACCTGGGAAGGAGTTCTTGCCGTGCAAGGCAG	1080
21-294	GGGATCAGTTGTTGATGCATGGGCCCCCAAACCTGGGAAGGAGTTCTTGCCGTGCAAGGCAG	1080
21-056	GGGATCAGTTGTTGATGCATGGGCCCCCAAACCTGGGAAGGAGTTCTTGCCGTGCAAGGCAG	1080
20-009	GGGATCAGTTGTTGATGCATGGGCCCCCAAACCTGGGAAGGAGTTCTTGCCGTGCAAGGCAG	1080
20-014	GGGATCAGTTGTTGATGCATGGGCCCCCAAACCTGGGAAGGAGTTCTTGCCGTGCAAGGCAG *****	1080
21-BMYV	TTCACATGATTCCGGCATCGAGTGTGCAAAATCGAAAGCAACTTTCTTTGCTCTGTCCG	1140
21-254	TTCACATGATTCCGGCATCGAGTGTGCAAAATCGAAAGCAACTTTCTTTGCTCTGTCCG	1140
20-BMYV	TTCACATGATTCCGGCATCGAGTGTGCAAAATCGAAAGCAACTTTCTTTGCTCTGTCCG	1140
20-010	TTCACATGATTCCGGCATCGAGTGTGCAAAATCGAAAGCAACTTTCTTTGCTCTGTCCG	1140
20-006	TTCACATGATTCCGGCATCGAGTGTGCAAAATCGAAAGCAACTTTCTTTGCTCTGTCCG	1140
19-BMYV	TTCACATGATTCCGGCATCGAGTGTGCAAAATCGAAAGCAACTTTCTTTGCTCTGTCCG	1140
21-097	TTCACATGATTCCGGCATCGAGTGTGCAAAATCGAAAGCAACTTTCTTTGCTCTGTCCG	1140
19-013	TTCACATGATTCCGGCATCGAGTGTGCAAAATCGAAAGCAACTTTCTTTGCTCTGTCCG	1140

21-294 TTCACATGATTCCGGCATCGAGTGTTGCAAAATCGAAAGCAACTTTCTTTGCTCTGTCCG 1140  
 21-056 TTCACATGATTCCGGCATCGAGTGTTGCAAAATCGAAAGCAACTTTCTTTGCTCTGTCCG 1140  
 20-009 TTCACATGATTCCGGCATCGAGTGTTGCAAAATCGAAAGCAACTTTCTTTGCTCTGTCCG 1140  
 20-014 TTCACATGATTCCGGCATCGAGTGTTGCAAAATCGAAAGCAACTTTCTTTGCTCTGTCCG 1140  
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 21-BMYV ATGGTGAATGGCATTCCCTCTAATGCCGAGCTCGTTGGCACATCCAAGTGGCGAAAATTC 1200  
 21-254 ATGGTGAATGGCATTCCCTCTAATGCCGAGCTCGTTGGCACATCCAAGTGGCGAAAATTC 1200  
 20-BMYV ATGGTGAATGGCATTCCCTCTAATGCCGAGCTCGTTGGCACATCCAAGTGGCGAAAATTC 1200  
 20-010 ATGGTGAATGGCATTCCCTCTAATGCCGAGCTCGTTGGCACATCCAAGTGGCGAAAATTC 1200  
 20-006 ATGGTGAATGGCATTCCCTCTAATGCCGAGCTCGTTGGCACATCCAAGTGGCGAAAATTC 1200  
 19-BMYV ATGGTGAATGGCATTCCCTCTAATGCCGAGCTCGTTGGCACATCCAAGTGGCGAAAATTC 1200  
 21-097 ATGGTGAATGGCATTCCCTCTAATGCCGAGCTCGTTGGCACATCCAAGTGGCGAAAATTC 1200  
 19-013 ATGGTGAATGGCATTCCCTCTAATGCCGAGCTCGTTGGCACATCCAAGTGGCGAAAATTC 1200  
 21-294 ATGGTGAATGGCATTCCCTCTAATGCCGAGCTCGTTGGCACATCCAAGTGGCGAAAATTC 1200  
 21-056 ATGGTGAATGGCATTCCCTCTAATGCCGAGCTCGTTGGCACATCCAAGTGGCGAAAATTC 1200  
 20-009 ATGGTGAATGGCATTCCCTCTAATGCCGAGCTCGTTGGCACATCCAAGTGGCGAAAATTC 1200  
 20-014 ATGGTGAATGGCATTCCCTCTAATGCCGAGCTCGTTGGCACATCCAAGTGGCGAAAATTC 1200  
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 21-BMYV TTTCTGTACTCAGTGACACAAAGAGTGGTCATTCCGGGCACTCCCTATTTCAATGGTAAGA 1260  
 21-254 TTTCTGTACTCAGTGACACAAAGAGTGGTCATTCCGGGCACTCCCTATTTCAATGGTAAGA 1260  
 20-BMYV TTTCTGTACTCAGTGACACAAAGAGTGGTCATTCCGGGCACTCCCTATTTCAATGGTAAGA 1260  
 20-010 TTTCTGTACTCAGTGACACAAAGAGTGGTCATTCCGGGCACTCCCTATTTCAATGGTAAGA 1260  
 20-006 TTTCTGTACTCAGTGACACAAAGAGTGGTCATTCCGGGCACTCCCTATTTCAATGGTAAGA 1260  
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 21-097 TTTCTGTACTCAGTGACACAAAGAGTGGTCATTCCGGGCACTCCCTATTTCAATGGTAAGA 1260  
 19-013 TTTCTGTACTCAGTGACACAAAGAGTGGTCATTCCGGGCACTCCCTATTTCAATGGTAAGA 1260  
 21-294 TTTCTGTACTCAGTGACACAAAGAGTGGTCATTCCGGGCACTCCCTATTTCAATGGTAAGA 1260  
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 20-009 TTTCTGTACTCAGTGACACAAAGAGTGGTCATTCCGGGCACTCCCTATTTCAATGGTAAGA 1260  
 20-014 TTTCTGTACTCAGTGACACAAAGAGTGGTCATTCCGGGCACTCCCTATTTCAATGGTAAGA 1260  
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 21-BMYV GTGTTCTTGGAGTTCACATAGGTTCTCCGAAAGAATTTGAGTCGGAAAATGTTAATTACA 1320  
 21-254 GTGTTCTTGGAGTTCACATAGGTTCTCCGAAAGAATTTGAGTCGGAAAATGTTAATTACA 1320  
 20-BMYV GTGTTCTTGGAGTTCACATAGGTTCTCCGAAAGAATTTGAGTCGGAAAATGTTAATTACA 1320  
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 21-294 GTGTTCTTGGAGTTCACATAGGTTCTCCGAAAGAATTTGAGTCGGAAAATGTTAATTACA 1320  
 21-056 GTGTTCTTGGAGTTCACATAGGTTCTCCGAAAGAATTTGAGTCGGAAAATGTTAATTACA 1320  
 20-009 GTGTTCTTGGAGTTCACATAGGTTCTCCGAAAGAATTTGAGTCGGAAAATGTTAATTACA 1320  
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 21-BMYV TGTCTCCTATAACCACGTTTTTCCTGGATTAACCAGCCCGAACTACATATTTGAAACCACAG 1380  
 21-254 TGTCTCCTATAACCACGTTTTTCCTGGATTAACCAGCCCGAACTACATATTTGAAACCACAG 1380  
 20-BMYV TGTCTCCTATAACCACGTTTTTCCTGGATTAACCAGCCCGAACTACATATTTGAAACCACAG 1380  
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 20-006 TGTCTCCTATAACCACGTTTTTCCTGGATTAACCAGCCCGAACTACATATTTGAAACCACAG 1380  
 19-BMYV TGTCTCCTATAACCACGTTTTTCCTGGATTAACCAGCCCGAACTACATATTTGAAACCACAG 1380  
 21-097 TGTCTCCTATAACCACGTTTTTCCTGGATTAACCAGCCCGAACTACATATTTGAAACCACAG 1380  
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 21-294 TGTCTCCTATAACCACGTTTTTCCTGGATTAACCAGCCCGAACTACATATTTGAAACCACAG 1380  
 21-056 TGTCTCCTATAACCACGTTTTTCCTGGATTAACCAGCCCGAACTACATATTTGAAACCACAG 1380  
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 20-014 TGTCTCCTATAACCACGTTTTTCCTGGATTAACCAGCCCGAACTACATATTTGAAACCACAG 1380  
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 21-BMYV CCCTTGCTGGAAAATTTTTTCAGTCAAGAGGAAGTCGAAGAGCTAATGGAAGACTTCTCTC 1440  
 21-254 CCCTTGCTGGAAAATTTTTTCAGTCAAGAGGAAGTCGAAGAGCTAATGGAAGACTTCTCTC 1440  
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 20-006 CCCTTGCTGGAAAATTTTTTCAGTCAAGAGGAAGTCGAAGAGCTAATGGAAGACTTCTCTC 1440  
 19-BMYV CCCTTGCTGGAAAATTTTTTCAGTCAAGAGGAAGTCGAAGAGCTAATGGAAGACTTCTCTC 1440

21-097	CCCTTGCTGGAAAATTTTTTCAGCCAAGAGGAAGTCGAAGAGCTAATGGAAGACTTCTCTC	1440
19-013	CCCTTGCTGGAAAATTTTTTCAGCCAAGAGGAAGTCGAAGAGCTAATGGAAGACTTCTCTC	1440
21-294	CCCTTGCTGGAAAATTTTTTCAGCCAAGAGGAAGTCGAAGAGCTAATGGAAGACTTCTCTC	1440
21-056	CCCTTGCTGGAAAATTTTTTCAGCCAAGAGGAAGTCGAAGAGCTAATGGAAGACTTCTCTC	1440
20-009	CCCTTGCTGGAAAATTTTTTCAGCCAAGAGGAAGTCGAAGAGCTAATGGAAGACTTCTCTC	1440
20-014	CCCTTGCTGGAAAATTTTTTCAGCCAAGAGGAAGTCGAAGAGCTAATGGAAGACTTCTCTC	1440
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21-254	TCCAAGAGATTTATTTCTATAGCGACGGCACGTGGGAAGTACATAAAAATATGAGGCTTGTC	1500
20-BMYV	TCCAAGAGATTTATTTCTATAGCGACGGCACGTGGGAAGTACATAAAAATATGAGGCTTGTC	1500
20-010	TCCAAGAGATTTATTTCTATAGCGACGGCACGTGGGAAGTACATAAAAATATGAGGCTTGTC	1500
20-006	TCCAAGAGATTTATTTCTATAGCGACGGCACGTGGGAAGTACATAAAAATATGAGGCTTGTC	1500
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21-254	CAGGTGAAGAGACATTTCCATGATGTGCTAACAGAGTCCTCCCCGATGCAGGGGGAAGGAA	1560
20-BMYV	CAGATGAAGAGACATTTCCATGATGTGCTAACAGAGTCCTCCCCGATGCAGGGGGAAGGAA	1560
20-010	CAGGTGAAGAGACATTTCCATGATGTGCTAACAGAGTCCTCCCCGATGCAGGGGGAAGGAA	1560
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19-BMYV	CAGGTGAAGAGACATTTCCATGATGTGCTAACAGAGTCCTCCCCGATGCAGGGGGAAGGAA	1560
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19-013	CAGGTGAAGAGACATTTCCATGATGTGCTAACAGAGTCCTCCCCGATGCAGGGGGAAGGAA	1560
21-294	CAGGTGAAGAGACATTTCCATGATGTGCTAACAGAGTCCTCCCCGATGCAGGGGGAAGGAA	1560
21-056	CAGGTGAAGAGACATTTCCATGATGTGCTAACAGAGTCCTCCCCGATGCAGGGGGAAGGAA	1560
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20-014	CAGGTGAAGAGACATTTCCATGATGTGCTAACAGAGTCCTCCCCGATGCAGGGGGAAGGAA	1560
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21-254	GGGCGGCTCCGACCGCCGGAACAACCGGAAACGCAAGCACCCACGAGAGATCCGCAGGAA	1620
20-BMYV	GGGCGGCTCCGACCGCCGGAACAACCGGAAACGCAAGCACCCACGAGAGATCCGCAGGAA	1620
20-010	GGGCGGCTCCGACCGCCGGAACAACCGGAAACGCAAGCACCCACGAGAGATCCGCAGGAA	1620
20-006	GGGCGGCTCCGACCGCCGGAACAACCGGAAACGCAAGCACCCACGAGAGATCCGCAGGAA	1620
19-BMYV	GGGCGGCTCCGACCGCCGGAACAACCGGAAACGCAAGCACCCACGAGAGATCCGCAGGAA	1620
21-097	GGGCGGCTCCGACCGCCGGAACAACCGGAAACGCAAGCACCCACGAGAGATCCGCAGGAA	1620
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21-056	GGGCGGCTCCGACCGCCGGAACAACCGGAAACGCAAGCACCCACGAGAGATCCGCAGGAA	1620
20-009	GGGCGGCTCCGACCGCCGGAACAACCGGAAACGCAAGCACCCACGAGAGATCCGCAGGAA	1620
20-014	GGGCGGCTCCGACCGCCGGAACAACCGGAAACGCAAGCACCCACGAGAGATCCGCAGGAA	1620
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21-BMYV	ATGGAAAAAGCCCTCGTGCTGCTCCTTCTACACCGCGGGAACCCCTTGGTGAAAACCTGCAC	1680
21-254	ATGGAAAAAGCCCTCGTGCTGCTCCTTCTACACCGCGGGAACCCCTTGGTGAAAACCTGCAC	1680
20-BMYV	ATGGAAAAAGCCCTCGTGCTGCTCCTTCTACACCGCGGGAACCCCTTGGTGAAAACCTGCAC	1680
20-010	ATGGAAAAAGCCCTCGTGCTGCTCCTTCTACACCGCGGGAACCCCTTGGTGAAAACCTGCAC	1680
20-006	ATGGAAAAAGCCCTCGTGCTGCTCCTTCTACACCGCGGGAACCCCTTGGTGAAAACCTGCAC	1680
19-BMYV	ATGGAAAAAGCCCTCGTGCTGCTCCTTCTACACCGCGGGAACCCCTTGGTGAAAACCTGCAC	1680
21-097	ATGGAAAAAGCCCTCGTGCTGCTCCTTCTACACCGCGGGAACCCCTTGGTGAAAACCTGCAC	1680
19-013	ATGGAAAAAGCCCTCGTGCTGCTCCTTCTACACCGCGGGAACCCCTTGGTGAAAACCTGCAC	1680
21-294	ATGGAAAAAGCCCTCGTGCTGCTCCTTCTACACCGCGGGAACCCCTTGGTGAAAACCTGCAC	1680
21-056	ATGGAAAAAGCCCTCGTGCTGCTCCTTCTACACCGCGGGAACCCCTTGGTGAAAACCTGCAC	1680
20-009	ATGGAAAAAGCCCTCGTGCTGCTCCTTCTACACCGCGGGAACCCCTTGGTGAAAACCTGCAC	1680
20-014	ATGGAAAAAGCCCTCGTGCTGCTCCTTCTACACCGCGGGAACCCCTTGGTGAAAACCTGCAC	1680
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21-BMYV	CGCAAGCCACGTACATTGTACCTCAAAGAGGAATATGACGAATGGCCGAGATGCTGGTG	1740
21-254	CGCAAGCCACGTACATTGTACCTCAAAGAGGAATATGACGAATGGCCGAGATGCTGGTG	1740
20-BMYV	CGCAAGCCACGTACATTGTACCTCAAAGAGGAATATGACGAATGGCCGAGATGCTGGTG	1740
20-010	CGCAAGCCACGTACATTGTACCTCAAAGAGGAATATGACGAATGGCCGAGATGCTGGTG	1740

20-006	CGCAAGCCACGTACATTGTACCTCAAAGAGGAATATGACGAATGGCCGAGATGCTGGTG	1740
19-BMYV	CGCAAGCCACGTACATTGTACCTCAAAGAGGAATATGACGAATGGCCGAGATGCTGGTG	1740
21-097	CGCAAGCCACGTACATTGTACCTCAAAGAGGAATATGACGAATGGCCGAGATGCTGGTG	1740
19-013	CGCAAGCCACGTACATTGTACCTCAAAGAGGAATATGACGAATGGCCGAGATGCTGGTG	1740
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21-056	CGCAAGCCACGTACATTGTACCTCAAAGAGGAATATGACGAATGGCCGAGATGCTGGTG	1740
20-009	CGCAAGCCACGTACATTGTACCTCAAAGAGGAATATGACGAATGGCCGAGATGCTGGTG	1740
20-014	CGCAAGCCACGTACATTGTACCTCAAAGAGGAATATGACGAATGGCCGAGATGCTGGTG	1740
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21-254	CCAAATTGCAGGCCACGACTGCCACTACCGATCAAATCTCAGAGATAAAGAAGGCTCTGA	1800
20-BMYV	CCAAATTGCAGGCCACGACTGCCACTACCGATCAAATCTCAGAGATAAAGAAGGCTCTGA	1800
20-010	CCAAATTGCAGGCCACGACTGCCACTACCGATCAAATCTCAGAGATAAAGAAGGCTCTGA	1800
20-006	CCAAATTGCAGGCCACGACTGCCACTACCGATCAAATCTCAGAGATAAAGAAGGCTCTGA	1800
19-BMYV	CCAAATTGCAGGCCACGACTGCCACTACCGATCAAATCTCAGAGATAAAGAAGGCTCTGA	1800
21-097	CCAAATTGCAGGCCACGACTGCCACTACCGATCAAATCTCAGAGATAAAGAAGGCTCTGA	1800
19-013	CCAAATTGCAGGCCACGACTGCCACTACCGATCAAATCTCAGAGATAAAGAAGGCTCTGA	1800
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21-056	CCAAATTGCAGGCCACGACTGCCACTACCGATCAAATCTCAGAGATAAAGAAGGCTCTGA	1800
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21-254	TAGACAAAATGGATTGAAATCGATCGAGAGACAAGTGGTAGAGACACTATCGTCGATGG	1860
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21-097	TAGACAAAATGGATTGAAATCGATCGAGAGACAAGTGGTAGAGACACTATCGTCGATGG	1860
19-013	TAGACAAAATGGATTGAAATCGATCGAGAGACAAGTGGTAGAGACACTATCGTCGATGG	1860
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21-056	TAGACAAAATGGATTGAAATCGATCGAGAGACAAGTGGTAGAGACACTATCGTCGATGG	1860
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21-BMYV	CCATGAAGAAGCCCCGCTCAAGAGGGCGGAGAAGATCCAAGAACAAGCAAACAATTTGG	1920
21-254	CCATGAAGAAGCCCCGCTCAAGAGGGCGGAGAAGATCCAAGAACAAGCAAACAATTTGG	1920
20-BMYV	CCATGAAGAAGCCCCGCTCAAGAGGGCGGAGAAGATCCAAGAACAAGCAAACAATTTGG	1920
20-010	CCATGAAGAAGCCCCGCTCAAGAGGGCGGAGAAGATCCAAGAACAAGCAAACAATTTGG	1920
20-006	CCATGAAGAAGCCCCGCTCAAGAGGGCGGAGAAGATCCAAGAACAAGCAAACAATTTGG	1920
19-BMYV	CCATGAAGAAGCCCCGCTCAAGAGGGCGGAGAAGATCCAAGAACAAGCAAACAATTTGG	1920
21-097	CCATGAAGAAGCCCCGCTCAAGAGGGCGGAGAAGATCCAAGAACAAGCAAACAATTTGG	1920
19-013	CCATGAAGAAGCCCCGCTCAAGAGGGCGGAGAAGATCCAAGAACAAGCAAACAATTTGG	1920
21-294	CCATGAAGAAGCCCCGCTCAAGAGGGCGGAGAAGATCCAAGAACAAGCAAACAATTTGG	1920
21-056	CCATGAAGAAGCCCCGCTCAAGAGGGCGGAGAAGATCCAAGAACAAGCAAACAATTTGG	1920
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20-014	CCATGAAGAAGCCCCGCTCAAGAGGGCGGAGAAGATCCAAGAACAAGCAAACAATTTGG	1920
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21-BMYV	ATGCTTCTTCAAACCCAGTACCCTGGGAAAGAGCCGAGAGGTCTGCCCCGGTTTCAT	1980
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20-BMYV	ACGCTTCTTCAAACCCAGTACCCTGGGAAAGAGCCGAGAGGTCTGCCCCGGTTTCAT	1980
20-010	ATGCTTCTTCAAACCCAGTACCCTGGGAAAGAGCCGAGAGGTCTGCCCCGGTTTCAT	1980
20-006	ACGCTTCTTCAAACCCAGTACCCTGGGAAAGAGCCGAGAGGTCTGCCCCGGTTTCAT	1980
19-BMYV	ACGCTTCTTCAAACCCAGTACCCTGGGAAAGAGCCGAGAGGTCTGCCCCGGTTTCAT	1980
21-097	ACGCTTCTTCAAACCCAGTACCCTGGGAAAGAGCCGAGAGGTCTGCCCCGGTTTCAT	1980
19-013	ATGCTTCTTCAAACCCAGTACCCTGGGAAAGAGCCGAGAGGTCTGCCCCGGTTTCAT	1980
21-294	ACGCTTCTTCAAACCCAGTACCCTGGGAAAGAGCCGAGAGGTCTGCCCCGGTTTCAT	1980
21-056	ACGCTTCTTCAAACCCAGTACCCTGGGAAAGAGCCGAGAGGTCTGCCCCGGTTTCAT	1980
20-009	ACGCTTCTTCAAACCCAGTACCCTGGGAAAGAGCCGAGAGGTCTGCCCCGGTTTCAT	1980
20-014	ACGCTTCTTCAAACCCAGTACCCTGGGAAAGAGCCGAGAGGTCTGCCCCGGTTTCAT	1980
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21-BMYV	CAAAGTCGGTGAGCTCCCAAGTTTTACTTCTCTAAACAAAAGGATGCTCGGATTGGGG	2040
21-254	CAAAGTCGGTGAGCTCCCAAGTTTTACTTCTCTAAACAAAAGGATGCTCGGATTGGGG	2040



20-BMYV	CAAAGTCGGTGAGCTCCCCAAGTTTTACTTCTCTAAACAAAAAGGATGCTCGGATTGGGG	2040
20-010	CAAAGTCGGTGAGCTCCCCAAGTTTTACTTCTCTAAACAAAAAGGATGCTCGGATTGGGG	2040
20-006	CAAAGTCGGTGAGCTCCCCAAGTTTTACTTCTCTAAACAAAAAGGATGCTCGGATTGGGG	2040
19-BMYV	CAAAGTCGGTGAGCTCCCCAAGTTTTACTTCTCTAAACAAAAAGGATGCTCGGATTGGGG	2040
21-097	CAAAGTCGGTGAGCTCCCCAAGTTTTACTTCTCTAAACAAAAAGGATGCTCGGATTGGGG	2040
19-013	CAAAGTCGGTGAGCTCCCCAAGTTTTACTTCTCTAAACAAAAAGGATGCTCGGATTGGGG	2040
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21-056	CAAAGTCGGTGAGCTCCCCAAGTTTTACTTCTCTAAACAAAAAGGATGCTCGGATTGGGG	2040
20-009	CAAAGTCGGTGAGCTCCCCAAGTTTTACTTCTCTAAACAAAAAGGATGCTCGGATTGGGG	2040
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21-254	CACGAAGCTCACCAGCCTCCACCCAGAATTGGAGGAGAAAACCCGAGGCTTCGGGTGGCC	2100
20-BMYV	CACGAAGCTCACCAGCCTCCACCCAGAATTGGAGGAGAAAACCCGAGGCTTCGGGTGGCC	2100
20-010	CACGAAGCTCACCAGCCTCCACCCAGAATTGGAGGAGAAAACCCGAGGCTTCGGGTGGCC	2100
20-006	CACGAAGCTCACCAGCCTCCACCCAGAATTGGAGGAGAAAACCCGAGGCTTCGGGTGGCC	2100
19-BMYV	CACGAAGCTCACCAGCCTCCACCCAGAATTGGAGGAGAAAACCCGAGGCTTCGGGTGGCC	2100
21-097	CACGAAGCTCACCAGCCTCCACCCAGAATTGGAGGAGAAAACCCGAGGCTTCGGGTGGCC	2100
19-013	CACGAAGCTCACCAGCCTCCACCCAGAATTGGAGGAGAAAACCCGAGGCTTCGGGTGGCC	2100
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20-009	CACGAAGCTCACCAGCCTCCACCCAGAATTGGAGGAGAAAACCCGAGGCTTCGGGTGGCC	2100
20-014	CACGAAGCTCACCAGCCTCCACCCAGAATTGGAGGAGAAAACCCGAGGCTTCGGGTGGCC	2100
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21-254	CAAGTTCGGGCCAGCGGCGGAACGAAATCCTTGCGGCTACAAGCCGCAAGATGGCTCGA	2160
20-BMYV	CAAGTTCGGGCCAGCGGCGGAACGAAATCCTTGCGGCTACAAGCCGCAAGATGGCTCGA	2160
20-010	CAAGTTCGGGCCAGCGGCGGAACGAAATCCTTGCGGCTACAAGCCGCAAGATGGCTCGA	2160
20-006	CAAGTTCGGGCCAGCGGCGGAACGAAATCCTTGCGGCTACAAGCCGCAAGATGGCTCGA	2160
19-BMYV	CAAGTTCGGGCCAGCGGCGGAACGAAATCCTTGCGGCTACAAGCCGCAAGATGGCTCGA	2160
21-097	CAAGTTCGGGCCAGCGGCGGAACGAAATCCTTGCGGCTACAAGCCGCAAGATGGCTCGA	2160
19-013	CAAGTTCGGGCCAGCGGCGGAACGAAATCCTTGCGGCTACAAGCCGCAAGATGGCTCGA	2160
21-294	CAAGTTCGGGCCAGCGGCGGAACGAAATCCTTGCGGCTACAAGCCGCAAGATGGCTCGA	2160
21-056	CAAGTTCGGGCCAGCGGCGGAACGAAATCCTTGCGGCTACAAGCCGCAAGATGGCTCGA	2160
20-009	CAAGTTCGGGCCAGCGGCGGAACGAAATCCTTGCGGCTACAAGCCGCAAGATGGCTCGA	2160
20-014	CAAGTTCGGGCCAGCGGCGGAACGAAATCCTTGCGGCTACAAGCCGCAAGATGGCTCGA	2160
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21-BMYV	ACGCGCCGAGCAAGTAAAAATCCCTTCGACTGAGGAAAGGGAGCGCGTCGTGAGGAAATG	2220
21-254	ACGCGCCGAGCAAGTAAAAATCCCTTCGACTGAGGAAAGGGAGCGCGTCGTGAGGAAATG	2220
20-BMYV	ACGCGCCGAGCAAGTAAAAATCCCTTCGACTGAGGAAAGGGAGCGCGTCGTGAGGAAATG	2220
20-010	ACGCGCCGAGCAAGTAAAAATCCCTTCGACTGAGGAAAGGGAGCGCGTCGTGAGGAAATG	2220
20-006	ACGCGCCGAGCAAGTAAAAATCCCTTCGACTGAGGAAAGGGAGCGCGTCGTGAGGAAATG	2220
19-BMYV	ACGCGCCGAGCAAGTAAAAATCCCTTCGACTGAGGAAAGGGAGCGCGTCGTGAGGAAATG	2220
21-097	ACGCGCCGAGCAAGTAAAAATCCCTTCGACTGAGGAAAGGGAGCGCGTCGTGAGGAAATG	2220
19-013	ACGCGCCGAGCAAGTAAAAATCCCTTCGACTGAGGAAAGGGAGCGCGTCGTGAGGAAATG	2220
21-294	ACGCGCCGAGCAAGTAAAAATCCCTTCGACTGAGGAAAGGGAGCGCGTCGTGAGGAAATG	2220
21-056	ACGCGCCGAGCAAGTAAAAATCCCTTCGACTGAGGAAAGGGAGCGCGTCGTGAGGAAATG	2220
20-009	ACGCGCCGAGCAAGTAAAAATCCCTTCGACTGAGGAAAGGGAGCGCGTCGTGAGGAAATG	2220
20-014	ACGCGCCGAGCAAGTAAAAATCCCTTCGACTGAGGAAAGGGAGCGCGTCGTGAGGAAATG	2220
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21-BMYV	TGTGGAAGCATTCTCGCCCACTCAAACACGAGGTCCCATGGCCACGAGAGGAAACAAACT	2280
21-254	TGTGGAAGCATTCTCGCCCACTCAAACACGAGGTCCCATGGCCACGAGAGGAAACAAACT	2280
20-BMYV	TGTGGAAGCATTCTCGCCCACTCAAACACGAGGTCCCATGGCCACGAGAGGAAACAAACT	2280
20-010	TGTGGAAGCATTCTCGCCCACTCAAACACGAGGTCCCATGGCCACGAGAGGAAACAAACT	2280
20-006	TGTGGAAGCATTCTCGCCCACTCAAACACGAGGTCCCATGGCCACGAGAGGAAACAAACT	2280
19-BMYV	TGTGGAAGCATTCTCGCCCACTCAAACACGAGGTCCCATGGCCACGAGAGGAAACAAACT	2280
21-097	TGTGGAAGCATTCTCGCCCACTCAAACACGAGGTCCCATGGCCACGAGAGGAAACAAACT	2280
19-013	TGTGGAAGCATTCTCGCCCACTCAAACACGAGGTCCCATGGCCACGAGAGGAAACAAACT	2280
21-294	TGTGGAAGCATTCTCGCCCACTCAAACACGAGGTCCCATGGCCACGAGAGGAAACAAACT	2280
21-056	TGTGGAAGCATTCTCGCCCACTCAAACACGAGGTCCCATGGCCACGAGAGGAAACAAACT	2280
20-009	TGTGGAAGCATTCTCGCCCACTCAAACACGAGGTCCCATGGCCACGAGAGGAAACAAACT	2280
20-014	TGTGGAAGCATTCTCGCCCACTCAAACACGAGGTCCCATGGCCACGAGAGGAAACAAACT	2280
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21-BMYV	GTCTTGGAACAATTTCCCTTGAAGATTTTAAAACGGCAGTTTTCTCTCTCGAGCTCGAAGC	2340
21-254	GTCTTGGAACAATTTCCCTTGAAGATTTTAAAACGGCAGTTTTCTCTCTCGAGCTCGAAGC	2340
20-BMYV	GTCTTGGAACAATTTCCCTTGAAGATTTTAAAACGGCAGTTTTCTCTCTCGAGCTCGAAGC	2340
20-010	GTCTTGGAACAATTTCCCTTGAAGATTTTAAAACGGCAGTTTTCTCTCTCGAGCTCGAAGC	2340
20-006	GTCTTGGAACAATTTCCCTTGAAGATTTTAAAACGGCAGTTTTCTCTCTCGAGCTCGAAGC	2340
19-BMYV	GTCTTGGAACAATTTCCCTTGAAGATTTTAAAACGGCAGTTTTCTCTCTCGAGCTCGAAGC	2340
21-097	GTCTTGGAACAATTTCCCTTGAAGATTTTAAAACGGCAGTTTTCTCTCTCGAGCTCGAAGC	2340
19-013	GTCTTGGAACAATTTCCCTTGAAGATTTTAAAACGGCAGTTTTCTCTCTCGAGCTCGAAGC	2340
21-294	GTCTTGGAACAATTTCCCTTGAAGATTTTAAAACGGCAGTTTTCTCTCTCGAGCTCGAAGC	2340
21-056	GTCTTGGAACAATTTCCCTTGAAGATTTTAAAACGGCAGTTTTCTCTCTCGAGCTCGAAGC	2340
20-009	GTCTTGGAACAATTTCCCTTGAAGATTTTAAAACGGCAGTTTTCTCTCTCGAGCTCGAAGC	2340
20-014	GTCTTGGAACAATTTCCCTTGAAGATTTTAAAACGGCAGTTTTCTCTCTCGAGCTCGAAGC	2340
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21-BMYV	CGGCGTAGGCGTCCCGTATGTTGCTTACGGTCGACGCACGCACAGAGGCTGGATTGAAGA	2400
21-254	CGGCGTAGGCGTCCCGTATGTTGCTTACGGTCGACGCACGCACAGAGGCTGGATTGAAGA	2400
20-BMYV	CGGCGTAGGCGTCCCGTATGTTGCTTACGGTCGACGCACGCACAGAGGCTGGATTGAAGA	2400
20-010	CGGCGTAGGCGTCCCGTATGTTGCTTACGGTCGACGCACGCACAGAGGCTGGATTGAAGA	2400
20-006	CGGCGTAGGCGTCCCGTATGTTGCTTACGGTCGACGCACGCACAGAGGCTGGATTGAAGA	2400
19-BMYV	CGGCGTAGGCGTCCCGTATGTTGCTTACGGTCGACGCACGCACAGAGGCTGGATTGAAGA	2400
21-097	CGGCGTAGGCGTCCCGTATGTTGCTTACGGTCGACGCACGCACAGAGGCTGGATTGAAGA	2400
19-013	CGGCGTAGGCGTCCCGTATGTTGCTTACGGTCGACGCACGCACAGAGGCTGGATTGAAGA	2400
21-294	CGGCGTAGGCGTCCCGTATGTTGCTTACGGTCGACGCACGCACAGAGGCTGGATTGAAGA	2400
21-056	CGGCGTAGGCGTCCCGTATGTTGCTTACGGTCGACGCACGCACAGAGGCTGGATTGAAGA	2400
20-009	CGGCGTAGGCGTCCCGTATGTTGCTTACGGTCGACGCACGCACAGAGGCTGGATTGAAGA	2400
20-014	CGGCGTAGGCGTCCCGTATGTTGCTTACGGTCGACGCACGCACAGAGGCTGGATTGAAGA	2400
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21-BMYV	TCCAGATCTGTTGCCGGTTTTAGCTCGTTTTACCTTCGATCGATTACAGAAGTTATCGGA	2460
21-254	TCCAGATCTGTTGCCGGTTTTAGCTCGTTTTACCTTCGATCGATTACAGAAGTTATCGGA	2460
20-BMYV	TCCAGATCTGTTGCCGGTTTTAGCTCGTTTTACCTTCGATCGATTACAGAAGTTATCGGA	2460
20-010	TCCAGATCTGTTGCCGGTTTTAGCTCGTTTTACCTTCGATCGATTACAGAAGTTATCGGA	2460
20-006	TCCAGATCTGTTGCCGGTTTTAGCTCGTTTTACCTTCGATCGATTACAGAAGTTATCGGA	2460
19-BMYV	TCCAGATCTGTTGCCGGTTTTAGCTCGTTTTACCTTCGATCGATTACAGAAGTTATCGGA	2460
21-097	TCCAGATCTGTTGCCGGTTTTAGCTCGTTTTACCTTCGATCGATTACAGAAGTTATCGGA	2460
19-013	TCCAGATCTGTTGCCGGTTTTAGCTCGTTTTACCTTCGATCGATTACAGAAGTTATCGGA	2460
21-294	TCCAGATCTGTTGCCGGTTTTAGCTCGTTTTACCTTCGATCGATTACAGAAGTTATCGGA	2460
21-056	TCCAGATCTGTTGCCGGTTTTAGCTCGTTTTACCTTCGATCGATTACAGAAGTTATCGGA	2460
20-009	TCCAGATCTGTTGCCGGTTTTAGCTCGTTTTACCTTCGATCGATTACAGAAGTTATCGGA	2460
20-014	TCCAGATCTGTTGCCGGTTTTAGCTCGTTTTACCTTCGATCGATTACAGAAGTTATCGGA	2460
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21-BMYV	GGTGAAATTTGAGCATATGAGCCCTGAACAATTTGGTTCAGGAAGGCCCTGTGTGACCCAAT	2520
21-254	GGTGAAATTTGAGCATATGAGCCCTGAACAATTTGGTTCAGGAAGGCCCTGTGTGACCCAAT	2520
20-BMYV	GGTGAAATTTGAGCATATGAGCCCTGAACAATTTGGTTCAGGAAGGCCCTGTGTGACCCAAT	2520
20-010	GGTGAAATTTGAGCATATGAGCCCTGAACAATTTGGTTCAGGAAGGCCCTGTGTGACCCAAT	2520
20-006	GGTGAAATTTGAGCATATGAGCCCTGAACAATTTGGTTCAGGAAGGCCCTGTGTGACCCAAT	2520
19-BMYV	GGTGAAATTTGAGCATATGAGCCCTGAACAATTTGGTTCAGGAAGGCCCTGTGTGACCCAAT	2520
21-097	GGTGAAATTTGAGCATATGAGCCCTGAACAATTTGGTTCAGGAAGGCCCTGTGTGACCCAAT	2520
19-013	GGTGAAATTTGAGCATATGAGCCCTGAACAATTTGGTTCAGGAAGGCCCTGTGTGACCCAAT	2520
21-294	GGTGAAATTTGAGCATATGAGCCCTGAACAATTTGGTTCAGGAAGGCCCTGTGTGACCCAAT	2520
21-056	GGTGAAATTTGAGCATATGAGCCCTGAACAATTTGGTTCAGGAAGGCCCTGTGTGACCCAAT	2520
20-009	GGTGAAATTTGAGCATATGAGCCCTGAACAATTTGGTTCAGGAAGGCCCTGTGTGACCCAAT	2520
20-014	GGTGAAATTTGAGCATATGAGCCCTGAACAATTTGGTTCAGGAAGGCCCTGTGTGACCCAAT	2520
	** *****	
21-BMYV	ACGGTTATTCGTA AAAAGGCGAGCCACACAAACAATCCAAACTTGATGAAGGACGCTACCG	2580
21-254	ACGGTTATTCGTA AAAAGGCGAGCCACACAAACAATCCAAACTTGATGAAGGACGCTACCG	2580
20-BMYV	ACGGTTATTCGTA AAAAGGCGAGCCACACAAACAATCCAAACTTGATGAAGGACGCTACCG	2580
20-010	ACGGTTATTCGTA AAAAGGCGAGCCACACAAACAATCCAAACTTGATGAAGGACGCTACCG	2580
20-006	ACGGTTATTCGTA AAAAGGCGAGCCACACAAACAATCCAAACTTGATGAAGGACGCTACCG	2580
19-BMYV	ACGGTTATTCGTA AAAAGGCGAGCCACACAAACAATCCAAACTTGATGAAGGACGCTACCG	2580
21-097	ACGGTTATTCGTA AAAAGGCGAGCCACACAAACAATCCAAACTTGATGAAGGACGCTACCG	2580
19-013	ACGGTTATTCGTA AAAAGGCGAGCCACACAAACAATCCAAACTTGATGAAGGACGCTACCG	2580
21-294	ACGGTTATTCGTA AAAAGGCGAGCCACACAAACAATCCAAACTTGATGAAGGACGCTACCG	2580
21-056	ACGGTTATTCGTA AAAAGGCGAGCCACACAAACAATCCAAACTTGATGAAGGACGCTACCG	2580
20-009	ACGGTTATTCGTA AAAAGGCGAGCCACACAAACAATCCAAACTTGATGAAGGACGCTACCG	2580
20-014	ACGGTTATTCGTA AAAAGGCGAGCCACACAAACAATCCAAACTTGATGAAGGACGCTACCG	2580

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21-BMYV	CCTCATCATGAGTGTCTCATTGGTTGACCAACTGGTAGCCCGGGTTCTGTTTCAAAATCA	2640
21-254	CCTCATCATGAGTGTCTCATTGGTTGATCAACTGGTAGCCCGGGTTCTGTTTCAAAATCA	2640
20-BMYV	CCTCATCATGAGTGTCTCATTGGTTGATCAACTGGTAGCCCGGGTTCTGTTTCAAAATCA	2640
20-010	CCTCATCATGAGTGTCTCATTGGTTGATCAACTGGTAGCCCGGGTTCTGTTTCAAAATCA	2640
20-006	CCTCATCATGAGTGTCTCATTGGTTGATCAACTGGTAGCCCGGGTTCTGTTTCAAAATCA	2640
19-BMYV	CCTCATCATGAGTGTCTCATTGGTTGATCAACTGGTAGCCCGGGTTCTGTTTCAAAATCA	2640
21-097	CCTCATCATGAGTGTCTCATTGGTTGATCAACTGGTAGCCCGGGTTCTGTTTCAAAATCA	2640
19-013	CCTCATCATGAGTGTCTCATTGGTTGATCAACTGGTAGCCCGGGTTCTGTTTCAAAATCA	2640
21-294	CCTCATCATGAGTGTCTCATTGGTTGATCAACTGGTAGCCCGGGTTCTGTTTCAAAATCA	2640
21-056	CCTCATCATGAGTGTCTCATTGGTTGATCAACTGGTAGCCCGGGTTCTGTTTCAAAATCA	2640
20-009	CCTCATCATGAGTGTCTCATTGGTTGATCAACTGGTAGCCCGGGTTCTGTTTCAAAATCA	2640
20-014	CCTCATCATGAGTGTCTCATTGGTTGATCAACTGGTAGCCCGGGTTCTGTTTCAAAATCA	2640

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21-BMYV	GAACAAGCGCGAGATCGCGCTCTGGAGGGCGATTCCCTCAAACCCGGATTTCGGATTGTC	2700
21-254	GAACAAGCGCGAGATCGCGCTCTGGAGGGCGATTCCCTCAAACCCGGATTTCGGATTGTC	2700
20-BMYV	GAACAAGCGCGAGATCGCGCTTTGGAGGGCGATTCCCTCAAACCCGGATTTCGGATTGTC	2700
20-010	GAACAAGCGCGAGATCGCGCTTTGGAGGGCGATTCCCTCAAACCCGGATTTCGGATTGTC	2700
20-006	GAACAAGCGCGAGATCGCGCTTTGGAGGGCGATTCCCTCAAACCCGGATTTCGGATTGTC	2700
19-BMYV	GAACAAGCGCGAGATCGCGCTTTGGAGGGCGATTCCCTCAAACCCGGATTTCGGATTGTC	2700
21-097	GAACAAGCGCGAGATCGCGCTTTGGAGGGCGATTCCCTCAAACCCGGATTTCGGATTGTC	2700
19-013	GAACAAGCGCGAGATCGCGCTTTGGAGGGCGATTCCCTCAAACCCGGATTTCGGATTGTC	2700
21-294	GAACAAGCGCGAGATCGCGCTTTGGAGGGCGATTCCCTCAAACCCGGATTTCGGATTGTC	2700
21-056	GAACAAGCGCGAGATCGCGCTTTGGAGGGCGATTCCCTCAAACCCGGATTTCGGATTGTC	2700
20-009	GAACAAGCGCGAGATCGCGCTTTGGAGGGCGATTCCCTCAAACCCGGATTTCGGATTGTC	2700
20-014	GAACAAGCGCGAGATCGCGCTTTGGAGGGCGATTCCCTCAAACCCGGATTTCGGATTGTC	2700

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21-BMYV	CACGGACGGACAAGTCGTCGATTTTCATGCAAGCATTATCGGCGCAGGTGGGAGTGAACAC	2760
21-254	CACGGACGGACAAGTCGTCGATTTTCATGCAAGCATTATCGGCGCAGGTGGGAGTGAACAC	2760
20-BMYV	CACGGACGGACAAGTCGTCGATTTTCATGCAAGCATTATCGGCGCAGGTGGGAGTGAACAC	2760
20-010	CACGGACGGACAAGTCGTCGATTTTCATGCAAGCATTATCGGCGCAGGTGGGAGTGAACAC	2760
20-006	CACAGACGGACAAGTCGTCGATTTTCATGCAAGCATTATCGGCGCAGGTGGGAGTGAACAC	2760
19-BMYV	CACAGACGGACAAGTCGTCGATTTTCATGCAAGCATTATCGGCGCAGGTGGGAGTGAACAC	2760
21-097	CACGGACGGACAAGTCGTCGATTTTCATGCAAGCATTATCGGCGCAGGTGGGAGTGAACAC	2760
19-013	CACGGACGGACAAGTCGTCGATTTTCATGCAAGCATTATCGGCGCAGGTGGGAGTGAACAC	2760
21-294	CACGGACGGACAAGTCGTCGATTTTCATGCAAGCATTATCGGCGCAGGTGGGAGTGAACAC	2760
21-056	CACGGACGGACAAGTCGTCGATTTTCATGCAAGCATTATCGGCGCAGGTGGGAGTGAACAC	2760
20-009	CACGGACGGACAAGTCGTCGATTTTCATGCAAGCATTATCGGCGCAGGTGGGAGTGAACAC	2760
20-014	CACGGACGGACAAGTCGTCGATTTTCATGCAAGCATTATCGGCGCAGGTGGGAGTGAACAC	2760

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21-BMYV	TGCTGAATTACTCCAAAATTGGAAATCCCACCTTATTCCTACAGATTGCTCTGGTTTTGA	2820
21-254	TGCTGAATTACTCCAAAATTGGAAATCCCACCTTATTCCTACAGATTGCTCTGGTTTTGA	2820
20-BMYV	TGCTGAATTACTCCAAAATTGGAAATCCCACCTTATTCCTACAGATTGCTCTGGTTTTGA	2820
20-010	TGCTGAATTACTCCAAAATTGGAAATCCCACCTTATTCCTACAGATTGCTCTGGTTTTGA	2820
20-006	TGCTGAATTACTCCAAAATTGGAAATCCCACCTTATTCCTACAGATTGCTCTGGTTTTGA	2820
19-BMYV	TGCTGAATTACTCCAAAATTGGAAATCCCACCTTATTCCTACAGATTGCTCTGGTTTTGA	2820
21-097	TGCTGAATTACTCCAAAATTGGAAATCCCACCTTATTCCTACAGATTGCTCTGGTTTTGA	2820
19-013	TGCTGAATTACTCCAAAATTGGAAATCCCACCTTATTCCTACAGATTGCTCTGGTTTTGA	2820
21-294	TGCTGAATTACTCCAAAATTGGAAATCCCACCTTATTCCTACAGATTGCTCTGGTTTTGA	2820
21-056	TGCTGAATTACTCCAAAATTGGAAATCCCACCTTATTCCTACAGATTGCTCTGGTTTTGA	2820
20-009	TGCTGAATTACTCCAAAATTGGAAATCCCACCTTATTCCTACAGATTGCTCTGGTTTTGA	2820
20-014	TGCTGAATTACTCCAAAATTGGAAATCCCACCTTATTCCTACAGATTGCTCTGGTTTTGA	2820

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21-BMYV	CTGGAGCGTTTCGGACTGGCTTCTAGAGGATGAAATGGAAGTCCGGAACAGGCTCACGTT	2880
21-254	CTGGAGCGTTTCGGACTGGCTTCTAGAGGATGAAATGGAAGTCCGGAACAGGCTCACGTT	2880
20-BMYV	CTGGAGCGTTTCGGACTGGCTTCTAGAGGATGAAATGGAAGTCCGGAACAGGCTCACGTT	2880
20-010	CTGGAGCGTTTCGGACTGGCTTCTAGAGGATGAAATGGAAGTCCGGAACAGGCTCACGTT	2880
20-006	CTGGAGCGTTTCGGACTGGCTTCTAGAGGATGAAATGGAAGTCCGGAACAGGCTCACGTT	2880
19-BMYV	CTGGAGCGTTTCGGACTGGCTTCTAGAGGATGAAATGGAAGTCCGGAACAGGCTCACGTT	2880
21-097	CTGGAGCGTTTCGGACTGGCTTCTAGAGGATGAAATGGAAGTCCGGAACAGGCTCACGTT	2880
19-013	CTGGAGCGTTTCGGACTGGCTTCTAGAGGATGAAATGGAAGTCCGGAACAGGCTCACGTT	2880
21-294	CTGGAGCGTTTCGGACTGGCTTCTAGAGGATGAAATGGAAGTCCGGAACAGGCTCACGTT	2880
21-056	CTGGAGCGTTTCGGACTGGCTTCTAGAGGATGAAATGGAAGTCCGGAACAGGCTCACGTT	2880

20-009	CTGGAGCGTTTCGGACTGGCTTCTAGAGGATGAAATGGAAGTCCGGAACAGGCTCACGTT	2880
20-014	CTGGAGCGTTTCGGACTGGCTTCTAGAGGATGAAATGGAAGTCCGGAACAGGCTCACGTT *****	2880
21-BMYV	GGATATAAATGATCTAACCAGGCGTCTGCGAGCTGGATGGCTTAAATGCCTCGCAAATAG	2940
21-254	GGATATAAATGATCTAACCAGGCGACTGCGAGCTGGATGGCTTAAATGCCTCGCAAATAG	2940
20-BMYV	GGACATAAATGATCTAACCAGGCGTCTGCGAGCTGGATGGCTTAAATGCCTCGCAAATAG	2940
20-010	GGACATAAATGATCTAACCAGGCGACTGCGAGCTGGATGGCTTAAATGCCTCGCAAATAG	2940
20-006	GGACATAAATGATCTAACCAGGCGTCTGCGAGCTGGATGGCTTAAATGCCTCGCAAATAG	2940
19-BMYV	GGACATAAATGATCTAACCAGGCGTCTGCGAGCTGGATGGCTTAAATGCCTCGCAAATAG	2940
21-097	GGACATAAATGATCTAACCAGGCGACTGCGAGCTGGATGGCTTAAATGCCTCGCAAATAG	2940
19-013	GGACATAAATGATCTAACCAGGCGTCTGCGAGCTGGATGGCTTAAATGCCTCGCAAATAG	2940
21-294	GGACATAAATGATCTAACCAGGCGTCTGCGAGCTGGATGGCTTAAATGCCTCGCAAATAG	2940
21-056	GGACATAAATGATCTAACCAGGCGTCTGCGAGCTGGATGGCTTAAATGCCTCGCAAATAG	2940
20-009	GGACATAAATGATCTAACCAGGCGTCTGCGAGCTGGATGGCTTAAATGCCTCGCAAATAG	2940
20-014	GGACATAAATGATCTAACCAGGCGTCTGCGAGCTGGATGGCTTAAATGCCTCGCAAATAG *** *****	2940
21-BMYV	TGTTCTCTGTCTATCAGATGGAACATTGCTCTCGCAGCAAGTGCCTGGTGTACAAAAGAG	3000
21-254	TGTTCTCTGTCTATCAGATGGAACATTGCTCTCGCAGCAAGTGCCTGGTGTACAAAAGAG	3000
20-BMYV	TGTTCTCTGTCTATCAGATGGAACATTGCTCTCGCAGCAAGTGCCTGGTGTACAAAAGAG	3000
20-010	TGTTCTCTGTCTATCAGATGGAACATTGCTCTCGCAGCAAGTGCCTGGTGTACAAAAGAG	3000
20-006	TGTTCTCTGTCTATCAGATGGAACATTGCTCTCGCAGCAAGTGCCTGGTGTACAAAAGAG	3000
19-BMYV	TGTTCTCTGTCTATCAGATGGAACATTGCTCTCGCAGCAAGTGCCTGGTGTACAAAAGAG	3000
21-097	TGTTCTCTGTCTATCAGATGGAACATTGCTCTCGCAGCAAGTGCCTGGTGTACAAAAGAG	3000
19-013	TGTTCTCTGTCTATCAGATGGAACATTGCTCTCGCAGCAAGTGCCTGGTGTACAAAAGAG	3000
21-294	TGTTCTCTGTCTATCAGATGGAACATTGCTCTCGCAGCAAGTGCCTGGTGTACAAAAGAG	3000
21-056	TGTTCTCTGTCTATCAGATGGAACATTGCTCTCGCAGCAAGTGCCTGGTGTACAAAAGAG	3000
20-009	TGTTCTCTGTCTATCAGATGGAACATTGCTCTCGCAGCAAGTGCCTGGTGTACAAAAGAG	3000
20-014	TGTTCTCTGTCTATCAGATGGAACATTGCTCTCGCAGCAAGTGCCTGGTGTACAAAAGAG *****	3000
21-BMYV	TGGCAGCTACAACACCTCCTCGTCTAACTCTAGAATTCGAGTGATGGCCGCTTACCACTC	3060
21-254	TGGCAGCTACAACACCTCCTCGTCTAATTCTAGAATTCGAGTGATGGCCGCTTACCACTC	3060
20-BMYV	TGGCAGCTACAACACCTCCTCGTCTAACTCTAGAATTCGAGTGATGGCTGCTTACCACTC	3060
20-010	TGGCAGCTACAACACCTCCTCGTCTAACTCTAGAATTCGAGTGATGGCCGCTTACCACTC	3060
20-006	TGGCAGCTACAACACCTCCTCGTCTAACTCTAGAATTCGAGTGATGGCCGCTTACCACTC	3060
19-BMYV	TGGCAGCTACAACACCTCCTCGTCTAACTCTAGAATTCGAGTGATGGCCGCTTACCACTC	3060
21-097	TGGCAGCTACAACACCTCCTCGTCTAACTCTAGAATTCGAGTGATGGCCGCTTACCACTC	3060
19-013	TGGCAGCTACAACACCTCCTCGTCTAACTCTAGAATTCGAGTGATGGCCGCTTACCACTC	3060
21-294	TGGCAGCTACAACACCTCCTCGTCTAACTCTAGAATTCGAGTGATGGCCGCTTACCACTC	3060
21-056	TGGCAGCTACAACACCTCCTCGTCTAACTCTAGAATTCGAGTGATGGCCGCTTACCACTC	3060
20-009	TGGCAGCTACAACACCTCCTCGTCTAACTCTAGAATTCGAGTGATGGCCGCTTACCACTC	3060
20-014	TGGCAGCTACAACACCTCCTCGTCTAACTCTAGAATTCGAGTGATGGCCGCTTACCACTC *****	3060
21-BMYV	CGGAGCCTCCTGGGCCATCGCCATGGGTGATGATGCCCTTGAATCTGTAGATGCAGACCT	3120
21-254	CGGAGCCTCCTGGGCCATCGCCATGGGTGATGATGCCCTTGAATCTGTAGATGCAGACCT	3120
20-BMYV	CGGAGCCTCCTGGGCCATCGCCATGGGTGATGATGCCCTTGAATCTGTAGATGCAGACCT	3120
20-010	CGGAGCCTCCTGGGCCATCGCCATGGGTGATGATGCCCTTGAATCTGTAGATGCAGACCT	3120
20-006	CGGAGCCTCCTGGGCCATCGCCATGGGTGATGATGCCCTTGAATCTGTAGATGCAGACCT	3120
19-BMYV	CGGAGCCTCCTGGGCCATCGCCATGGGTGATGATGCCCTTGAATCTGTAGATGCAGACCT	3120
21-097	CGGAGCCTCCTGGGCCATCGCCATGGGTGATGATGCCCTTGAATCTGTAGATGCAGACCT	3120
19-013	CGGAGCCTCCTGGGCCATCGCCATGGGTGATGATGCCCTTGAATCTGTAGATGCAGACCT	3120
21-294	CGGAGCCTCCTGGGCCATCGCCATGGGTGATGATGCCCTTGAATCTGTAGATGCAGACCT	3120
21-056	CGGAGCCTCCTGGGCCATCGCCATGGGTGATGATGCCCTTGAATCTGTAGATGCAGACCT	3120
20-009	CGGAGCCTCCTGGGCCATCGCCATGGGTGATGATGCCCTTGAATCTGTAGATGCAGACCT	3120
20-014	CGGAGCCTCCTGGGCCATCGCCATGGGTGATGATGCCCTTGAATCTGTAGATGCAGACCT *****	3120
21-BMYV	AAGTCGATACTCATCCTTAGGTTTCAAAGTCGAGGTTTCTTCACAACCTGGAATTCTGCTC	3180
21-254	AAGTCGATACTCATCCTTAGGTTTCAAAGTCGAGGTTTCTTCACAACCTGGAATTCTGCTC	3180
20-BMYV	AAGTCGATACTCATCCTTAGGTTTCAAAGTCGAGGTTTCTTCACAACCTGGAATTCTGCTC	3180
20-010	AAGTCGATACTCATCCTTAGGTTTCAAAGTCGAGGTTTCTTCACAACCTGGAATTCTGCTC	3180
20-006	AAGTCGATACTCATCCTTAGGTTTCAAAGTCGAGGTTTCTTCACAACCTGGAATTCTGCTC	3180
19-BMYV	AAGTCGATACTCATCCTTAGGTTTCAAAGTCGAGGTTTCTTCACAACCTGGAATTCTGCTC	3180
21-097	AAGTCGATACTCATCCTTAGGTTTCAAAGTCGAGGTTTCTTCACAACCTGGAATTCTGCTC	3180
19-013	AAGTCGATACTCATCCTTAGGTTTCAAAGTCGAGGTTTCTTCACAACCTGGAATTCTGCTC	3180

21-294	AAGTCGATACTCATCCTTAGGCTTCAAAGTCGAGGTTTCTTCACAACCTGGAATTCTGCTC	3180
21-056	AAGTCGATACTCATCCTTAGGTTTCAAAGTCGAGGTTTCTTCACAACCTGGAATTCTGCTC	3180
20-009	AAGTCGATACTCATCCTTAGGTTTCAAAGTCGAGGTTTCTTCACAACCTGGAATTCTGCTC	3180
20-014	AAGTCGATACTCATCCTTAGGCTTCAAAGTCGAGGTTTCTTCACAACCTGGAATTCTGCTC	3180
	*****	
21-BMYV	TCACATTTTTGAGGAGGAGAACCCTGCGCGTTCCGGTAAACAAAGCTAAAATGCTTTATAA	3240
21-254	TCACATTTTTGAGGAGGAGAACCCTGCGCGTTCCGGTAAACAAAGCTAAAATGCTTTATAA	3240
20-BMYV	TCACATTTTTGAGGAGGAGAACCCTGCGCGTTCCGGTAAACAAAGCTAAAATGCTTTATAA	3240
20-010	TCACATTTTTGAGGAGGAGAACCCTGCGCGTTCCGGTAAACAAAGCTAAAATGCTTTATAA	3240
20-006	TCACATTTTTGAGGAGGAGAACCCTGCGCGTTCCGGTAAACAAAGCTAAAATGCTTTATAA	3240
19-BMYV	TCACATTTTTGAGGAGGAGAACCCTGCGCGTTCCGGTAAACAAAGCTAAAATGCTTTATAA	3240
21-097	TCACATTTTTGAGGAGGAGAACCCTGCGCGTTCCGGTAAACAAAGCTAAAATGCTTTATAA	3240
19-013	TCACATTTTTGAGGAGGAGAACCCTGCGCGTTCCGGTAAACAAAGCTAAAATGCTTTATAA	3240
21-294	TCACATTTTTGAGGAGGAGAACCCTGCGCGTTCCGGTAAACAAAGCTAAAATGCTTTATAA	3240
21-056	TCACATTTTTGAGGAGGAGAACCCTGCGCGTTCCGGTAAACAAAGCTAAAATGCTTTATAA	3240
20-009	TCACATTTTTGAGGAGGAGAACCCTGCGCGTTCCGGTAAACAAAGCTAAAATGCTTTATAA	3240
20-014	TCACATTTTTGAGGAGGAGAACCCTGCGCGTTCCGGTAAACAAAGCTAAAATGCTTTATAA	3240
	*****	
21-BMYV	ATTGATACATGGTTATGAACCGGAATGTGGCAACCTTGAAGTTCGACGAACCTATCTTGC	3300
21-254	ATTGATACATGGTTATGAACCGGAATGTGGCAACCTTGAAGTTCGACGAACCTATCTTGC	3300
20-BMYV	ATTGATACATGGTTATGAACCGGAATGTGGCAACCTTGAAGTTCGACGAACCTATCTTGC	3300
20-010	ATTGATACATGGTTATGAACCGGAATGTGGCAACCTTGAAGTTCGACGAACCTATCTTGC	3300
20-006	ATTGATACATGGTTATGAACCGGAATGTGGCAACCTTGAAGTTCGACGAACCTATCTTGC	3300
19-BMYV	ATTGATACATGGTTATGAACCGGAATGTGGCAACCTTGAAGTTCGACGAACCTATCTTGC	3300
21-097	ATTGATACATGGTTATGAACCGGAATGTGGCAACCTTGAAGTTCGACGAACCTATCTTGC	3300
19-013	ATTGATACATGGTTATGAACCGGAATGTGGCAACCTTGAAGTTCGACGAACCTATCTTGC	3300
21-294	ATTGATACATGGTTATGAACCGGAATGTGGCAACCTTGAAGTTCGACGAACCTATCTTGC	3300
21-056	ATTGATACATGGTTATGAACCGGAATGTGGCAACCTTGAAGTTCGACGAACCTATCTTGC	3300
20-009	ATTGATACATGGTTATGAACCGGAATGTGGCAACCTTGAAGTTCGACGAACCTATCTTGC	3300
20-014	ATTGATACATGGTTATGAACCGGAATGTGGCAACCTTGAAGTTCGACGAACCTATCTTGC	3300
	*****	
21-BMYV	AGCTTGTTTCTCAATTTTAAACGAGCTGAGATCCGATCAAGAACTCGTTGCCTCCCTCTA	3360
21-254	AGCTTGTTTCTCAATTTTAAACGAGCTGAGATCCGATCAAGAACTCGTTGCCTCCCTCTA	3360
20-BMYV	AGCTTGTTTCTCAATTTTAAACGAGCTGAGATCCGATCAAGAACTCGTTGCCTCCCTCTA	3360
20-010	AGCTTGTTTCTCAATTTTAAACGAGCTGAGATCCGATCAAGAACTCGTTGCCTCCCTCTA	3360
20-006	AGCTTGTTTCTCAATTTTAAACGAGCTGAGATCCGATCAAGAACTCGTTGCCTCCCTCTA	3360
19-BMYV	AGCTTGTTTCTCAATTTTAAACGAGCTGAGATCCGATCAAGAACTCGTTGCCTCCCTCTA	3360
21-097	AGCTTGTTTCTCAATTTTAAACGAGCTGAGATCCGATCAAGAACTCGTTGCCTCCCTCTA	3360
19-013	AGCTTGTTTCTCAATTTTAAACGAGCTGAGATCCGATCAAGAACTCGTTGCCTCCCTCTA	3360
21-294	AGCTTGTTTCTCAATTTTAAACGAGCTGAGATCCGATCAAGAACTCGTTGCCTCCCTCTA	3360
21-056	AGCTTGTTTCTCAATTTTAAACGAGCTGAGATCCGATCAAGAACTCGTTGCCTCCCTCTA	3360
20-009	AGCTTGTTTCTCAATTTTAAACGAGCTGAGATCCGATCAAGAACTCGTTGCCTCCCTCTA	3360
20-014	AGCTTGTTTCTCAATTTTAAACGAGCTGAGATCCGATCAAGAACTCGTTGCCTCCCTCTA	3360
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21-BMYV	TCAGTGGCTGGTCCCTCCAGTGCAGCCACAAAAGATATAACGAGGGACAATATAAACAGC	3420
21-254	TCAGTGGCTGGTCCCTCCAGTGCAGCCACAAAAGATATAACGAGGGACAATATAAACAGC	3420
20-BMYV	TCAGTGGCTGGTCCCTCCAGTGCAGCCACAAAAGATATAACGAGGGACAATATAAACAGC	3420
20-010	CCAGTGGCTGGTCCCTCCAGTGCAGCCACAAAAGATATAACGAGGGACAATATAAACAGC	3420
20-006	TCAGTGGCTGGTCCCTCCAGTGCAGCCACAAAAGATATAACGAGGGACAATATAAACAGC	3420
19-BMYV	TCAGTGGCTGGTCCCTCCAGTGCAGCCACAAAAGATATAACGAGGGACAATATAAACAGC	3420
21-097	TCAGTGGCTGGTCCCTCCAGTGCAGCCACAAAAGATATAACGAGGGACAATATAAACAGC	3420
19-013	TCAGTGGCTGGTCCCTCCAGTGCAGCCACAAAAGATATAACGAGGGACAATATAAACAGC	3420
21-294	TCAGTGGCTGGTCCCTCCAGTGCAGCCACAAAAGATATAACGAGGGACAATATAAACAGC	3420
21-056	TCAGTGGCTGGTCCCTCCAGTGCAGCCACAAAAGATATAACGAGGGACAATATAAACAGC	3420
20-009	TCAGTGGCTGGTCCCTCCAGTGCAGCCACAAAAGATATAACGAGGGACAATATAAACAGC	3420
20-014	TCAGTGGCTGGTCCCTCCAGTGCAGCCACAAAAGATATAACGAGGGACAATATAAACAGC	3420
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21-BMYV	CGGGTAAACATCAGTTGCAAACGCCGGAAGTTTAAAGTCTGATTACATAACAAGCCAAAA	3480
21-254	CGGGTAAACATCAGTTGCAAACGCCGGAAGTTTAAAGTCTGATTACATAACAAGCCAAAA	3480
20-BMYV	CGGGTAAACATCAGTTGCAAACGCCGGAAGTTTAAAGTCTGATTACATAACAAGCCAAAA	3480
20-010	CGGGTAAACATCAGTTGCAAACGCCGGAAGTTTAAAGTCTGATTACATAACAAGCCAAAA	3480
20-006	CGGGTAAACATCAGTTGCAAACGCCGGAAGTTTAAAGTCTGATTACATAACAAGCCAAAA	3480
19-BMYV	CGGGTAAACATCAGTTGCAAACGCCGGAAGTTTAAAGTCTGATTACATAACAAGCCAAAA	3480

21-097	CGGGTAAACATCAGTTGCAAACGCCGGAAGTTTAAAGTCTGATTACATAACAAGCCAAA	3480
19-013	CGGGTAAACATCAGTTGCAAACGCCGGAAGTTTAAAGTCTGATTACATAACAAGCCAAA	3480
21-294	CGGGTAAACATCAGTTGCAAACGCCGGAAGTTTAAAGTCTGATTACATAACAAGCCAAA	3480
21-056	CGGGTAAACATCAGTTGCAAACGCCGGAAGTTTAAAGTCTGATTACATAACAAGCCAAA	3480
20-009	CGGGTAAACATCAGTTGCAAACGCCGGAAGTTTAAAGTCTGATTACATAACAAGCCAAA	3480
20-014	CGGGTAAACATCAGTTGCAAACGCCGGAAGTTTAAAGTCTGATTACATAACAAGCCAAA	3480
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21-BMYV	TAGATTTCAAGTTTTTAGCAGGATTTTCAAGTGGTCTATGTCAGCAATACCTGTAACGGT	3540
21-254	TAGATTTCAAGTTTTTAGCAGGATTTTCAAGTGGTCTATGTCAGCAATACCTGTAACGGT	3540
20-BMYV	TAGATTTCAAGTTTTTAGCAGGATTTTCAAGTGGTCTATGTCAGCAATACCTGTAACGGT	3540
20-010	TAGATTTCAAGTTTTTAGCAGGATTTTCAAGTGGTCTATGTCAGCAATACCTGTAACGGT	3540
20-006	TAGATTTCAAGTTTTTAGCAGGATTTTCAAGTGGTCTATGTCAGCAATACCTGTAACGGT	3540
19-BMYV	TAGATTTCAAGTTTTTAGCAGGATTTTCAAGTGGTCTATGTCAGCAATACCTGTAACGGT	3540
21-097	TAGATTTCAAGTTTTTAGCAGGATTTTCAAGTGGTCTATGTCAGCAATACCTGTAACGGT	3540
19-013	TAGATTTCAAGTTTTTAGCAGGATTTTCAAGTGGTCTATGTCAGCAATACCTGTAACGGT	3540
21-294	TAGATTTCAAGTTTTTAGCAGGATTTTCAAGTGGTCTATGTCAGCAATACCTGTAACGGT	3540
21-056	TAGATTTCAAGTTTTTAGCAGGATTTTCAAGTGGTCTATGTCAGCAATACCTGTAACGGT	3540
20-009	TAGATTTCAAGTTTTTAGCAGGATTTTCAAGTGGTCTATGTCAGCAATACCTGTAACGGT	3540
20-014	TAGATTTCAAGTTTTTAGCAGGATTTTCAAGTGGTCTATGTCAGCAATACCTGTAACGGT	3540
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21-BMYV	AGTTGGCTTGTATTTTCGTCTACCTTAAGATTTCTCACCACGTCAGATCAATTGTTAATGA	3600
21-254	AGTTGGCTTGTATTTTCGTCTACCTTAAGATTTCTCACCACGTCAGATCAATTGTTAATGA	3600
20-BMYV	AGTTGGCTTGTATTTTCGTCTACCTTAAGATTTCTCACCACGTCAGATCAATTGTTAATGA	3600
20-010	AGTTGGCTTGTATTTTCGTCTACCTTAAGATTTCTCACCACGTCAGATCAATTGTTAATGA	3600
20-006	AGTTGGCTTGTATTTTCGTCTACCTTAAGATTTCTCACCACGTCAGATCAATTGTTAATGA	3600
19-BMYV	AGTTGGCTTGTATTTTCGTCTACCTTAAGATTTCTCACCACGTCAGATCAATTGTTAATGA	3600
21-097	AGTTGGCTTGTATTTTCGTCTACCTTAAGATTTCTCACCACGTCAGATCAATTGTTAATGA	3600
19-013	AGTTGGCTTGTATTTTCGTCTACCTTAAGATTTCTCACCACGTCAGATCAATTGTTAATGA	3600
21-294	AGTTGGCTTGTATTTTCGTCTACCTTAAGATTTCTCACCACGTCAGATCAATTGTTAATGA	3600
21-056	AGTTGGCTTGTATTTTCGTCTACCTTAAGATTTCTCACCACGTCAGATCAATTGTTAATGA	3600
20-009	AGTTGGCTTGTATTTTCGTCTACCTTAAGATTTCTCACCACGTCAGATCAATTGTTAATGA	3600
20-014	AGTTGGCTTGTATTTTCGTCTACCTTAAGATTTCTCACCACGTCAGATCAATTGTTAATGA	3600
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21-BMYV	ATACGGTCGTGGGTAGGAGAACGATCAATGGAAGAAGACGACCACGTAGGCAAACACGAC	3660
21-254	ATACGGTCGTGGGTAGGAGAACGATCAATGGAAGAAGACGACCACGTAGGCAAACACGAC	3660
20-BMYV	ATACGGTCGTGGGTAGGAGAACGATCAATGGAAGAAGACGACCACGTAGGCAAACACGAC	3660
20-010	ATACGGTCGTGGGTAGGAGAACGATCAATGGAAGAAGACGACCACGTAGGCAAACACGAC	3660
20-006	ATACGGTCGTGGGTAGGAGAACGATCAATGGAAGAAGACGACCACGTAGGCAAACACGAC	3660
19-BMYV	ATACGGTCGTGGGTAGGAGAACGATCAATGGAAGAAGACGACCACGTAGGCAAACACGAC	3660
21-097	ATACGGTCGTGGGTAGGAGAACGATCAATGGAAGAAGACGACCACGTAGGCAAACACGAC	3660
19-013	ATACGGTCGTGGGTAGGAGAACGATCAATGGAAGAAGACGACCACGTAGGCAAACACGAC	3660
21-294	ATACGGTCGTGGGTAGGAGAACGATCAATGGAAGAAGACGACCACGTAGGCAAACACGAC	3660
21-056	ATACGGTCGTGGGTAGGAGAACGATCAATGGAAGAAGACGACCACGTAGGCAAACACGAC	3660
20-009	ATACGGTCGTGGGTAGGAGAACGATCAATGGAAGAAGACGACCACGTAGGCAAACACGAC	3660
20-014	ATACGGTCGTGGGTAGGAGAACGATCAATGGAAGAAGACGACCACGTAGGCAAACACGAC	3660
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21-BMYV	GCGCTCAGCGCTCTCAGCCAGTGGTTGTGGTCCAAGCCTCTCGGACAACACAACGCCGAC	3720
21-254	GCGCTCAGCGCTCTCAGCCAGTGGTTGTGGTCCAAGCCTCTCGGACAACACAACGCCGAC	3720
20-BMYV	GCGCTCAGCGCTCTCAGCCAGTGGTTGTGGTCCAAGCCTCTCGGACAACACAACGCCGAC	3720
20-010	GCGCTCAGCGCTCTCAGCCAGTGGTTGTGGTCCAAGCCTCTCGGACAACACAACGCCGAC	3720
20-006	GCGCTCAGCGCTCTCAGCCAGTGGTTGTGGTCCAAGCCTCTCGGACAACACAACGCCGAC	3720
19-BMYV	GCGCTCAGCGCTCTCAGCCAGTGGTTGTGGTCCAAGCCTCTCGGACAACACAACGCCGAC	3720
21-097	GCGCTCAGCGCTCTCAGCCAGTGGTTGTGGTCCAAGCCTCTCGGACAACACAACGCCGAC	3720
19-013	GCGCTCAGCGCTCTCAGCCAGTGGTTGTGGTCCAAGCCTCTCGGACAACACAACGCCGAC	3720
21-294	GCGCTCAGCGCTCTCAGCCAGTGGTTGTGGTCCAAGCCTCTCGGACAACACAACGCCGAC	3720
21-056	GCGCTCAGCGCTCTCAGCCAGTGGTTGTGGTCCAAGCCTCTCGGACAACACAACGCCGAC	3720
20-009	GCGCTCAGCGCTCTCAGCCAGTGGTTGTGGTCCAAGCCTCTCGGACAACACAACGCCGAC	3720
20-014	GCGCTCAGCGCTCTCAGCCAGTGGTTGTGGTCCAAGCCTCTCGGACAACACAACGCCGAC	3720
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21-BMYV	CTAGACGACGACGAAGAGGCAACAACCGGACAAGAAGAACTGTTTCTACCAGAGGAACAG	3780
21-254	CTAGACGACGACGAAGAGGCAACAACCGGACAAGAAGAACTGTTTCTACCAGAGGAACAG	3780
20-BMYV	CTAGACGACGACGAAGAGGTAACAACCGGACAAGAAGAACTGTTTCTACCAGAGGAACAG	3780
20-010	CTAGACGACGACGAAGAGGCAACAACCGGACAAGAAGAACTGTTTCTACCAGAGGAACAG	3780

20-006	CTAGACGACGACGAAGAGGGCAACAACCGGACAAGAAGAAGTGTTCCTACCAGAGGAACAG	3780
19-BMYV	CTAGACGACGACGAAGAGGGCAACAACCGGACAAGAAGAAGTGTTCCTACCAGAGGAACAG	3780
21-097	CTAGACGACGACGAAGAGGGCAACAACCGGACAAGAAGAAGTGTTCCTACCAGAGGAACAG	3780
19-013	CTAGACGACGACGAAGAGGGCAACAACCGGACAAGAAGAAGTGTTCCTACCAGAGGAACAG	3780
21-294	CTAGACGACGACGAAGAGGGCAACAACCGGACAAGAAGAAGTGTTCCTACCAGAGGAACAG	3780
21-056	CTAGACGACGACGAAGAGGGCAACAACCGGACAAGAAGAAGTGTTCCTACCAGAGGAACAG	3780
20-009	CTAGACGACGACGAAGAGGGCAACAACCGGACAAGAAGAAGTGTTCCTACCAGAGGAACAG	3780
20-014	CTAGACGACGACGAAGAGGGCAACAACCGGACAAGAAGAAGTGTTCCTACCAGAGGAACAG	3780
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21-BMYV	GTTTCGAGCGAGACATTCGTTTTCTCAAAGACAATCTCGCGGAAGTTCAGCGGAGCAA	3840
21-254	GTTTCGAGCGAGACATTCGTTTTCTCAAAGACAATCTCGCGGAAGTTCAGCGGAGCAA	3840
20-BMYV	GTTTCGAGCGAGACATTCGTTTTCTCAAAGACAATCTCGCGGAAGTTCAGCGGAGCAA	3840
20-010	GTTTCGAGCGAGACATTCGTTTTCTCAAAGACAATCTCGCGGAAGTTCAGCGGAGCAA	3840
20-006	GTTTCGAGCGAGACATTCGTTTTCTCAAAGACAATCTCGCGGAAGTTCAGCGGAGCAA	3840
19-BMYV	GTTTCGAGCGAGACATTCATTTTTCTCAAAGACAATCTCGCGGAAGTTCAGCGGAGCAA	3840
21-097	GTTTCGAGCGAGACATTCGTTTTCTCAAAGACAATCTCGCGGAAGTTCAGCGGAGCAA	3840
19-013	GTTTCGAGCGAGACATTCGTTTTCTCAAAGACAATCTCGCGGAAGTTCAGCGGAGCAA	3840
21-294	GTTTCGAGCGAGACATTCGTTTTCTCAAAGACAATCTCGCGGAAGTTCAGCGGAGCAA	3840
21-056	GTTTCGAGCGAGACATTCGTTTTCTCAAAGACAATCTCGCGGAAGTTCAGCGGAGCAA	3840
20-009	GTTTCGAGCGAGACATTCGTTTTCTCAAAGACAATCTCGCGGAAGTTCAGCGGAGCAA	3840
20-014	GTTTCGAGCGAGACATTCGTTTTCTCAAAGACAATCTCGCGGAAGTTCAGCGGAGCAA	3840
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21-BMYV	TCACGTTCCGGCCGAGTCTATCAGACTGCCCGGCATTCGCTGATGGAATGCTCAAGGCCT	3900
21-254	TCACGTTCCGGCCGAGTCTATCAGACTGCCCGGCATTCGCTGATGGAATGCTCAAGGCCT	3900
20-BMYV	TCACGTTCCGGCCGAGTCTATCAGACTGCCCGGCATTCGCTGATGGAATGCTCAAGGCCT	3900
20-010	TCACGTTCCGGCCGAGTCTATCAGACTGCCCGGCATTCGCTGATGGAATGCTCAAGGCCT	3900
20-006	TCACGTTCCGGCCGAGTCTATCAGACTGCCCGGCATTCGCTGATGGAATGCTCAAGGCCT	3900
19-BMYV	TCACGTTCCGGCCGAGTCTATCAGACTGCCCGGCATTCGCTGATGGAATGCTCAAGGCCT	3900
21-097	TCACGTTCCGGCCGAGTCTATCAGACTGCCCGGCATTCGCTGATGGAATGCTCAAGGCCT	3900
19-013	TCACGTTCCGGCCGAGTCTATCAGACTGCCCGGCATTCGCTGATGGAATGCTCAAGGCCT	3900
21-294	TCACGTTCCGGCCGAGTCTATCAGACTGCCCGGCATTCGCTGATGGAATGCTCAAGGCCT	3900
21-056	TCACGTTCCGGCCGAGTCTATCAGACTGCCCGGCATTCGCTGATGGAATGCTCAAGGCCT	3900
20-009	TCACGTTCCGGCCGAGTCTATCAGACTGCCCGGCATTCGCTGATGGAATGCTCAAGGCCT	3900
20-014	TCACGTTCCGGCCGAGTCTATCAGACTGCCCGGCATTCGCTGATGGAATGCTCAAGGCCT	3900
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21-BMYV	ACCATGAGTATAAAAATCTCAATGGTCATTTTGGAGTTCGCTCCTCCGAGGCCCTTTCCCAA	3960
21-254	ACCATGAGTATAAAAATCTCAATGGTCATTTTGGAGTTCGCTCCTCCGAGGCCCTTTCCCAA	3960
20-BMYV	ACCATGAGTATAAAAATCTCAATGGTCATTTTGGAGTTCGCTCCTCCGAGGCCCTTTCCCAA	3960
20-010	ACCATGAGTATAAAAATCTCAATGGTCATTTTGGAGTTCGCTCCTCCGAGGCCCTTTCCCAA	3960
20-006	ACCATGAGTATAAAAATCTCAATGGTCATTTTGGAGTTCGCTCCTCCGAGGCCCTTTCCCAA	3960
19-BMYV	ACCATGAGTATAAAAATCTCAATGGTCATTTTGGAGTTCGCTCCTCCGAGGCCCTTTCCCAA	3960
21-097	ACCATGAGTATAAAAATCTCAATGGTCATTTTGGAGTTCGCTCCTCCGAGGCCCTTTCCCAA	3960
19-013	ACCATGAGTATAAAAATCTCAATGGTCATTTTGGAGTTCGCTCCTCCGAGGCCCTTTCCCAA	3960
21-294	ACCATGAGTATAAAAATCTCAATGGTCATTTTGGAGTTCGCTCCTCCGAGGCCCTTTCCCAA	3960
21-056	ACCATGAGTATAAAAATCTCAATGGTCATTTTGGAGTTCGCTCCTCCGAGGCCCTTTCCCAA	3960
20-009	ACCATGAGTATAAAAATCTCAATGGTCATTTTGGAGTTCGCTCCTCCGAGGCCCTTTCCCAA	3960
20-014	ACCATGAGTATAAAAATCTCAATGGTCATTTTGGAGTTCGCTCCTCCGAGGCCCTTTCCCAA	3960
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21-BMYV	ATTCCGGTTCCATCGCTTACGAGCTGGACCCACACTGTAAACTCAGTGCCCTTTTCATCAA	4020
21-254	ATTCCGGTTCCATCGCTTACGAGCTGGACCCACACTGTAAACTCAGTGCCCTTTTCATCAA	4020
20-BMYV	ATTCCGGTTCCATCGCTTACGAGCTGGACCCACACTGTAAACTCAGTGCCCTTTTCATCAA	4020
20-010	ATTCCGGTTCCATCGCTTACGAGCTGGACCCACACTGTAAACTCAGTGCCCTTTTCATCAA	4020
20-006	ATTCCGGTTCCATCGCTTACGAGCTGGACCCACACTGTAAACTCAGTGCCCTTTTCATCAA	4020
19-BMYV	ATTCCGGTTCCATCGCTTACGAGCTGGACCCACACTGTAAACTCAGTGCCCTTTTCATCAA	4020
21-097	ATTCCGGTTCCATCGCTTACGAGCTGGACCCACACTGTAAACTCAGTGCCCTTTTCATCAA	4020
19-013	ATTCCGGTTCCATCGCTTACGAGCTGGACCCACACTGTAAACTCAGTGCCCTTTTCATCAA	4020
21-294	ATTCCGGTTCCATCGCTTACGAGCTGGACCCACACTGTAAACTCAGTGCCCTTTTCATCAA	4020
21-056	ATTCCGGTTCCATCGCTTACGAGCTGGACCCACACTGTAAACTCAGTGCCCTTTTCATCAA	4020
20-009	ATTCCGGTTCCATCGCTTACGAGCTGGACCCACACTGTAAACTCAGTGCCCTTTTCATCAA	4020
20-014	ATTCCGGTTCCATCGCTTACGAGCTGGACCCACACTGTAAACTCAGTGCCCTTTTCATCAA	4020
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21-BMYV	CCATTAACAAGTTCGGGATCACAACCCGGCAGGAGGGCATTTCGACGCTTTACATCA	4080
21-254	CCATTAACAAGTTCGGGATCACAACCCGGCAGGAGGGCATTTCGACGCTTTACATCA	4080

20-BMYV	CCATTAACAAGTTCGGGATCACAAAACCCGGCAGGAGGGCATTTCAGCGTCTTACATCA	4080
20-010	CCATTAACAAGTTCGGGATCACAAAACCCGGCAGGAGGGCATTTCAGCGTCTTACATCA	4080
20-006	CCATTAACAAGTTCGGGATCACAAAACCCGGCAGGAGGGCATTTCAGCGTCTTACATCA	4080
19-BMYV	CCATTAACAAGTTCGGGATCACAAAACCCGGCAGGAGGGCATTTCAGCGTCTTACATCA	4080
21-097	CCATTAACAAGTTCGGGATCACAAAACCCGGCAGGAGGGCATTTCAGCGTCTTACATCA	4080
19-013	CCATTAACAAGTTCGGGATCACAAAACCCGGCAGGAGGGCATTTCAGCGTCTTACATCA	4080
21-294	CCATTAACAAGTTCGGGATCACAAAACCCGGCAGGAGGGCATTTCAGCGTCTTACATCA	4080
21-056	CCATTAACAAGTTCGGGATCACAAAACCCGGCAGGAGGGCATTTCAGCGTCTTACATCA	4080
20-009	CCATTAACAAGTTCGGGATCACAAAACCCGGCAGGAGGGCATTTCAGCGTCTTACATCA	4080
20-014	CCATTAACAAGTTCGGGATCACAAAACCCGGCAGGAGGGCATTTCAGCGTCTTACATCA	4080
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21-BMYV	ACGGGGCGGACTGGCATGACGTTGCCAAGGACCAATTCAGGATCCTCTACAAAGGCAATG	4140
21-254	ACGGGACGGACTGGCATGACGTTGCCAAGGACCAATTCAGGATCCTCTACAAAGGCAATG	4140
20-BMYV	ACGGGGCGGACTGGCATGACGTTGCCAAGGACCAATTCAGGATCCTCTACAAAGGCAATG	4140
20-010	ACGGGGCGGACTGGCATGACGTTGCCAAGGACCAATTCAGGATCCTCTACAAAGGCAATG	4140
20-006	ACGGGGCGGACTGGCATGACGTTGCCAAGGACCAATTCAGGATCCTCTACAAAGGCAATG	4140
19-BMYV	ACGGGGCGGACTGGCATGACGTTGCCAAGGACCAATTCAGGATCCTCTACAAAGGCAATG	4140
21-097	ACGGGGCGGACTGGCATGACGTTGCCAAGGACCAATTCAGGATCCTCTACAAAGGCAATG	4140
19-013	ACGGGGCGGACTGGCATGACGTTGCCAAGGACCAATTCAGGATCCTCTACAAAGGCAATG	4140
21-294	ACGGGGCGGACTGGCATGACGTTGCCAAGGACCAATTCAGGATCCTCTACAAAGGCAATG	4140
21-056	ACGGGGCGGACTGGCATGACGTTGCCAAGGACCAATTCAGGATCCTCTACAAAGGCAATG	4140
20-009	ACGGGGCGGACTGGCATGACGTTGCCAAGGACCAATTCAGGATCCTCTACAAAGGCAATG	4140
20-014	ACGGGGCGGACTGGCATGACGTTGCCAAGGACCAATTCAGGATCCTCTACAAAGGCAATG	4140
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21-BMYV	GTTCTTCATCGATAGCTGGTTCCTTTTAGAATCACCATGAAGTGCCAGTTCACAATCCCA	4200
21-254	GTTCTTCATCGATAGCTGGTTCCTTTTAGAATCACCATGAAGTGCCAGTTCACAATCCGA	4200
20-BMYV	GTTCTTCATCGATAGCTGGTTCCTTTTAGAATCACCATGAAGTGCCAGTTCACAATCCGA	4200
20-010	GTTCTTCATCGATAGCTGGTTCCTTTTAGAATCACCATGAAGTGCCAGTTCACAATCCGA	4200
20-006	GTTCTTCATCGATAGCTGGTTCCTTTTAGAATCACCATGAAGTGCCAGTTCACAATCCCA	4200
19-BMYV	GTTCTTCATCGATAGCTGGTTCCTTTTAGAATCACCATGAAGTGCCAGTTCACAATCCGA	4200
21-097	GTTCTTCATCGATAGCTGGTTCCTTTTAGAATCACCATGAAGTGCCAGTTCACAATCCGA	4200
19-013	GTTCTTCATCGATAGCTGGTTCCTTTTAGAATCACCATGAAGTGCCAGTTCACAATCCGA	4200
21-294	GTTCTTCATCGATAGCTGGTTCCTTTTAGAATCACCATGAAGTGCCAGTTCACAATCCGA	4200
21-056	GTTCTTCATCGATAGCTGGTTCCTTTTAGAATCACCATGAAGTGCCAGTTCACAATCCGA	4200
20-009	GTTCTTCATCGATAGCTGGTTCCTTTTAGAATCACCATGAAGTGCCAGTTCACAATCCGA	4200
20-014	GTTCTTCATCGATAGCTGGTTCCTTTTAGAATCACCATGAAGTGCCAGTTCACAATCCGA	4200
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21-BMYV	AATAGGTAGACAAGGAACCCGGCCCTAGCCAGGGCCTTCTCCCTCTCCACAACCCACAC	4260
21-254	AATAGGTAGACAAGGAACCCGGCCCTAGCCAGGGCCTTCTCCCTCTCCACAACCCACAC	4260
20-BMYV	AATAGGTAGACAAGGAACCCGGCCCTAGCCAGGGCCTTCTCCCTCTCCACAACCCACAC	4260
20-010	AATAGGTAGACAAGGAACCCGGCCCTAGCCAGGGCCTTCTCCCTCTCCACAACCCACAC	4260
20-006	AATAGGTAGACAAGGAACCCGGCCCTAGCCAGGGCCTTCTCCCTCTCCACAACCCACAC	4260
19-BMYV	AATAGGTAGACAAGGAACCCGGCCCTAGCCAGGGCCTTCTCCCTCTCCACAACCCACAC	4260
21-097	AATAGGTAGACAAGGAACCCGGCCCTAGCCAGGGCCTTCTCCCTCTCCACAACCCACAC	4260
19-013	AATAGGTAGACAAGGAACCCGGCCCTAGCCAGGGCCTTCTCCCTCTCCACAACCCACAC	4260
21-294	AATAGGTAGACAAGGAACCCGGCCCTAGCCAGGGCCTTCTCCCTCTCCACAACCCACAC	4260
21-056	AATAGGTAGACAAGGAACCCGGCCCTAGCCAGGGCCTTCTCCCTCTCCACAACCCACAC	4260
20-009	AATAGGTAGACAAGGAACCCGGCCCTAGCCAGGGCCTTCTCCCTCTCCACAACCCACAC	4260
20-014	AATAGGTAGACAAGGAACCCGGCCCTAGCCAGGGCCTTCTCCCTCTCCACAACCCACAC	4260
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21-BMYV	CCTCAAAGAAATATCGTTTTATCGTCTATACTGGTGTCCCTGTGACCCGTATAATGGCCC	4320
21-254	CCTCAAAGAAATATCGTTTTATCGTCTATACTGGTGTCCCTGTGACCCGTATAATGGCCC	4320
20-BMYV	CCTCAAAGAAATATCGTTTTATCGTCTATACTGGTGTCCCTGTGACCCGTATAATGGCCC	4320
20-010	CCTCAAAGAAATATCGTTTTATCGTCTATACTGGTGTCCCTGTGACCCGTATAATGGCCC	4320
20-006	CCTCAAAGAAATATCGTTTTATCGTCTATACTGGTGTCCCTGTGACCCGTATAATGGCCC	4320
19-BMYV	CCTCAAAGAAATATCGTTTTATCGTCTATACTGGTGTCCCTGTGACCCGTATAATGGCCC	4320
21-097	CCTCAAAGAAATATCGTTTTATCGTCTATACTGGTGTCCCTGTGACCCGTATAATGGCCC	4320
19-013	CCTCAAAGAAATATCGTTTTATCGTCTATACTGGTGTCCCTGTGACCCGTATAATGGCCC	4320
21-294	CCTCAAAGAAATATCGTTTTATCGTCTATACTGGTGTCCCTGTGACCCGTATAATGGCCC	4320
21-056	CCTCAAAGAAATATCGTTTTATCGTCTATACTGGTGTCCCTGTGACCCGTATAATGGCCC	4320
20-009	CCTCAAAGAAATATCGTTTTATCGTCTATACTGGTGTCCCTGTGACCCGTATAATGGCCC	4320
20-014	CCTCAAAGAAATATCGTTTTATCGTCTATACTGGTGTCCCTGTGACCCGTATAATGGCCC	4320
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21-BMYV	AATCCACTGATGACGCCATCTCTCTGTACGACATGCCTTCTCAACGGTTTCGCTACATAG	4380
21-254	AATCCACTGATGACGCCATCTCTCTGTACGACATGCCTTCCCAACGGTTTCGCTACATAG	4380
20-BMYV	AATCCACTGATGACGCCATCTCTCTGTACGACATGCCTTCCCAACGGTTTCGCTACATAG	4380
20-010	AATCCACTGATGACGCCATCTCTCTGTACGACATGCCTTCCCAACGGTTTCGCTACATAG	4380
20-006	AATCCACTGATGACGCCATCTCTCTGTACGACATGCCTTCCCAACGGTTTCGCTACATAG	4380
19-BMYV	AATCCACTGATGACGCCATCTCTCTGTACGACATGCCTTCCCAACGGTTTCGCTACATAG	4380
21-097	AATCCACTGATGACGCCATCTCTCTGTACGACATGCCTTCCCAACGGTTTCGCTACATAG	4380
19-013	AATCCACTGATGACGCCATCTCTCTGTACGACATGCCTTCCCAACGGTTTCGCTACATAG	4380
21-294	AATCCACTGATGACGCCATCTCTCTGTACGACATGCCTTCCCAACGGTTTCGCTACATAG	4380
21-056	AATCCACTGATGACGCCATCTCTCTGTACGACATGCCTTCCCAACGGTTTCGCTACATAG	4380
20-009	AATCCACTGATGACGCCATCTCTCTGTACGACATGCCTTCCCAACGGTTTCGCTACATAG	4380
20-014	AATCCACTGATGACGCCATCTCTCTGTACGACATGCCTTCCCAACGGTTTCGCTACATAG	4380
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21-BMYV	AAGACGAGAATATGAACTGGACAAACCTCGATTCTCGATGGTATTTCCAGAATTCCTTTGA	4440
21-254	AAGACGAGAATATGAACTGGACAAACCTCGATTCTCGATGGTATTTCCAGAATTCCTTTGA	4440
20-BMYV	AAGACGAGAATATGAACTGGACAAACCTCGATTCTCGATGGTATTTCCAGAATTCCTTTGA	4440
20-010	AAGACGAGAATATGAACTGGACAAACCTCGATTCTCGATGGTATTTCCAGAATTCCTTTGA	4440
20-006	AAGACGAGAATATGAACTGGACAAACCTCGATTCTCGATGGTATTTCCAGAATTCCTTTGA	4440
19-BMYV	AAGACGAGAATATGAACTGGACAAACCTCGATTCTCGATGGTATTTCCAGAATTCCTTTGA	4440
21-097	AAGACGAGAATATGAACTGGACAAACCTCGATTCTCGATGGTATTTCCAGAATTCCTTTGA	4440
19-013	AAGACGAGAATATGAACTGGACAAACCTCGATTCTCGATGGTATTTCCAGAATTCCTTTGA	4440
21-294	AAGACGAGAATATGAACTGGACAAACCTCGATTCTCGATGGTATTTCCAGAATTCCTTTGA	4440
21-056	AAGACGAGAATATGAACTGGACAAACCTCGATTCTCGATGGTATTTCCAGAATTCCTTTGA	4440
20-009	AAGACGAGAATATGAACTGGACAAACCTCGATTCTCGATGGTATTTCCAGAATTCCTTTGA	4440
20-014	AAGACGAGAATATGAACTGGACAAACCTCGATTCTCGATGGTATTTCCAGAATTCCTTTGA	4440
	*****	
21-BMYV	AAGCCATCCCAATGATAATAGTACCAGTCCCTCAAGGTGAGTGGACTGTGGAAATTTTCGA	4500
21-254	AAGCCATCCCAATGATAATAGTACCAGTCCCTCAAGGTGAGTGGACTGTGGAAATTTTCGA	4500
20-BMYV	AAGCCATCCCAATGATAATAGTACCAGTCCCTCAAGGTGAGTGGACTGTGGAAATTTTCGA	4500
20-010	AAGCCATCCCAATGATAATAGTACCAGTCCCTCAAGGTGAGTGGACTGTGGAAATTTTCGA	4500
20-006	AAGCCATCCCAATGATAATAGTACCAGTCCCTCAAGGTGAGTGGACTGTGGAAATTTTCGA	4500
19-BMYV	AAGCCATCCCAATGATAATAGTACCAGTCCCTCAAGGTGAGTGGACTGTGGAAATTTTCGA	4500
21-097	AAGCCATCCCAATGATAATAGTACCAGTCCCTCAAGGTGAGTGGACTGTGGAAATTTTCGA	4500
19-013	AAGCCATCCCAATGATAATAGTACCAGTCCCTCAAGGTGAGTGGACTGTGGAAATTTTCGA	4500
21-294	AAGCCATCCCAATGATAATAGTACCAGTCCCTCAAGGTGAGTGGACTGTGGAAATTTTCGA	4500
21-056	AAGCCATCCCAATGATAATAGTACCAGTCCCTCAAGGTGAGTGGACTGTGGAAATTTTCGA	4500
20-009	AAGCCATCCCAATGATAATAGTACCAGTCCCTCAAGGTGAGTGGACTGTGGAAATTTTCGA	4500
20-014	AAGCCATCCCAATGATAATAGTACCAGTCCCTCAAGGTGAGTGGACTGTGGAAATTTTCGA	4500
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21-BMYV	TGGAGGGGTATCAACCAACCTCAAGCACCACAGATCCTAATAAGGACAAACAAGATGGTC	4560
21-254	TGGAGGGGTATCAACCAACCTCAAGCACCACAGATCCTAATAAGGACAAACAAGATGGTC	4560
20-BMYV	TGGAGGGGTATCAACCAACCTCAAGCACCACAGATCCTAATAAGGACAAACAAGATGGTC	4560
20-010	TGGAGGGGTATCAACCAACCTCAAGCACCACAGATCCTAATAAGGACAAACAAGATGGTC	4560
20-006	TGGAGGGGTATCAACCAACCTCAAGCACCACAGATCCTAATAAGGACAAACAAGATGGTC	4560
19-BMYV	TGGAGGGGTATCAACCAACCTCAAGCACCACAGATCCTAATAAGGACAAACAAGATGGTC	4560
21-097	TGGAGGGGTATCAACCAACCTCAAGCACCACAGATCCTAATAAGGACAAACAAGATGGTC	4560
19-013	TGGAGGGGTATCAACCAACCTCAAGCACCACAGATCCTAATAAGGACAAACAAGATGGTC	4560
21-294	TGGAGGGGTATCAACCAACCTCAAGCACCACAGATCCTAATAAGGACAAACAAGATGGTC	4560
21-056	TGGAGGGGTATCAACCAACCTCAAGCACCACAGATCCTAATAAGGACAAACAAGATGGTC	4560
20-009	TGGAGGGGTATCAACCAACCTCAAGCACCACAGATCCTAATAAGGACAAACAAGATGGTC	4560
20-014	TGGAGGGGTATCAACCAACCTCAAGCACCACAGATCCTAATAAGGACAAACAAGATGGTC	4560
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21-BMYV	TTATTGCATACAATGATGACCTCAAGGAGGGTTGGAATGTAGGGGTTTATAACAATGTGG	4620
21-254	TTATTGCATATAATGATGACCTCAAGGAGGGTTGGAATGTAGGGGTTTATAACAATGTGG	4620
20-BMYV	TTATTGCATACAATGATGACCTCAAGGAGGGTTGGAATGTAGGGGTTTATAACAATGTGG	4620
20-010	TTATTGCATATAATGATGACCTCAAGGAGGGTTGGAATGTAGGGGTTTATAACAATGTGG	4620
20-006	TTATTGCATACAATGATGACCTCAAGGAGGGTTGGAATGTAGGGGTTTATAACAATGTGG	4620
19-BMYV	TTATTGCATATAATGATGACCTCAAGGAGGGTTGGAATGTAGGGGTTTATAACAATGTGG	4620
21-097	TTATTGCATATAATGATGACCTCAAGGAGGGTTGGAATGTAGGGGTTTATAACAATGTGG	4620
19-013	TTATTGCATATAATGATGACCTCAAGGAGGGTTGGAATGTAGGGGTTTATAACAATGTGG	4620
21-294	TTATTGCATACAATGATGACCTCAAGGAGGGTTGGAATGTAGGGGTTTATAACAATGTGG	4620
21-056	TTATTGCATATAATGATGACCTCAAGGAGGGTTGGAATGTAGGGGTTTATAACAATGTGG	4620
20-009	TTATTGCATATAATGATGACCTCAAGGAGGGTTGGAATGTAGGGGTTTATAACAATGTGG	4620
20-014	TTATTGCATATAATGATGACCTCAAGGAGGGTTGGAATGTAGGGGTTTATAACAATGTGG	4620

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21-BMYV AGATAACCAACAATAAGGCTGATAACACTTTGAAGTACGGCCATCCAGACATGGAGCTCA 4680  
21-254 AGATAACCAACAATAAGGCTGATAACACTTTGAAGTACGGCCATCCAGACATGGAGCTCA 4680  
20-BMYV AGATAACCAACAATAAGGCTGATAACACTTTGAAGTACGGCCATCCAGACATGGAGCTCA 4680  
20-010 AGATAACCAACAATAAGGCTGATAACACTTTGAAGTACGGCCATCCAGACATGGAGCTCA 4680  
20-006 AGATAACCAACAATAAGGCTGATAACACTTTGAAGTACGGCCATCCAGACATGGAGCTCA 4680  
19-BMYV AGATAACCAACAATAAGGCTGATAACACTTTGAAGTACGGCCATCCAGACATGGAGCTCA 4680  
21-097 AGATAACCAACAATAAGGCTGATAACACTTTGAAGTACGGCCATCCAGACATGGAGCTCA 4680  
19-013 AGATAACCAACAATAAGGCTGATAACACTTTGAAGTACGGCCATCCAGACATGGAGCTCA 4680  
21-294 AGATAACCAACAATAAGGCTGATAACACTTTGAAGTACGGCCATCCAGACATGGAGCTCA 4680  
21-056 AGATAACCAACAATAAGGCTGATAACACTTTGAAGTACGGCCATCCAGACATGGAGCTCA 4680  
20-009 AGATAACCAACAATAAGGCTGATAACACTTTGAAGTACGGCCATCCAGACATGGAGCTCA 4680  
20-014 AGATAACCAACAATAAGGCTGATAACACTTTGAAGTACGGCCATCCAGACATGGAGCTCA 4680  
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21-BMYV ATAGTTGTCATTTTAATCAAGGACAATGTTTGGAAAGAGATGGAGATTTGACTTGTCCAG 4740  
21-254 ATAGTTGTCATTTTAATCAAGGACAATGTTTGGAAAGAGATGGAGATTTGACTTGTCCAG 4740  
20-BMYV ATAGTTGTCATTTTAATCAAGGACAATGTTTGGAAAGAGATGGAGATTTGACTTGTCCAG 4740  
20-010 ATAGTTGTCATTTTAATCAAGGACAATGTTTGGAAAGAGATGGAGATTTGACTTGTCCAG 4740  
20-006 ATAGTTGTCATTTTAATCAAGGACAATGTTTGGAAAGAGATGGAGATTTGACTTGTCCAG 4740  
19-BMYV ATAGTTGTCATTTTAATCAAGGACAATGTTTGGAAAGAGATGGAGATTTGACTTGTCCAG 4740  
21-097 ATAGTTGTCATTTTAATCAAGGACAATGTTTGGAAAGAGATGGAGATTTGACTTGTCCAG 4740  
19-013 ATAGTTGTCATTTTAATCAAGGACAATGTTTGGAAAGAGATGGAGATTTGACTTGTCCAG 4740  
21-294 ATAGTTGTCATTTTAATCAAGGACAATGTTTGGAAAGAGATGGAGATTTGACTTGTCCAG 4740  
21-056 ATAGTTGTCATTTTAATCAAGGACAATGTTTGGAAAGAGATGGAGATTTGACTTGTCCAG 4740  
20-009 ATAGTTGTCATTTTAATCAAGGACAATGTTTGGAAAGAGATGGAGATTTGACTTGTCCAG 4740  
20-014 ATAGTTGTCATTTTAATCAAGGACAATGTTTGGAAAGAGATGGAGATTTGACTTGTCCAG 4740  
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21-BMYV TTAAAACAACTGGTGACAATGCCTCCTTCTTTATTGTTGGTCCCGTGTCCAGAAGCAAT 4800  
21-254 TTAAAACAACTGGTGACAATGCCTCCTTCTTTATTGTTGGTCCCGTGTCCAGAAGCAAT 4800  
20-BMYV TTAAAACAACTGGTGACAATGCCTCCTTCTTTATTGTTGGTCCCGTGTCCAGAAGCAAT 4800  
20-010 TTAAAACAACTGGTGACAATGCCTCCTTCTTTATTGTTGGTCCCGTGTCCAGAAGCAAT 4800  
20-006 TTAAAACAACTGGTGACAATGCCTCCTTCTTTATTGTTGGTCCCGTGTCCAGAAGCAAT 4800  
19-BMYV TTAAAACAACTGGTGACAATGCCTCCTTCTTTATTGTTGGTCCCGTGTCCAGAAGCAAT 4800  
21-097 TTAAAACAACTGGTGACAATGCCTCCTTCTTTATTGTTGGTCCCGTGTCCAGAAGCAAT 4800  
19-013 TTAAAACAACTGGTGACAATGCCTCCTTCTTTATTGTTGGTCCCGTGTCCAGAAGCAAT 4800  
21-294 TTAAAACAACTGGTGACAATGCCTCCTTCTTTATTGTTGGTCCCGTGTCCAGAAGCAAT 4800  
21-056 TTAAAACAACTGGTGACAATGCCTCCTTCTTTATTGTTGGTCCCGTGTCCAGAAGCAAT 4800  
20-009 TTAAAACAACTGGTGACAATGCCTCCTTCTTTATTGTTGGTCCCGTGTCCAGAAGCAAT 4800  
20-014 TTAAAACAACTGGTGACAATGCCTCCTTCTTTATTGTTGGTCCCGTGTCCAGAAGCAAT 4800  
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21-BMYV CCAAATACAATTATGCCGTTTCATACGGAGCTTGGACAGATCGGATGATGGAGATAGGGA 4860  
21-254 CCAAATACAATTATGCCGTTTCATACGGAGCTTGGACAGATCGGATGATGGAGATAGGGA 4860  
20-BMYV CCAAATACAATTATGCCGTTTCATACGGAGCTTGGACAGATCGGATGATGGAGATAGGGA 4860  
20-010 CCAAATACAATTATGCCGTTTCATACGGAGCTTGGACAGATCGGATGATGGAGATAGGGA 4860  
20-006 CCAAATACAATTATGCCGTTTCATACGGAGCTTGGACAGATCGGATGATGGAGATAGGGA 4860  
19-BMYV CCAAATACAATTATGCCGTTTCATACGGAGCTTGGACAGATCGGATGATGGAGATAGGGA 4860  
21-097 CCAAATACAATTATGCCGTTTCATACGGAGCTTGGACAGATCGGATGATGGAGATAGGGA 4860  
19-013 CCAAATACAATTATGCCGTTTCATACGGAGCTTGGACAGATCGGATGATGGAGATAGGGA 4860  
21-294 CCAAATACAATTATGCCGTTTCATACGGAGCTTGGACAGATCGGATGATGGAGATAGGGA 4860  
21-056 CCAAATACAATTATGCCGTTTCATACGGAGCTTGGACAGATCGGATGATGGAGATAGGGA 4860  
20-009 CCAAATACAATTATGCCGTTTCATACGGAGCTTGGACAGATCGGATGATGGAGATAGGGA 4860  
20-014 CCAAATACAATTATGCCGTTTCATACGGAGCTTGGACAGATCGGATGATGGAGATAGGGA 4860  
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21-BMYV TGATAGCCATAGCACTTGTATGAACAAGGCTCATCCGGTTCGCAAAGATAGAAAGACCAA 4920  
21-254 TGATAGCCATAGCACTTGTATGAACAAGGCTCATCCGGTTCGCAAAGATAGAAAGACCAA 4920  
20-BMYV TGATAGCCATAGCACTTGTATGAACAAGGCTCATCCGGTTCGCAAAGATAGAAAGACCAA 4920  
20-010 TGATAGCCATAGCACTTGTATGAACAAGGCTCATCCGGTTCGCAAAGATAGAAAGACCAA 4920  
20-006 TGATAGCCATAGCACTTGTATGAACAAGGCTCATCCGGTTCGCAAAGATAGAAAGACCAA 4920  
19-BMYV TGATAGCCATAGCACTTGTATGAACAAGGCTCATCCGGTTCGCAAAGATAGAAAGACCAA 4920  
21-097 TGATAGCCATAGCACTTGTATGAACAAGGCTCATCCGGTTCGCAAAGATAGAAAGACCAA 4920  
19-013 TGATAGCCATAGCACTTGTATGAACAAGGCTCATCCGGTTCGCAAAGATAGAAAGACCAA 4920  
21-294 TGATAGCCATAGCACTTGTATGAACAAGGCTCATCCGGTTCGCAAAGATAGAAAGACCAA 4920  
21-056 TGATAGCCATAGCACTTGTATGAACAAGGCTCATCCGGTTCGCAAAGATAGAAAGACCAA 4920

20-009	TGATAGCCATAGCACTTGATGAACAAGGCTCATCCGGTTCGGCAAAGATAGAAAGACCAA	4920
20-014	TGATAGCCATAGCACTTGATGAACAAGGCTCATCCGGTTCGGCAAAGATAGAAAGACCAA *****	4920
21-BMYV	AGAGAGTCGGGCACTCCATGGCAGTCTCAACCTGGGAGACTATAAACTTACCGGAGAAGG	4980
21-254	AGAGAGTCGGGCACTCCATGGCAGTCTCAACCTGGGAGACTATAAACTTACCGGAGAAGG	4980
20-BMYV	AGAGAGTCGGGCACTCCATGGCAGTCTCAACCTGGGAGACTATAAACTTACCGGAGAAGG	4980
20-010	AGAGAGTCGGGCACTCCATGGCAGTCTCAACCTGGGAGACTATAAACTTACCGGAGAAGG	4980
20-006	AGAGAGTCGGGCACTCCATGGCAGTCTCAACCTGGGAGACTATAAACTTACCGGAGAAGG	4980
19-BMYV	AGAGAGTCGGGCACTCCATGGCAGTCTCAACCTGGGAGACTATAAACTTACCGGAGAAGG	4980
21-097	AGAGAGTCGGGCACTCCATGGCAGTCTCAACCTGGGAGACTATAAACTTACCGGAGAAGG	4980
19-013	AGAGAGTCGGGCACTCCATGGCAGTCTCAACCTGGGAGACTATAAACTTACCGGAGAAGG	4980
21-294	AGAGAGTCGGGCACTCCATGGCAGTCTCAACCTGGGAGACTATAAACTTACCGGAGAAGG	4980
21-056	AGAGAGTCGGGCACTCCATGGCAGTCTCAACCTGGGAGACTATAAACTTACCGGAGAAGG	4980
20-009	AGAGAGTCGGGCACTCCATGGCAGTCTCAACCTGGGAGACTATAAACTTACCGGAGAAGG	4980
20-014	AGAGAGTCGGGCACTCCATGGCAGTCTCAACCTGGGAGACTATAAACTTACCGGAGAAGG *****	4980
21-BMYV	AAAACCTCCGGTGAATTCAAAACCGATCAAAGACAAGATCTCAAACCTCCTCCACATCTG	5040
21-254	AAAACCTCCGGTGAATTCAAAACCGATCAAAGACAAGATCTCAAACCTCCTCCACATCTG	5040
20-BMYV	AAAACCTCCGGTGAATTCAAAACCGATCAAAGACAAGATCTCAAACCTCCTCCACATCTG	5040
20-010	AAAACCTCCGGTGAATTCAAAACCGATCAAAGACAAGATCTCAAACCTCCTCCACATCTG	5040
20-006	AAAACCTCCGGTGAATTCAAAACCGATCAAAGACAAGATCTCAAACCTCCTCCACATCTG	5040
19-BMYV	AAAACCTCCGGTGAATTCAAAACCGATCAAAGACAAGATCTCAAACCTCCTCCACATCTG	5040
21-097	AAAACCTCCGGTGAATTCAAAACCGATCAAAGACAAGATCTCAAACCTCCTCCACATCTG	5040
19-013	AAAACCTCCGGTGAATTCAAAACCGATCAAAGACAAGATCTCAAACCTCCTCCACATCTG	5040
21-294	AAAACCTCCGGTGAATTCAAAACCGATCAAAGACAAGATCTCAAACCTCCTCCACATCTG	5040
21-056	AAAACCTCCGGTGAATTCAAAACCGATCAAAGACAAGATCTCAAACCTCCTCCACATCTG	5040
20-009	AAAACCTCCGGTGAATTCAAAACCGATCAAAGACAAGATCTCAAACCTCCTCCACATCTG	5040
20-014	AAAACCTCCGGTGAATTCAAAACCGATCAAAGACAAGATCTCAAACCTCCTCCACATCTG *****	5040
21-BMYV	GTGGGAGTTCCGATATGCCGGATATCGTTCAAGGAGGCTTACCCCTTCCCATTGAAGAAG	5100
21-254	GTGGGAGTTCCGATATGCTGGATATCGTTCAAGGAGGCTTACCCCTTCCCATTGAAGAAG	5100
20-BMYV	GTGGGAGTTCCGATATGCCGGATATCGTTCAAGGAGGCTTACCCCTTCCCATTGAAGAAG	5100
20-010	GTGGGAGTTCCGATATGCTGGATATCGTTCAAGGAGGCTTACCCCTTCCCATTGAAGAAG	5100
20-006	GTGGGAGTTCCGATATGCTGGATATCGTTCAAGGAGGCTTACCCCTTCCCATTGAAGAAG	5100
19-BMYV	GTGGGAGTTCCGATATGCCGGATATCGTTCAAGGAGGCTTACCCCTTCCCATTGAAGAAG	5100
21-097	GTGGGAGTTCCGATATGCCGGATATCGTTCAAGGAGGCTTACCCCTTCCCATTGAAGAAG	5100
19-013	GTGGGAGTTCCGATATGCCGGATATCGTTCAAGGAGGCTTACCCCTTCCCATTGAAGAAG	5100
21-294	GTGGGAGTTCCGATATGCTGGATATCGTTCAAGGAGGCTTACCCCTTCCCATTGAAGAAG	5100
21-056	GTGGGAGTTCCGATATGCCGGATATCGTTCAAGGAGGCTTACCCCTTCCCATTGAAGAAG	5100
20-009	GTGGGAGTTCCGATATGCCGGATATCGTTCAAGGAGGCTTACCCCTTCCCATTGAAGAAG	5100
20-014	GTGGGAGTTCCGATATGCCGGATATCGTTCAAGGAGGCTTACCCCTTCCCATTGAAGAAG *****	5100
21-BMYV	ACATTCCTGATTTTCATCAGGGATGACCCCTTGGTCCAACATACCGGCCAAGACTTCGCGGG	5160
21-254	ACATTCCTGATTTTCATCAGGGATGACCCCTTGGTCCAACATACCGGCCAAGACTTCGCGGG	5160
20-BMYV	ACATTCCTGATTTTCATCAGGGATGACCCCTTGGTCCAACATACCGGCCAAGACTTCGCGGG	5160
20-010	ACATTCCTGATTTTCATCAGGGATGACCCCTTGGTCCAACATACCGGCCAAGACTTCGCGGG	5160
20-006	ACATTCCTGATTTTCATCAGGGATGACCCCTTGGTCCAACATACCGGCCAAGACTTCGCGGG	5160
19-BMYV	ACATTCCTGATTTTCATCAGGGATGACCCCTTGGTCCAACATACCGGCCAAGACTTCGCGGG	5160
21-097	ACATTCCTGATTTTCATCAGGGATGACCCCTTGGTCCAACATACCGGCCAAGACTTCGCGGG	5160
19-013	ACATTCCTGATTTTCATCAGGGATGACCCCTTGGTCCAACATACCGGCCAAGACTTCGCGGG	5160
21-294	ACATTCCTGATTTTCATCAGGGATGACCCCTTGGTCCAACATACCGGCCAAGACTTCGCGGG	5160
21-056	ACATTCCTGATTTTCATCAGGGATGACCCCTTGGTCCAACATACCGGCCAAGACTTCGCGGG	5160
20-009	ACATTCCTGATTTTCATCAGGGATGACCCCTTGGTCCAACATACCGGCCAAGACTTCGCGGG	5160
20-014	ACATTCCTGATTTTCATCAGGGATGACCCCTTGGTCCAACATACCGGCCAAGACTTCGCGGG *****	5160
21-BMYV	AAGACGAGGCTGCGTCATCAAAGAGTGGTTTTAAACCCCAATTGAAGCCTCCTGGCTTGC	5220
21-254	AAGACGAGGCTGCGTCATCAAAGAGTGGTTTTAAACCCCAATTGAAGCCTCCTGGCTTGC	5220
20-BMYV	AAGACGAGGCTGCGTCATCAAAGAGTGGTTTTAAACCCCAATTGAAGCCTCCTGGCTTGC	5220
20-010	AAGACGAGGCTGCGTCATCAAAGAGTGGTTTTAAACCCCAATTGAAGCCTCCTGGCTTGC	5220
20-006	AAGACGAGGCTGCGTCATCAAAGAGTGGTTTTAAACCCCAATTGAAGCCTCCTGGCTTGC	5220
19-BMYV	AAGACGAGGCTGCGTCATCAAAGAGTGGTTTTAAACCCCAATTGAAGCCTCCTGGCTTGC	5220
21-097	AAGACGAGGCTGCGTCATCAAAGAGTGGTTTTAAACCCCAATTGAAGCCTCCTGGCTTGC	5220
19-013	AAGACGAGGCTGCGTCATCAAAGAGTGGTTTTAAACCCCAATTGAAGCCTCCTGGCTTGC	5220

21-294 AAGACGAGGCTGCGTCATCAAAGAGTGGTTTTAAACCCCAATTGAAGCCTCCTGGCTTGC 5220  
21-056 AAGACGAGGCTGCGTCATCAAAGAGTGGTTTTAAACCCCAATTGAAGCCTCCTGGCTTGC 5220  
20-009 AAGACGAGGCTGCGTCATCAAAGAGTGGTTTTAAACCCCAATTGAAGCCTCCTGGCTTGC 5220  
20-014 AAGACGAGGCTGCGTCATCAAAGAGTGGTTTTAAACCCCAATTGAAGCCTCCTGGCTTGC 5220  
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21-BMYV CAAAGCCACAACCGGTCAGAACGATCCGAAACTTCGATCCAGAACCTGACTTGGTTGAGG 5280  
21-254 CAAAGCCACAACCGGTCAGAACGATCCGAAACTTCGATCCAGAACCTGACTTGGTTGAGG 5280  
20-BMYV CAAAGCCACAACCGGTCAGAACGATCCGAAACTTCGATCCAGAACCTGACTTGGTTGAGG 5280  
20-010 CAAAGCCACAACCGGTCAGAACGATCCGAAACTTCGATCCAGAACCTGACTTGGTTGAGG 5280  
20-006 CAAAGCCACAACCGGTCAGAACGATCCGAAACTTCGATCCAGAACCTGACTTGGTTGAGG 5280  
19-BMYV CAAAGCCACAACCGGTCAGAACGATCCGAAACTTCGATCCAGAACCTGACTTGGTTGAGG 5280  
21-097 CAAAGCCACAACCGGTCAGAACGATCCGAAACTTCGATCCAGAACCTGACTTGGTTGAGG 5280  
19-013 CAAAGCCACAACCGGTCAGAACGATCCGAAACTTCGATCCAGAACCTGACTTGGTTGAGG 5280  
21-294 CAAAGCCACAACCGGTCAGAACGATCCGAAACTTCGATCCAGAACCTGACTTGGTTGAGG 5280  
21-056 CAAAGCCACAACCGGTCAGAACGATCCGAAACTTCGATCCAGAACCTGACTTGGTTGAGG 5280  
20-009 CAAAGCCACAACCGGTCAGAACGATCCGAAACTTCGATCCAGAACCTGACTTGGTTGAGG 5280  
20-014 CAAAGCCACAACCGGTCAGAACGATCCGAAACTTCGATCCAGAACCTGACTTGGTTGAGG 5280  
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21-BMYV CATGGCGACCTGACGTGAACCCCGGATATTCGAAGGACGTTGGCAGCGGCCACTGTTA 5340  
21-254 CATGGCGACCTGACGTGAACCCCGGATATTCGAAGGACGTTGGCAGCGGCCACTGTTA 5340  
20-BMYV CATGGCGACCTGACGTGAACCCCGGATATTCGAAGGACGTTGGCAGCGGCCACTGTTA 5340  
20-010 CATGGCGACCTGACGTGAACCCCGGATATTCGAAGGACGTTGGCAGCGGCCACTGTTA 5340  
20-006 CATGGCGACCTGACGTGAACCCCGGATATTCGAAGGACGTTGGCAGCGGCCACTGTTA 5340  
19-BMYV CATGGCGACCTGACGTGAACCCCGGATATTCGAAGGACGTTGGCAGCGGCCACTGTTA 5340  
21-097 CATGGCGACCTGACGTGAACCCCGGATATTCGAAGGACGTTGGCAGCGGCCACTGTTA 5340  
19-013 CATGGCGACCTGACGTGAACCCCGGATATTCGAAGGACGTTGGCAGCGGCCACTGTTA 5340  
21-294 CATGGCGACCTGACGTGAACCCCGGATATTCGAAGGACGTTGGCAGCGGCCACTGTTA 5340  
21-056 CATGGCGACCTGACGTGAACCCCGGATATTCGAAGGACGTTGGCAGCGGCCACTGTTA 5340  
20-009 CATGGCGACCTGACGTGAACCCCGGATATTCGAAGGACGTTGGCAGCGGCCACTGTTA 5340  
20-014 CATGGCGACCTGACGTGAACCCCGGATATTCGAAGGACGTTGGCAGCGGCCACTGTTA 5340  
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21-BMYV TGTACGGGGGTTCCGTTAATGAAGGCCGGTCTATGATTGACAAGCGTGATAAAGCTGTGT 5400  
21-254 TGTACGGGGGTTCCGTTAATGAAGGCCGGTCTATGATTGACAAGCGTGATAAAGCTGTGT 5400  
20-BMYV TGTACGGGGGTTCCGTTAATGAAGGCCGGTCTATGATTGACAAGCGTGATAAAGCTGTGT 5400  
20-010 TGTACGGGGGTTCCGTTAATGAAGGCCGGTCTATGATTGACAAGCGTGATAAAGCTGTGT 5400  
20-006 TGTACGGGGGTTCCGTTAATGAAGGCCGGTCTATGATTGACAAGCGTGATAAAGCTGTGT 5400  
19-BMYV TGTACGGGGGTTCCGTTAATGAAGGCCGGTCTATGATTGACAAGCGTGATAAAGCTGTGT 5400  
21-097 TGTACGGGGGTTCCGTTAATGAAGGCCGGTCTATGATTGACAAGCGTGATAAAGCTGTGT 5400  
19-013 TGTACGGGGGTTCCGTTAATGAAGGCCGGTCTATGATTGACAAGCGTGATAAAGCTGTGT 5400  
21-294 TGTACGGGGGTTCCGTTAATGAAGGCCGGTCTATGATTGACAAGCGTGATAAAGCTGTGT 5400  
21-056 TGTACGGGGGTTCCGTTAATGAAGGCCGGTCTATGATTGACAAGCGTGATAAAGCTGTGT 5400  
20-009 TGTACGGGGGTTCCGTTAATGAAGGCCGGTCTATGATTGACAAGCGTGATAAAGCTGTGT 5400  
20-014 TGTACGGGGGTTCCGTTAATGAAGGCCGGTCTATGATTGACAAGCGTGATAAAGCTGTGT 5400  
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21-BMYV TAGACGGCCGCAAGAGTTGGGGATCTTCCTTGGCGTCCTCCTTGACGGGAGGCACGCTTA 5460  
21-254 TAGACGGCCGCAAGAGTTGGGGATCTTCCTTGGCGTCCTCCTTGACGGGAGGCACGCTTA 5460  
20-BMYV TAGACGGCCGCAAGAGTTGGGGATCTTCCTTGGCGTCCTCCTTGACGGGAGGCACGCTTA 5460  
20-010 TAGACGGCCGCAAGAGTTGGGGATCTTCCTTGGCGTCCTCCTTGACGGGAGGCACGCTTA 5460  
20-006 TAGACGGCCGCAAGAGTTGGGGATCTTCCTTGGCGTCCTCCTTGACGGGAGGCACGCTTA 5460  
19-BMYV TAGACGGCCGCAAGAGTTGGGGATCTTCCTTGGCGTCCTCCTTGACGGGAGGCACGCTTA 5460  
21-097 TAGACGGCCGCAAGAGTTGGGGATCTTCCTTGGCGTCCTCCTTGACGGGAGGCACGCTTA 5460  
19-013 TAGACGGCCGCAAGAGTTGGGGATCTTCCTTGGCGTCCTCCTTGACGGGAGGCACGCTTA 5460  
21-294 TAGACGGCCGCAAGAGTTGGGGATCTTCCTTGGCGTCCTCCTTGACGGGAGGCACGCTTA 5460  
21-056 TAGACGGCCGCAAGAGTTGGGGATCTTCCTTGGCGTCCTCCTTGACGGGAGGCACGCTTA 5460  
20-009 TAGACGGCCGCAAGAGTTGGGGATCTTCCTTGGCGTCCTCCTTGACGGGAGGCACGCTTA 5460  
20-014 TAGACGGCCGCAAGAGTTGGGGATCTTCCTTGGCGTCCTCCTTGACGGGAGGCACGCTTA 5460  
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21-BMYV AGGCCTCTGCAAAGTCAGAGAAGCTTGCCAAACTCACTTCGAGTGAAAGGGCGCAGTTCA 5520  
21-254 AGGCCTCTGCAAAGTCAGAGAAGCTTGCCAAACTCACTTCGAGTGAAAGGGCGCAGTTCA 5520  
20-BMYV AGGCCTCTGCAAAGTCAGAGAAGCTTGCCAAACTCACTTCGAGTGAAAGGGCGCAGTTCA 5520  
20-010 AGGCCTCTGCAAAGTCAGAGAAGCTTGCCAAACTCACTTCGAGTGAAAGGGCGCAGTTCA 5520  
20-006 AGGCCTCTGCAAAGTCAGAGAAGCTTGCCAAACTCACTTCGAGTGAAAGGGCGCAGTTCA 5520  
19-BMYV AGGCCTCTGCAAAGTCAGAGAAGCTTGCCAAACTCACTTCGAGTGAAAGGGCGCAGTTCA 5520

21-097 AGGCCTCTGCAAAGTCAGAGAAGCTTGCCAAACTCACTTCGAGTGAAAGGGCGCAGTTCA 5520  
 19-013 AGGCCTCTGCAAAGTCAGAGAAGCTTGCCAAACTCACTTCGAGTGAAAGGGCGCAGTTCA 5520  
 21-294 AGGCCTCTGCAAAGTCAGAGAAGCTTGCCAAACTCACTTCGAGTGAAAGGGCGCAGTTCA 5520  
 21-056 AGGCCTCTGCAAAGTCAGAGAAGCTTGCCAAACTCACTTCGAGTGAAAGGGCGCAGTTCA 5520  
 20-009 AGGCCTCTGCAAAGTCAGAGAAGCTTGCCAAACTCACTTCGAGTGAAAGGGCGCAGTTCA 5520  
 20-014 AGGCCTCTGCAAAGTCAGAGAAGCTTGCCAAACTCACTTCGAGTGAAAGGGCGCAGTTCA 5520  
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21-BMYV AACGAATTAAGCGCCAGCAAGGTGCCACACGAGCTTCAGAATTTTTAGAACAACTTCTGG 5580  
 21-254 AACGAATTAAGCGCCAGCAAGGTGCCACACGAGCTTCAGAATTTTTAGAACAACTTCTGG 5580  
 20-BMYV AACGAATTAAGCGCCAGCAAGGTGCCACACGAGCTTCAGAATTTTTAGAACAACTTCTGG 5580  
 20-010 AACGAATTAAGCGCCAGCAAGGTGCCACACGAGCTTCAGAATTTTTAGAACAACTTCTGG 5580  
 20-006 AACGAATTAAGCGCCAGCAAGGTGCCACACGAGCTTCAGAATTTTTAGAACAACTTCTGG 5580  
 19-BMYV AACGAATTAAGCGCCAGCAAGGTGCCACACGAGCTTCAGAATTTTTAGAACAACTTCTGG 5580  
 21-097 AACGAATTAAGCGCCAGCAAGGTGCCACACGAGCTTCAGAATTTTTAGAACAACTTCTGG 5580  
 19-013 AACGAATTAAGCGCCAGCAAGGTGCCACACGAGCTTCAGAATTTTTAGAACAACTTCTGG 5580  
 21-294 AACGAATTAAGCGCCAGCAAGGTGCCACACGAGCTTCAGAATTTTTAGAACAACTTCTGG 5580  
 21-056 AACGAATTAAGCGCCAGCAAGGTGCCACACGAGCTTCAGAATTTTTAGAACAACTTCTGG 5580  
 20-009 AACGAATTAAGCGCCAGCAAGGTGCCACACGAGCTTCAGAATTTTTAGAACAACTTCTGG 5580  
 20-014 AACGAATTAAGCGCCAGCAAGGTGCCACACGAGCTTCAGAATTTTTAGAACAACTTCTGG 5580  
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21-BMYV CTGGCACAAACCCTGACCCAAGGTCTTGATGAACCTTTCCCAATCATCACAGTCAAGCCC 5640  
 21-254 CTGGCACAAACCCTGACCCAAGGTCTTGATGAACCTTTCCCAATCATCACAGTCAAGCCC 5640  
 20-BMYV CTGGCACAAACCCTGACCCAAGGTCTTGATGAACCTTTCCCAATCATCACAGTCAAGCCC 5640  
 20-010 CTGGCACAAACCCTGACCCAAGGTCTTGATGAACCTTTCCCAATCATCACAGTCAAGCCC 5640  
 20-006 CTGGCACAAACCCTGACCCAAGGTCTTGATGAACCTTTCCCAATCATCACAGTCAAGCCC 5640  
 19-BMYV CTGGCACAAACCCTGACCCAAGGTCTTGATGAACCTTTCCCAATCATCACAGTCAAGCCC 5640  
 21-097 CTGGCACAAACCCTGACCCAAGGTCTTGATGAACCTTTCCCAATCATCACAGTCAAGCCC 5640  
 19-013 CTGGCACAAACCCTGACCCAAGGTCTTGATGAACCTTTCCCAATCATCACAGTCAAGCCC 5640  
 21-294 CTGGCACAAACCCTGACCCAAGGTCTTGATGAACCTTTCCCAATCATCACAGTCAAGCCC 5640  
 21-056 CTGGCACAAACCCTGACCCAAGGTCTTGATGAACCTTTCCCAATCATCACAGTCAAGCCC 5640  
 20-009 CTGGCACAAACCCTGACCCAAGGTCTTGATGAACCTTTCCCAATCATCACAGTCAAGCCC 5640  
 20-014 CTGGCACAAACCCTGACCCAAGGTCTTGATGAACCTTTCCCAATCATCACAGTCAAGCCC 5640  
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21-BMYV GTGACTTTAAACGCGGAACGACTCCGAAAGGATAGGCAACGAGTGTTTTACGCTGGGATA 5700  
 21-254 GTGACTTTAAACGCGGAACGACTCCGAAAGGATAGGCAACGAGTGTTTTACGCTGGGATA 5700  
 20-BMYV GTGACTTTAAACGCGGAACGACTCCGAAAGGATAGGCAACGAGTGTTTTACGCTGGGATA 5700  
 20-010 GTGACTTTAAACGCGGAACGACTCCGAAAGGATAGGCAACGAGTGTTTTACGCTGGGATA 5700  
 20-006 GTGACTTTAAACGCGGAACGACTCCGAAAGGATAGGCAACGAGTGTTTTACGCTGGGATA 5700  
 19-BMYV GTGACTTTAAACGCGGAACGACTCCGAAAGGATAGGCAACGAGTGTTTTACGCTGGGATA 5700  
 21-097 GTGACTTTAAACGCGGAACGACTCCGAAAGGATAGGCAACGAGTGTTTTACGCTGGGATA 5700  
 19-013 GTGACTTTAAACGCGGAACGACTCCGAAAGGATAGGCAACGAGTGTTTTACGCTGGGATA 5700  
 21-294 GTGACTTTAAACGCGGAACGACTCCGAAAGGATAGGCAACGAGTGTTTTACGCTGGGATA 5700  
 21-056 GTGACTTTAAACGCGGAACGACTCCGAAAGGATAGGCAACGAGTGTTTTACGCTGGGATA 5700  
 20-009 GTGACTTTAAACGCGGAACGACTCCGAAAGGATAGGCAACGAGTGTTTTACGCTGGGATA 5700  
 20-014 GTGACTTTAAACGCGGAACGACTCCGAAAGGATAGGCAACGAGTGTTTTACGCTGGGATA 5700  
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21-BMYV ACTCCCTACGGCACTTCGGTGT 5722  
 21-254 ACTCCCTACGGCACTTCGGTGT 5722  
 20-BMYV ACTCCCTACGGCACTTCGGTGT 5722  
 20-010 ACTCCCTACGGCACTTCGGTGT 5722  
 20-006 ACTCCCTACGGCACTTCGGTGT 5722  
 19-BMYV ACTCCCTACGGCACTTCGGTGT 5722  
 21-097 ACTCCCTACGGCACTTCGGTGT 5722  
 19-013 ACTCCCTACGGCACTTCGGTGT 5722  
 21-294 ACTCCCTACGGCACTTCGGTGT 5722  
 21-056 ACTCCCTACGGCACTTCGGTGT 5722  
 20-009 ACTCCCTACGGCACTTCGGTGT 5722  
 20-014 ACTCCCTACGGCACTTCGGTGT 5722  
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## Appendix D – BChV consensus sequence alignment

BChV consensus sequences produced using BCFtools (see Chapter 5 section 5.3.2), and aligned using ClustalOmega.

CLUSTAL O(1.2.4) multiple sequence alignment

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19_016          ACAAAGAATAGCAGGAGGACAGTGAATGAACTTTGAGATTTGCTTCAAACGAACAGCG      60
21-256          ACAAAGAATAGCAGGAGGACAGTGAATGAACTTTGAGATTTGCTTCAAACGAACAGCG      60
21_024          ACAAAGAATAGCAGGAGGACAGTGAATGAACTTTGAGATTTGCTTCAAACGAACAGCG      60
20-021          ACAAAGAATAGCAGGAGGACAGTGAATGAACTTTGAGATTTGCTTCAAACGAACAGCG      60
BBRO21-BChV    ACAAAGAATAGCAGGAGGACAGTGAATGAACTTTGAGATTTGCTTCAAACGAACAGCG      60
21-039          ACAAAGAATAGCAGGAGGACAGTGAATGAACTTTGAGATTTGCTTCAAACGAACAGCG      60
21-020          ACAAAGAATAGCAGGAGGACAGTGAATGAACTTTGAGATTTGCTTCAAACGAACAGCG      60
19_015          ACAAAGAATAGCAGGAGGACAGTGAATGAACTTTGAGATTTGCTTCAAACGAACAGCG      60
21-196          ACAAAGAATAGCAGGAGGACAGTGAATGAACTTTGAGATTTGCTTCAAACGAACAGCG      60
21-187          ACAAAGAATAGCAGGAGGACAGTGAATGAACTTTGAGATTTGCTTCAAACGAACAGCG      60
21-056          ACAAAGAATAGCAGGAGGACAGTGAATGAACTTTGAGATTTGCTTCAAACGAACAGCG      60
21_290          ACAAAGAATAGCAGGAGGACAGTGAATGAACTTTGAGATTTGCTTCAAACGAACAGCG      60
21-121          ACAAAGAATAGCAGGAGGACAGTGAATGAACTTTGAGATTTGCTTCAAACGAACAGCG      60
19_005          ACAAAGAATAGCAGGAGGACAGTGAATGAACTTTGAGATTTGCTTCAAACGAACAGCG      60
21-294          ACAAAGAATAGCAGGAGGACAGTGAATGAACTTTGAGATTTGCTTCAAACGAACAGCG      60
21-179          ACAAAGAATAGCAGGAGGACAGTGAATGAACTTTGAGATTTGCTTCAAACGAACAGCG      60
20-009          ACAAAGAATAGCAGGAGGACAGTGAATGAACTTTGAGATTTGCTTCAAACGAACAGCG      60
21-071          ACAAAGAATAGCAGGAGGACAGTGAATGAACTTTGAGATTTGCTTCAAACGAACAGCG      60
20-018-2       ACAAAGAATAGCAGGAGGACAGTGAATGAACTTTGAGATTTGCTTCAAACGAACAGCG      60
20-014          ACAAAGAATAGCAGGAGGACAGTGAATGAACTTTGAGATTTGCTTCAAACGAACAGCG      60
21_278          ACAAAGAATAGCAGGAGGACAGTGAATGAACTTTGAGATTTGCTTCAAACGAACAGCG      60
21-111          ACAAAGAATAGCAGGAGGACAGTGAATGAACTTTGAGATTTGCTTCAAACGAACAGCG      60
20-016          ACAAAGAATAGCAGGAGGACAGTGAATGAACTTTGAGATTTGCTTCAAACGAACAGCG      60
20-015          ACAAAGAATAGCAGGAGGACAGTGAATGAACTTTGAGATTTGCTTCAAACGAACAGCG      60
20-012          ACAAAGAATAGCAGGAGGACAGTGAATGAACTTTGAGATTTGCTTCAAACGAACAGCG      60
19_011          ACAAAGAATAGCAGGAGGACAGTGAATGAACTTTGAGATTTGCTTCAAACGAACAGCG      60
19-002          ACAAAGAATAGCAGGAGGACAGTGAATGAACTTTGAGATTTGCTTCAAACGAACAGCG      60
19_010          ACAAAGAATAGCAGGAGGACAGTGAATGAACTTTGAGATTTGCTTCAAACGAACAGCG      60
20-017          ACAAAGAATAGCAGGAGGACAGTGAATGAACTTTGAGATTTGCTTCAAACGAACAGCG      60
20-018-1       ACAAAGAATAGCAGGAGGACAGTGAATGAACTTTGAGATTTGCTTCAAACGAACAGCG      60
*****

19_016          AACTACTAGTGACGAGCGAAGACACTTGCCACTAAAAGAGAGATCATTATAATCGGAA      120
21-256          AACTACTAGTGACGAGCGAAGACACTTGCCACTAAAAGAGAGATCATTATAATCGGAA      120
21_024          AACTACTAGTGACGAGCGAAGACACTTGCCACTAAAAGAGAGATCATTATAATCGGAA      120
20-021          AACTACTAGTGACGAGCGAAGACACTTGCCACTAAAAGAGAGATCATTATAATCGGAA      120
BBRO21-BChV    AACTACTAGTGACGAGCGAAGACACTTGCCACTAAAAGAGAGATCATTATAATCGGAA      120
21-039          AACTACTAGTGACGAGCGAAGACACTTGCCACTAAAAGAGAGATCATTATAATCGGAA      120
21-020          AACTACTAGTGACGAGCGAAGACACTTGCCACTAAAAGAGAGATCATTATAATCGGAA      120
19_015          AACTACTAGTGACGAGCGAAGACACTTGCCACTAAAAGAGAGATCATTATAATCGGAA      120
21-196          AACTACTAGTGACGAGCGAAGACACTTGCCACTAAAAGAGAGATCATTATAATCGGAA      120
21-187          AACTACTAGTGACGAGCGAAGACACTTGCCACTAAAAGAGAGATCATTATAATCGGAA      120
21-056          AACTACTAGTGACGAGCGAAGACACTTGCCACTAAAAGAGAGATCATTATAATCGGAA      120
21_290          AACTACTAGTGACGAGCGAAGACACTTGCCACTAAAAGAGAGATCATTATAATCGGAA      120
21-121          AACTACTAGTGACGAGCGAAGACACTTGCCACTAAAAGAGAGATCATTATAATCGGAA      120
19_005          AACTACTAGTGACGAGCGAAGACACTTGCCACTAAAAGAGAGATCATTATAATCGGAA      120
21-294          AACTACTAGTGACGAGCGAAGACACTTGCCACTAAAAGAGAGATCATTATAATCGGAA      120
21-179          AACTACTAGTGACGAGCGAAGACACTTGCCACTAAAAGAGAGATCATTATAATCGGAA      120
20-009          AACTACTAGTGACGAGCGAAGACACTTGCCACTAAAAGAGAGATCATTATAATCGGAA      120
21-071          AACTACTAGTGACGAGCGAAGACACTTGCCACTAAAAGAGAGATCATTATAATCGGAA      120
20-018-2       AACTACTAGTGACGAGCGAAGACACTTGCCACTAAAAGAGAGATCATTATAATCGGAA      120
20-014          AACTACTAGTGACGAGCGAAGACACTTGCCACTAAAAGAGAGATCATTATAATCGGAA      120
21_278          AACTACTAGTGACGAGCGAAGACACTTGCCACTAAAAGAGAGATCATTATAATCGGAA      120
21-111          AACTACTAGTGACGAGCGAAGACACTTGCCACTAAAAGAGAGATCATTATAATCGGAA      120
20-016          AACTACTAGTGACGAGCGAAGACACTTGCCACTAAAAGAGAGATCATTATAATCGGAA      120
20-015          AACTACTAGTGACGAGCGAAGACACTTGCCACTAAAAGAGAGATCATTATAATCGGAA      120
20-012          AACTACTAGTGACGAGCGAAGACACTTGCCACTAAAAGAGAGATCATTATAATCGGAA      120
19_011          AACTACTAGTGACGAGCGAAGACACTTGCCACTAAAAGAGAGATCATTATAATCGGAA      120
19-002          AACTACTAGTGACGAGCGAAGACACTTGCCACTAAAAGAGAGATCATTATAATCGGAA      120
19_010          AACTACTAGTGACGAGCGAAGACACTTGCCACTAAAAGAGAGATCATTATAATCGGAA      120
20-017          AACTACTAGTGACGAGCGAAGACACTTGCCACTAAAAGAGAGATCATTATAATCGGAA      120
20-018-1       AACTACTAGTGACGAGCGAAGACACTTGCCACTAAAAGAGAGATCATTATAATCGGAA      120
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19_016          GATTTCTCACGCAATCCCTCAACTTTTGCACCATTTCAAATATGGACACCAAGTTGAAC      180

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20-018-1	CAGAAAAAGCTGTCAGATTACAGCTACAAAGAACTTGTAAATACACGTCTTGCAAAGAGGC *****	420
19_016	TACAACGACACCCAGAAATACTTGCCACAACCTAGCATCAATGGATTTTCGAAAAGCACTGG	480
21-256	TACAACGACACCCAGAAATACTTGCCACAACCTAGCATCAATGGATTTTCGAAAAGCACTGG	480
21_024	TACAACGACACCCAGAAATACTTGCCACAACCTAGCATCAATGGATTTTCGAAAAGCACTGG	480
20-021	TACAACGACACCCAGAAATACTTGCCACAACCTAGCATCAATGGATTTTCGAAAAGCACTGG	480
BBRO21-BChV	TACAACGACACCCAGAAATACTTGCCACAACCTAGCATCAATGGATTTTCGAAAAGCACTGG	480
21-039	TACAACGACACCCAGAAATACTTGCCACAACCTAGCATCAATGGATTTTCGAAAAGCACTGG	480
21-020	TACAACGACACCCAGAAATACTTGCCACAACCTAGCATCAATGGATTTTCGAAAAGCACTGG	480
19_015	TACAACGACACCCAGAAATACTTGCCACAACCTAGCATCAATGGATTTTCGAAAAGCACTGG	480
21-196	TACAACGACACCCAGAAATACTTGCCACAACCTAGCATCAATGGATTTTCGAAAAGCACTGG	480
21-187	TACAACGACACCCAGAAATACTTGCCACAACCTAGCATCAATGGATTTTCGAAAAGCACTGG	480
21-056	TACAACGACACCCAGAAATACTTGCCACAACCTAGCATCAATGGATTTTCGAAAAGCACTGG	480
21_290	TACAACGACACCCAGAAATACTTGCCACAACCTAGCATCAATGGATTTTCGAAAAGCACTGG	480
21-121	TACAACGACACCCAGAAATACTTGCCACAACCTAGCATCAATGGATTTTCGAAAAGCACTGG	480
19_005	TACAACGACACCCAGAAATACTTGCCACAACCTAGCATCAATGGATTTTCGAAAAGCACTGG	480
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21-179	TACAACGACACCCAGAAATACTTGCCACAACCTAGCATCAATGGATTTTCGAAAAGCACTGG	480
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21-071	TACAACGACACCCAGAAATACTTGCCACAACCTAGCATCAATGGATTTTCGAAAAGCACTGG	480
20-018-2	TACAACGACACCCAGAAATACTTGCCACAACCTAGCATCAATGGATTTTCGAAAAGCACTGG	480
20-014	TACAACGACACCCAGAAATACTTGCCACAACCTAGCATCAATGGATTTTCGAAAAGCACTGG	480
21_278	TACAACGACACCCAGAAATACTTGCCACAACCTAGCATCAATGGATTTTCGAAAAGCACTGG	480
21-111	TACAACGACACCCAGAAATACTTGCCACAACCTAGCATCAATGGATTTTCGAAAAGCACTGG	480
20-016	TACAACGACACCCAGAAATACTTGCCACAACCTAGCATCAATGGATTTTCGAAAAGCACTGG	480
20-015	TACAACGACACCCAGAAATACTTGCCACAACCTAGCATCAATGGATTTTCGAAAAGCACTGG	480
20-012	TACAACGACACCCAGAAATACTTGCCACAACCTAGCATCAATGGATTTTCGAAAAGCACTGG	480
19_011	TACAACGACACCCAGAAATACTTGCCACAACCTAGCATCAATGGATTTTCGAAAAGCACTGG	480
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19_010	TACAACGACACCCAGAAATACTTGCCACAACCTAGCATCAATGGATTTTCGAAAAGCACTGG	480
20-017	TACAACGACACCCAGAAATACTTGCCACAACCTAGCATCAATGGATTTTCGAAAAGCACTGG	480
20-018-1	TACAACGACACCCAGAAATACTTGCCACAACCTAGCATCAATGGATTTTCGAAAAGCACTGG *****	480
19_016	GATACCATCTCAGGGCAATTAACAGGATTGAATATCCTGGAAAGATTGATGTGGGGCCTC	540
21-256	GATACCATCTCAGGGCAATTAACAGGATTGAATATCCTGGAAAGATTGATGTGGGGCCTC	540
21_024	GATACCATCTCAGGGCAATTAACAGGATTGAATATCCTGGAAAGATTGATGTGGGGCCTC	540
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BBRO21-BChV	GATACCATCTCAGGGCAATTAACAGGATTGAATATCCTGGAAAGATTGATGTGGGGCCTC	540
21-039	GATACCATCTCAGGGCAATTAACAGGATTGAATATCCTGGAAAGATTGATGTGGGGCCTC	540
21-020	GATACCATCTCAGGGCAATTAACAGGATTGAATATCCTGGAAAGATTGATGTGGGGCCTC	540
19_015	GATACCATCTCAGGGCAATTAACAGGATTGAATATCCTGGAAAGATTGATGTGGGGCCTC	540
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21-187	GATACCATCTCAGGGCAATTAACAGGATTGAATATCCTGGAAAGATTGATGTGGGGCCTC	540
21-056	GATACCATCTCAGGGCAATTAACAGGATTGAATATCCTGGAAAGATTGATGTGGGGCCTC	540
21_290	GATACCATCTCAGGGCAATTAACAGGATTGAATATCCTGGAAAGATTGATGTGGGGCCTC	540
21-121	GATACCATCTCAGGGCAATTAACAGGATTGAATATCCTGGAAAGATTGATGTGGGGCCTC	540
19_005	GATACCATCTCAGGGCAATTAACAGGATTGAATATCCTGGAAAGATTGATGTGGGGCCTC	540
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21-179	GATACCATCTCAGGGCAATTAACAGGATTGAATATCCTGGAAAGATTGATGTGGGGCCTC	540
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21-071	GATACCATCTCAGGGCAATTAACAGGATTGAATATCCTGGAAAGATTGATGTGGGGCCTC	540
20-018-2	GATACCATCTCAGGGCAATTAACAGGATTGAATATCCTGGAAAGATTGATGTGGGGCCTC	540
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21-111	GATACCATCTCAGGGCAATTAACAGGATTGAATATCCTGGAAAGATTGATGTGGGGCCTC	540
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20-015	GATACCATCTCAGGGCAATTAACAGGATTGAATATCCTGGAAAGATTGATGTGGGGCCTC	540
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19_011	GATACCATCTCAGGGCAATTAACAGGATTGAATATCCTGGAAAGATTGATGTGGGGCCTC	540
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19_010	GATACCATCTCAGGGCAATTAACAGGATTGAATATCCTGGAAAGATTGATGTGGGGCCTC	540
20-017	GATACCATCTCAGGGCAATTAACAGGATTGAATATCCTGGAAAGATTGATGTGGGGCCTC	540
20-018-1	GATACCATCTCAGGGCAATTAACAGGATTGAATATCCTGGAAAGATTGATGTGGGGCCTC *****	540
19_016	ATACGGTTATGGGGTTATGTAATCTGGGTTGTCAGCTCATCCACGATGAGCTTCTTGATG	600
21-256	ATACGGTTATGGGGTTATGTAATCTGGGTTGTCAGCTCATCCACGATGAGCTTCTTGATG	600
21_024	ATACGGTTATGGGGTTATGTAATCTGGGTTGTCAGCTCATCCACGATGAGCTTCTTGATG	600
20-021	ATACGGTTATGGGGTTATGTAATCTGGGTTGTCAGCTCATCCACGATGAGCTTCTTGATG	600
BBRO21-BChV	ATACGGTTATGGGGTTATGTAATCTGGGTTGTCAGCTCATCCACGATGAGCTTCTTGATG	600
21-039	ATACGGTTATGGGGTTATGTAATCTGGGTTGTCAGCTCATCCACGATGAGCTTCTTGATG	600
21-020	ATACGGTTATGGGGTTATGTAATCTGGGTTGTCAGCTCATCCACGATGAGCTTCTTGATG	600
19_015	ATACGGTTATGGGGTTATGTAATCTGGGTTGTCAGCTCATCCACGATGAGCTTCTTGATG	600



21-111	CGGGCAGCTCAATTATTCTTCAAAAATATTACAACCTATGCCTACCTGTGTTGGCGGCAAGG	720
20-016	CGGGCAGCTCAATTATTCTTCAAAAATATTACAACCTATGCCTACCTGTGTTGGCGGCAAGG	720
20-015	CGGGCAGCTCAATTATTCTTCAAAAATATTACAACCTATGCCTACCTGTGTTGGCGGCAAGG	720
20-012	CGGGCAGCTCAATTATTCTTCAAAAATATTACAACCTATGCCTACCTGTGTTGGCGGCAAGG	720
19_011	CGGGCAGCTCAATTATTCTTCAAAAATATTACAACCTATGCCTACCTGTGTTGGCGGCAAGG	720
19-002	CGGGCAGCTCAATTATTCTTCAAAAATATTACAACCTATGCCTACCTGTGTTGGCGGCAAGG	720
19_010	CGGGCAGCTCAATTATTCTTCAAAAATATTACAACCTATGCCTACCTGTGTTGGCGGCAAGG	720
20-017	CGGGCAGCTCAATTATTCTTCAAAAATATTACAACCTATGCCTACCTGTGTTGGCGGCAAGG	720
20-018-1	CGGGCAGCTCAATTATTCTTCAAAAATATTACAACCTATGCCTACCTGTGTTGGCGGCAAGG *****	720
19_016	ATGGTGGTGAGCGCGTTACCACGATGAAGAGAGTTTGCATAGAGAGACCTAAATCCTAT	780
21-256	ATGGTGGTGAGCGCGTTACCACGATGAAGAGAGTTTGCATAGAGAGACCTAAATCCTAT	780
21_024	ATGGTGGTGAGCGCGTTACCACGATGAAGAGAGTTTGCATAGAGAGACCTAAATCCTAT	780
20-021	ATGGTGGTGAGCGCGTTACCACGATGAAGAGAGTTTGCATAGAGAGACCTAAATCCTAT	780
BBRO21-BChV	ATGGTGGTGAGCGCGTTACCACGATGAAGAGAGTTTGCATAGAGAGACCTAAATCCTAT	780
21-039	ATGGTGGTGAGCGCGTTACCACGATGAAGAGAGTTTGCATAGAGAGACCTAAATCCTAT	780
21-020	ATGGTGGTGAGCGCGTTACCACGATGAAGAGAGTTTGCATAGAGAGACCTAAATCCTAT	780
19_015	ATGGTGGTGAGCGCGTTACCACGATGAAGAGAGTTTGCATAGAGAGACCTAAATCCTAT	780
21-196	ATGGTGGTGAGCGCGTTACCACGATGAAGAGAGTTTGCATAGAGAGACCTAAATCCTAT	780
21-187	ATGGTGGTGAGCGCGTTACCACGATGAAGAGAGTTTGCATAGAGAGACCTAAATCCTAT	780
21-056	ATGGTGGTGAGCGCGTTACCACGATGAAGAGAGTTTGCATAGAGAGACCTAAATCCTAT	780
21_290	ATGGTGGTGAGCGCGTTACCACGATGAAGAGAGTTTGCATAGAGAGACCTAAATCCTAT	780
21-121	ATGGTGGTGAGCGCGTTACCACGATGAAGAGAGTTTGCATAGAGAGACCTAAATCCTAT	780
19_005	ATGGTGGTGAGCGCGTTACCACGATGAAGAGAGTTTGCATAGAGAGACCTAAATCCTAT	780
21-294	ATGGTGGTGAGCGCGTTACCACGATGAAGAGAGTTTGCATAGAGAGACCTAAATCCTAT	780
21-179	ATGGTGGTGAGCGCGTTACCACGATGAAGAGAGTTTGCATAGAGAGACCTAAATCCTAT	780
20-009	ATGGTGGTGAGCGCGTTACCACGATGAAGAGAGTTTGCATAGAGAGACCTAAATCCTAT	780
21-071	ATGGTGGTGAGCGCGTTACCACGATGAAGAGAGTTTGCATAGAGAGACCTAAATCCTAT	780
20-018-2	ATGGTGGTGAGCGCGTTACCACGATGAAGAGAGTTTGCATAGAGAGACCTAAATCCTAT	780
20-014	ATGGTGGTGAGCGCGTTACCACGATGAAGAGAGTTTGCATAGAGAGACCTAAATCCTAT	780
21_278	ATGGTGGTGAGCGCGTTACCACGATGAAGAGAGTTTGCATAGAGAGACCTAAATCCTAT	780
21-111	ATGGTGGTGAGCGCGTTACCACGATGAAGAGAGTTTGCATAGAGAGACCTAAATCCTAT	780
20-016	ATGGTGGTGAGCGCGTTACCACGATGAAGAGAGTTTGCATAGAGAGACCTAAATCCTAT	780
20-015	ATGGTGGTGAGCGCGTTACCACGATGAAGAGAGTTTGCATAGAGAGACCTAAATCCTAT	780
20-012	ATGGTGGTGAGCGCGTTACCACGATGAAGAGAGTTTGCATAGAGAGACCTAAATCCTAT	780
19_011	ATGGTGGTGAGCGCGTTACCACGATGAAGAGAGTTTGCATAGAGAGACCTAAATCCTAT	780
19-002	ATGGTGGTGAGCGCGTTACCACGATGAAGAGAGTTTGCATAGAGAGACCTAAATCCTAT	780
19_010	ATGGTGGTGAGCGCGTTACCACGATGAAGAGAGTTTGCATAGAGAGACCTAAATCCTAT	780
20-017	ATGGTGGTGAGCGCGTTACCACGATGAAGAGAGTTTGCATAGAGAGACCTAAATCCTAT	780
20-018-1	ATGGTGGTGAGCGCGTTACCACGATGAAGAGAGTTTGCATAGAGAGACCTAAATCCTAT *****	780
19_016	GTA AAAAGAGTGTGCAGTCCGGGGGTTCACTACATGGGCTGTACCAATGAAACCCCCAAAG	840
21-256	GTA AAAAGAGTGTGCAGTCCGGGGGTTCACTACATGGGCTGTACCAATGAAACCCCCAAAG	840
21_024	GTA AAAAGAGTGTGCAGTCCGGGGGTTCACTACATGGGCTGTACCAATGAAACCCCCAAAG	840
20-021	GTA AAAAGAGTGTGCAGTCCGGGGGTTCACTACATGGGCTGTACCAATGAAACCCCCAAAG	840
BBRO21-BChV	GTA AAAAGAGTGTGCAGTCCGGGGGTTCACTACATGGGCTGTACCAATGAAACCCCCAAAG	840
21-039	GTA AAAAGAGTGTGCAGTCCGGGGGTTCACTACATGGGCTGTACCAATGAAACCCCCAAAG	840
21-020	GTA AAAAGAGTGTGCAGTCCGGGGGTTCACTACATGGGCTGTACCAATGAAACCCCCAAAG	840
19_015	GTA AAAAGAGTGTGCAGTCCGGGGGTTCACTACATGGGCTGTACCAATGAAACCCCCAAAG	840
21-196	GTA AAAAGAGTGTGCAGTCCGGGGGTTCACTACATGGGCTGTACCAATGAAACCCCCAAAG	840
21-187	GTA AAAAGAGTGTGCAGTCCGGGGGTTCACTACATGGGCTGTACCAATGAAACCCCCAAAG	840
21-056	GTA AAAAGAGTGTGCAGTCCGGGGGTTCACTACATGGGCTGTACCAATGAAACCCCCAAAG	840
21_290	GTA AAAAGAGTGTGCAGTCCGGGGGTTCACTACATGGGCTGTACCAATGAAACCCCCAAAG	840
21-121	GTA AAAAGAGTGTGCAGTCCGGGGGTTCACTACATGGGCTGTACCAATGAAACCCCCAAAG	840
19_005	GTA AAAAGAGTGTGCAGTCCGGGGGTTCACTACATGGGCTGTACCAATGAAACCCCCAAAG	840
21-294	GTA AAAAGAGTGTGCAGTCCGGGGGTTCACTACATGGGCTGTACCAATGAAACCCCCAAAG	840
21-179	GTA AAAAGAGTGTGCAGTCCGGGGGTTCACTACATGGGCTGTACCAATGAAACCCCCAAAG	840
20-009	GTA AAAAGAGTGTGCAGTCCGGGGGTTCACTACATGGGCTGTACCAATGAAACCCCCAAAG	840
21-071	GTA AAAAGAGTGTGCAGTCCGGGGGTTCACTACATGGGCTGTACCAATGAAACCCCCAAAG	840
20-018-2	GTA AAAAGAGTGTGCAGTCCGGGGGTTCACTACATGGGCTGTACCAATGAAACCCCCAAAG	840
20-014	GTA AAAAGAGTGTGCAGTCCGGGGGTTCACTACATGGGCTGTACCAATGAAACCCCCAAAG	840
21_278	GTA AAAAGAGTGTGCAGTCCGGGGGTTCACTACATGGGCTGTACCAATGAAACCCCCAAAG	840
21-111	GTA AAAAGAGTGTGCAGTCCGGGGGTTCACTACATGGGCTGTACCAATGAAACCCCCAAAG	840
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19_011	GTA AAAAGAGTGTGCAGTCCGGGGGTTCACTACATGGGCTGTACCAATGAAACCCCCAAAG	840
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19_010	GTA AAAAGAGTGTGCAGTCCGGGGGTTCACTACATGGGCTGTACCAATGAAACCCCCAAAG	840
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20-018-1	GTA AAAAGAGTGTGCAGTCCGGGGGTTCACTACATGGGCTGTACCAATGAAACCCCCAAAG *****	840
19_016	AATTCGATTTTGCTCATAAGTCATGACGACGGTTACACGCAGGTTATGCAACTGCGTG	900
21-256	AATTCGATTTTGCTCATAAGTCATGACGACGGTTACACGCAGGTTATGCAACTGCGTG	900





20-017	GTCATGGGATGCAAAGCCTTAAACTTGTCTACTAGAGACAGCTTGGCGAAGGGGCCAGCC	1140
20-018-1	GTCATGGGATGCAAAGCCTTAAACTTGTCTACTAGAGACAGCTTGGCGAAGGGGCCAGCC *****	1140
19_016	ACCATTTACACTTTTGGTGATAATGGATGGACTCTTTCACAAGCCTCGATCAGTGGCGCA	1200
21-256	ACTATTTACACTTTTGGTGATAATGGATGGACTCTTTCACAAGCCTCGATTAGTGGCGCA	1200
21_024	ACCATTTACACTTTTGGTGATAATGGATGGACTCTTTCACAAGCCTCGATTAGTGGCGCA	1200
20-021	ACCATTTACACTTTTGGTGATAATGGATGGACTCTTTCACAAGCCTCGATTAGTGGCGCA	1200
BBRO21-BChV	ACCATTTACACTTTTGGTGATAATGGATGGACTCTTTCACAAGCCTCGATTAGTGGCGCA	1200
21-039	ACCATTTACACTTTTGGTGATAATGGATGGACTCTTTCACAAGCCTCGATTAGTGGCGCA	1200
21-020	ACCATTTACACTTTTGGTGATAATGGATGGACTCTTTCACAAGCCTCGATTAGTGGCGCA	1200
19_015	ACCATTTACACTTTTGGTGATAATGGATGGACTCTTTCACAAGCCTCGATTAGTGGCGCA	1200
21-196	ACCATTTACACTTTTGGTGATAATGGATGGACTCTTTCACAAGCCTCGATTAGTGGCGCA	1200
21-187	ACCATTTACACTTTTGGTGATAATGGATGGACTCTTTCACAAGCCTCGATTAGTGGCGCA	1200
21-056	ACCATTTACACTTTTGGTGATAATGGATGGACTCTTTCACAAGCCTCGATTAGTGGCGCA	1200
21_290	ACCATTTACACTTTTGGTGATAATGGATGGACTCTTTCACAAGCCTCGATTAGTGGCGCA	1200
21-121	ACCATTTACACTTTTGGTGATAATGGATGGACTCTTTCACAAGCCTCGATTAGTGGCGCA	1200
19_005	ACCATTTACACTTTTGGTGATAATGGATGGACTCTTTCACAAGCCTCGATTAGTGGCGCA	1200
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21-179	ACCATTTACACTTTTGGTGATAATGGATGGACTCTTTCACAAGCCTCGATTAGTGGCGCA	1200
20-009	ACCATTTACACTTTTGGTGATAATGGATGGACTCTTTCACAAGCCTCGATTAGTGGCGCA	1200
21-071	ACTATTTACACTTTTGGTGATAATGGATGGACTCTTTCACAAGCCTCGATTAGTGGCGCA	1200
20-018-2	ACCATTTACACTTTTGGTGATAATGGATGGACTCTTTCACAAGCCTCGATTAGTGGCGCA	1200
20-014	ACCATTTACACTTTTGGTGATAATGGATGGACTCTTTCACAAGCCTCGATTAGTGGCGCA	1200
21_278	ACCATTTACACTTTTGGTGATAATGGATGGACTCTTTCACAAGCCTCGATTAGTGGCGCA	1200
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19_011	ACCATTTACACTTTTGGTGATAATGGATGGACTCTTTCACAAGCCTCGATTAGTGGCGCA	1200
19-002	ACCATTTACACTTTTGGTGATAATGGATGGACTCTTTCACAAGCCTCGATTAGTGGCGCA	1200
19_010	ACCATTTACACTTTTGGTGATAATGGATGGACTCTTTCACAAGCCTCGATTAGTGGCGCA	1200
20-017	ACCATTTACACTTTTGGTGATAATGGATGGACTCTTTCACAAGCCTCGATTAGTGGCGCA	1200
20-018-1	ACCATTTACACTTTTGGTGATAATGGATGGACTCTTTCACAAGCCTCGATTAGTGGCGCA ** *****	1200
19_016	TACGATAAAAAATAAAGCATCCGTAAGTACTGAGCATAAACCATAAAGGGCATAAGTGGAGCCCCG	1260
21-256	TACGATAAAAAATAAAGCATCCGTAAGTACTGAGCATAAACCATAAAGGGCATAAGTGGAGCCCCG	1260
21_024	TACGATAAAAAATAAAGCATCCGTAAGTACTGAGCATAAACCATAAAGGGCATAAGTGGAGCCCCG	1260
20-021	TACGATAAAAAATAAAGCATCCGTAAGTACTGAGCATAAACCATAAAGGGCATAAGTGGAGCCCCG	1260
BBRO21-BChV	TACGATAAAAAATAAAGCATCCGTAAGTACTGAGCATAAACCATAAAGGGCATAAGTGGAGCCCCG	1260
21-039	TACGATAAAAAATAAAGCATCCGTAAGTACTGAGCATAAACCATAAAGGGCATAAGTGGAGCCCCG	1260
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19_015	TACGATAAAAAATAAAGCATCCGTAAGTACTGAGCATAAACCATAAAGGGCATAAGTGGAGCCCCG	1260
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21-187	TACGATAAAAAATAAAGCATCCGTAAGTACTGAGCATAAACCATAAAGGGCATAAGTGGAGCCCCG	1260
21-056	TACGATAAAAAATAAAGCATCCGTAAGTACTGAGCATAAACCATAAAGGGCATAAGTGGAGCCCCG	1260
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19_005	TACGATAAAAAATAAAGCATCCGTAAGTACTGAGCATAAACCATAAAGGGCATAAGTGGAGCCCCG	1260
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20-018-2	TACGATAAAAAATAAAGCATCCGTAAGTACTGAGCATAAACCATAAAGGGCATAAGTGGAGCCCCG	1260
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19_016	TACATTTAGTGGCAAAAACGTCATAGGAATCCACTCAGGAGGAGACGTCGTAGACAATGTG	1320
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21_024	TACATTTAGTGGCAAAAACGTCATAGGAATCCACTCAGGAGGAGACGTCGTAGACAATGTG	1320
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21-039	TACATTTAGTGGCAAAAACGTCATAGGAATCCACTCAGGAGGAGACGTCGTAGACAATGTG	1320
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19_015	TACATTTAGTGGCAAAAACGTCATAGGAATCCACTCAGGAGGAGACGTCGTAGACAATGTG	1320
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20-012	GTTTTGACTGGAGTGTAGCGGACTGGATGCTCGAAGATGACATGGAAGTCCCGCAATCGCT	2880
19_011	GTTTTGACTGGAGTGTAGCGGACTGGATGCTCGAAGATGACATGGAAGTCCCGCAATCGCT	2880
19-002	GTTTTGACTGGAGTGTAGCGGACTGGATGCTCGAAGATGACATGGAAGTCCCGCAATCGCT	2880
19_010	GTTTTGACTGGAGTGTAGCGGACTGGATGCTCGAAGATGACATGGAAGTCCCGCAATCGCT	2880
20-017	GTTTTGACTGGAGTGTAGCGGACTGGATGCTCGAAGATGACATGGAAGTCCCGCAATCGCT	2880
20-018-1	GTTTTGACTGGAGTGTAGCGGACTGGATGCTCGAAGATGACATGGAAGTCCCGCAATCGCT *****	2880
19_016	TGACCAGAAACAACAACCACACAACAAAACGATTACGATCAGTGTGGTTAAAAATGCATAA	2940
21-256	TGACCAGAAACAATAAACCACACAACAAAACGATTACGATCAGTGTGGTTAAAAATGCATAA	2940
21_024	TGACCAGAAACAACAACCACACAACAAAACGATTACGATCAGTGTGGTTAAAAATGCATAA	2940
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21-187	TGACCAGAAACAACAACCACACAACAAAACGATTACGATCAGTGTGGTTAAAAATGCATAA	2940
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21_290	TGACCAGAAACAACAACCACACAACAAAACGATTACGATCAGTGTGGTTAAAAATGCATAA	2940
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19_005	TGACCAGAAACAACAACCACACAACAAAACGATTACGATCAGTGTGGTTAAAAATGCATAA	2940
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21-179	TGACCAGAAACAACAACCACACAACAAAACGATTACGATCAGTGTGGTTAAAAATGCATAA	2940
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19_010	TGACCAGAAACAACAACCACACAACAAAACGATTACGATCAGTGTGGTTAAAAATGCATAA	2940
20-017	TGACCAGAAACAACAACCACACAACAAAACGATTACGATCAGTGTGGTTAAAAATGCATAA	2940
20-018-1	TGACCAGAAACAACAACCACACAACAAAACGATTACGATCAGTGTGGTTAAAAATGCATAA *****	2940
19_016	GCAATTCAGTCTTATGCCTTTCTGATGGGTGCCTTCTTTCACAGAGAGTGCCTGGAGTTC	3000
21-256	GCAATTCAGTCTTATGCCTTTCTGATGGGCGCCTTCTTTCACAGAGAGTGCCTGGAGTTC	3000
21_024	GCAATTCAGTCTTATGCCTTTCTGATGGGTGCCTTCTTTCACAGAGAGTGCCTGGAGTTC	3000
20-021	GCAATTCAGTCTTATGCCTTTCTGATGGGTGCCTTCTTTCACAGAGAGTGCCTGGAGTTC	3000
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21-039	GCAATTCAGTCTTATGCCTTTCTGATGGGTGCCTTCTTTCACAGAGAGTGCCTGGAGTTC	3000
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19_015	GCAATTCAGTCTTATGCCTTTCTGATGGGTGCCTTCTTTCACAGAGAGTGCCTGGAGTTC	3000
21-196	GCAATTCAGTCTTATGCCTTTCTGATGGGTGCCTTCTTTCACAGAGAGTGCCTGGAGTTC	3000
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21_290	GCAATTCAGTCTTATGCCTTTCTGATGGGTGCCTTCTTTCACAGAGAGTGCCTGGAGTTC	3000
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21-179	GCAATTCAGTCTTATGCCTTTCTGATGGGTGCCTTCTTTCACAGAGAGTGCCTGGAGTTC	3000
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21-071	GCAATTCAGTCTTATGCCTTTCTGATGGGTGCCTTCTTTCACAGAGAGTGCCTGGAGTTC	3000
20-018-2	GCAATTCAGTCTTATGCCTTTCTGATGGGTGCCTTCTTTCACAGAGAGTGCCTGGAGTTC	3000
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21_278	GCAATTCAGTCTTATGCCTTTCTGATGGGTGCCTTCTTTCACAGAGAGTGCCTGGAGTTC	3000
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19_010	GCAATTCAGTCTTATGCCTTTCTGATGGGTGCCTTCTTTCACAGAGAGTGCCTGGAGTTC	3000
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20-018-1	GCAATTCAGTCTTATGCCTTTCTGATGGGTGCCTTCTTTCACAGAGAGTGCCTGGAGTTC * *****	3000
19_016	AGAAAAGTGAAGTTACAATACTTCTTCTTCAAACCTCCCGTATCAGAGTTATGGCTGCAT	3060
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21_024	AGAAAAGTGAAGTTACAATACTTCTTCTTCAAACCTCCCGTATCAGAGTTATGGCTGCAT	3060
20-021	AGAAAAGTGAAGTTACAATACTTCTTCTTCAAACCTCCCGTATCAGAGTTATGGCTGCAT	3060
BBRO21-BChV	AGAAAAGTGAAGTTACAATACTTCTTCTTCAAACCTCCCGTATCAGAGTTATGGCTGCAT	3060





































21-111 TTGGTTGAATCGTGGAGACCTGATGTGAACCCCGGATATTC AAGGAAGACGTGGCAGCC 5340  
 20-016 TTGGTTGAATCGTGGAGACCTGATGTGAACCCCGGATATTC AAGGAAGACGTGGCAGCC 5340  
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 20-017 TTGGTTGAATCGTGGAGACCTGATGTGAACCCCGGATATTC AAGGAAGACGTGGCAGCC 5340  
 20-018-1 TTGGTTGAATCGTGGAGACCTGATGTGAACCCCGGATATTC AAGGAAGACGTGGCAGCC 5340

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 20-018-2 GCCACTATTCTTTATGGAGTTCTATCAAAGACGGCCGGTCTATGATCGATAAACCGGAT 5400  
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 21-256 AAAGCTGTGTTAGACGGCCGTAAGCATTGGGGTTCTTCCTTAGCTTCCTCCTTAACAGGA 5460  
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 BBRO21-BChV AAAGCTGTGTTAGACGGCCGTAAGCATTGGGGTTCTTCCTTAGCTTCCTCCTTAACAGGA 5460  
 21-039 AAAGCTGTGTTAGACGGCCGTAAGCATTGGGGTTCTTCCTTAGCTTCCTCCTTAACAGGA 5460  
 21-020 AAAGCTGTGTTAGACGGCCGTAAGCATTGGGGTTCTTCCTTAGCTTCCTCCTTAACAGGA 5460  
 19\_015 AAAGCTGTGTTAGACGGCCGTAAGCATTGGGGTTCTTCCTTAGCTTCCTCCTTAACAGGA 5460  
 21-196 AAAGCTGTGTTAGACGGCCGTAAGCATTGGGGTTCTTCCTTAGCTTCCTCCTTAACAGGA 5460  
 21-187 AAAGCTGTGTTAGACGGCCGTAAGCATTGGGGTTCTTCCTTAGCTTCCTCCTTAACAGGA 5460  
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 21\_290 AAAGCTGTGTTAGACGGCCGTAAGCATTGGGGTTCTTCCTTAGCTTCCTCCTTAACAGGA 5460  
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 19\_005 AAAGCTGTGTTAGACGGCCGTAAGCATTGGGGTTCTTCCTTAGCTTCCTCCTTAACAGGA 5460  
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 21-179 AAAGCTGTGTTAGACGGCCGTAAGCATTGGGGTTCTTCCTTAGCTTCCTCCTTAACAGGA 5460  
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 21\_278 AAAGCTGTGTTAGACGGCCGTAAGCATTGGGGTTCTTCCTTAGCTTCCTCCTTAACAGGA 5460  
 21-111 AAAGCTGTGTTAGACGGCCGTAAGCATTGGGGTTCTTCCTTAGCTTCCTCCTTAACAGGA 5460  
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 19\_010 AAAGCTGTGTTAGACGGCCGTAAGCATTGGGGTTCTTCCTTAGCTTCCTCCTTAACAGGA 5460  
 20-017 AAAGCTGTGTTAGACGGCCGTAAGCATTGGGGTTCTTCCTTAGCTTCCTCCTTAACAGGA 5460  
 20-018-1 AAAGCTGTGTTAGACGGCCGTAAGCATTGGGGTTCTTCCTTAGCTTCCTCCTTAACAGGA 5460

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19\_016 GGAACGCTTAAGCCTCTGCAAAGTCGGAGAAGCTTGCTAAACTCACCTCGCGTGAGAGG 5520  
 21-256 GGAACGCTTAAGCCTCTGCAAAGTCGGAGAAGCTTGCTAAACTCACCTCGCGTGAGAGG 5520





20-017	GACTCCCGAAAGGATAGGCACGAATGTTCCCCTTATTTAAAGGGTTATACAGTAGGATCC	5760
20-018-1	GACTCCCGAAAGGATAGGCACGAATGTTCCCCTTATTTAAAGGGTTATACAGTAGGATCC	5760
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19_016	TACGGCACTTCGGTGT	5776
21-256	TACGGCACTTCGGTGT	5776
21_024	TACGGCACTTCGGTGT	5776
20-021	TACGGCACTTCGGTGT	5776
BBRO21-BChV	TACGGCACTTCGGTGT	5776
21-039	TACGGCACTTCGGTGT	5776
21-020	TACGGCACTTCGGTGT	5776
19_015	TACGGCACTTCGGTGT	5776
21-196	TACGGCACTTCGGTGT	5776
21-187	TACGGCACTTCGGTGT	5776
21-056	TACGGCACTTCGGTGT	5776
21_290	TACGGCACTTCGGTGT	5776
21-121	TACGGCACTTCGGTGT	5776
19_005	TACGGCACTTCGGTGT	5776
21-294	TACGGCACTTCGGTGT	5776
21-179	TACGGCACTTCGGTGT	5776
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21-071	TACGGCACTTCGGTGT	5776
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21-111	TACGGCACTTCGGTGT	5776
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19-002	TACGGCACTTCGGTGT	5776
19_010	TACGGCACTTCGGTGT	5776
20-017	TACGGCACTTCGGTGT	5776
20-018-1	TACGGCACTTCGGTGT	5776
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