Cryptic viruses in Black-grass: Investigating their role in plant stress tolerance

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Abstract

Cryptic viruses are a group of persistent plant viruses characterised by a lack of disease symptoms, very low virus titer and lifelong persistence in individual hosts. These characteristics highlight a very close relationship between cryptic viruses and their hosts and so, our main hypothesis is that they are mutualistic symbionts of their hosts. Our main aim was to study the effect of viral cryptic infections on the tolerance of plants to abiotic stress. We worked with three cryptic viruses recently discovered in black-grass (*Alopecurus myosuroides*) populations: *Alopecurus myosuroides* partitivirus 1 (AMPV1), *Alopecurus myosuroides* partitivirus 2 (AMPV2) and *Alopecurus myosuroides varicosavirus* 1 (AMVV1). We started by characterizing these viruses. AMPV1 and AMPV2 are widespread in the studied populations and vertically transmitted. The titer of each virus varies across a wide range, both between and inside all tested populations. This increases population plasticity, the possible negative and positive effects of the viruses being distributed at different “strength levels” across the populations, increasing its survival potential against changing environmental conditions, and thus, potentially improving population fitness. AMVV1 shows variable incidence and titer in the populations and vertical transmission. It is possible that this variability might be due to different levels of resistance to this virus or/and to the efficiency of the transmission. Bioassays were carried out to analyse the viral effect on plant tolerance to drought stress. Although no significant effect on tolerance was observed, different trends were associated with each virus. AMVV1 appears to act as a conditional mutualist, hindering growth under normal conditions but alleviating these negative effects under stress. AMPV1 affects the growth pattern of its host regardless of the environmental conditions, favouring the development of short plants with many tillers. This could increase the plant’s fitness as it might increase its competitiveness in grassland. In contrast, AMPV2 acts as an antagonist, negatively affecting the growth of its host, an effect that is exacerbated under stress.

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List of abbreviations and acronyms

ACV	*Alfalfa cryptic virus*
AgMV	*Agropyron mosaic virus*
AMPV1	*Alopecurus myosuroides partitivirus 1*
AMPV2	*Alopecurus myosuroides partitivirus 2*
AMVV1	*Alopecurus myosuroides varicosavirus 1*
BCV	*Beet cryptic virus*
BMV	*Brome mosaic virus*
BSV	*Banana streak virus*
BYDV	*Barley yellow dwarf virus*
BYV	*Beet yellows virus*
CaMV	*Cauliflower mosaic virus*
CarCV	*Carnation cryptic virus*
CfCP	*Cocksfoot mottle virus*
CHV-1	*Cryphonectria hypovirus 1*
CMV	*Cucumber mosaic virus*
CP	Coat protein
CPV	*Cryptosporidium parvum virus*
CSV	*Cocksfoot streak virus*
CThTV	*Curvularia thermal tolerance virus*
DNA	Deoxyribonucleic acid
dsDNA	Double-stranded DNA viruses
dsRNA	Double-stranded RNA viruses
DW	Dry weight sample
ELISA	Enzyme-linked immunosorbent assay
EM	Electron microscope
FDW	Freeze-dry weight sample
FW	Fresh weight sample
GBV-C	*Hepatitis G virus C*
HDA	Helicase-dependent amplification
HIV	*Human immunodeficiency virus*
H-PV1	High AMPV1 titer group
H-PV2	High AMPV2 titer group
HSV	*Herpes simplex virus*

H-VV1	High AMVV1 titer group
ICTV	International committee on taxonomy of viruses
LAMP	Loop-mediated isothermal amplification
LBVaV	*Lettuce big vein associated virus*
LFIA	Lateral flow immunoassay
LOD	Limit of detection
LBVD	Lettuce big vein disease
LYSV	*Leek yellow stripe virus*
MLBVV	*Mirafiori lettuce big-vein virus*
MS	Murashige & Skoog media
NGS	Next-generation sequencing
NTSR	Non-target site resistance
OYDV	*Onion yellow dwarf virus*
PCR	Polymerase chain reaction
PCV-1	*Pepper cryptic virus 1*
PVX	*Potato virus X*
qPCR	Quantitative PCR
RCA	Rolling circle amplification
RCaVV	*Red clover associated varicosavirus*
RCV	*Ryegrass cryptic virus*
RdRP	RNA-dependent RNA polymerase
RgMV	*Ryegrass mosaic virus*
RIA	Radioimmunoassay
RNA	Ribonucleic acid
RPA	Recombinase polymerase amplification
RT-PCR	Reverse transcription PCR
RT-qPCR	Reverse Transcription qPCR
TMV	*Tobacco mosaic virus*
TRV	*Tobacco rattle virus*
TSR	Target site-based resistance
TStV	*Tobacco stunt virus*
TuMV	*Turnip mosaic virus*
VCV	*Vicia cryptic virus*
WCCV1	*White clover cryptic virus 1*

Chapter 1: Introduction

1.1 Virus

1.1.1 Definition and characteristics

A virus is an obligate intracellular parasite that uses cellular systems for its own replication (Hull, 2009; Villareal, 2005; Cann, 2012; Modrow *et al.*, 2013; Gaur *et al.*, 2016).

Viruses do not code for their own ribosomes, cell's protein-synthesis machinery, nor metabolic pathways for energy production. Therefore, they are entirely dependent on those of their host. The lack of ribosomes is one of the main features that distinguishes viruses from the other domains of life (Hull, 2009; Cann, 2012; Modrow *et al*., 2013; Gaur *et al*., 2016).

Virus particles or virions are composed of nucleic acid, which is the infectious element, and a protein coat or capsid that envelops it, acting as a protective barrier and having cell recognition and binding functions. Some viruses have an extra lipid layer surrounding the capsid, known as envelope and derived from the host's cell membrane. Virions contain only one type of nucleic acid, DNA or RNA, that can be single- or double-stranded. This nucleic acid can code for several different genes depending on the virus species, but, fundamentally, most viruses code for a polymerase and coat protein (CP). Unlike cells, virions do not grow or multiply by division; the different constituents of the viral particle are synthetized independently and assembled spontaneously, forming new virions. During viral replication, there is no membrane separation between the virus and its host's cell contents (Hull, 2009; Cann, 2012; Modrow *et al*., 2013; Gaur *et al*., 2016).

Viruses display high rates of genetic variability due to the high frequency of mutations during the replication process, as well as to recombination events, loss or acquisition of genetic material, etc. This results in the production of high numbers of genetic variants for one virus, giving rise to the concept of quasi-species (Hull, 2009; Gaur *et al*., 2016). Roger Hull (2009) defines a quasi-species as "a population structure in which collections of closely related genomes are subjected to a continuous process of genetic variation, competition, and selection". As other organisms, viruses have adapted to specific habitats and hosts (Villareal, 2005; Modrow *et al*., 2013).

1.1.2 Virus-like or subviral agents.

The so-called virus-like agents comprise entities that are more similar to viruses than to any other organisms and yet, do not exactly fit within the above definition and characteristics (Hull, 2009; Cann, 2012; Modrow *et al*., 2013). Three subviral agents can be distinguished: satellite viruses, viroids and prions.

Satellite viruses are dependent on the presence of a helper virus for their replication, as they do not code for a polymerase. Because they essentially hijack the transcriptional machinery of their helper viruses, satellite viruses can interfere, to variable degrees, with their replication, sometimes reducing disease symptoms. Nevertheless, satellite viruses can cause distinct disease symptoms, resulting in a more virulent disease when added to those of the helper virus. They measure between 300 to 1000 nucleotides and code for their own CPs (Hull, 2009; Cann, 2012; Modrow *et al*., 2013).

Viroids are small, 200-400 nucleotides, circular RNA molecules with a high degree of secondary structure found in plants. They are considered the smallest known self-replicating genetic unit. They do not code for any protein, depending on the host's cellular RNA polymerase for their replication. While they do not possess any kind of protective coat, their small size and secondary structure allows them to persist in the environment enough time to be transmitted to a new host (Hull, 2009; Cann, 2012; Modrow *et al*., 2013).

Prions, from "proteinaceous infectious particles", are infectious protein molecules with no nucleic acid component, responsible for neurodegenerative diseases. Prions are endogenous proteins whose pathology is caused by the change from the non-pathological prion protein isoform (PrPC), in a α-helical conformation, to the pathological isoform (PrPSc), in a β-sheet conformation. Prion transmission is limited but can happen both in an intra- and inter-species way, the latter being even rarer due to species barrier (Cann, 2012; Modrow *et al*., 2013) While no plant-infecting prions have been described, Pritzkow *et al.* (2015) found that plants were able to act as vectors for animal-infecting prions.

1.2 Historical overview

Tobacco mosaic virus (TMV) was the first virus to be described and studied, being fundamental to the establishment of viruses as a distinct infectious entity. Tobacco mosaic disease was first described by Adolf Mayer in 1882 (Bos, 1999; Hull, 2009; Cann, 2012); he demonstrated the sap's infectivity and excluded both fungus and

bacteria as the causal agents of the disease, instead speculating with the idea of a "soluble, enzyme-like *contagium"* as the agent. However, in his 1886 paper on tobacco mosaic disease, he stated that the latter had a bacterial origin which had yet to be isolated. In 1892, Dimitrii Ivanovsky demonstrated that the sap from infected tobacco plants remained infectious after passage through a Chamberland filter, at the time believed to retain all microorganisms, thus, describing the first "filterable infectious agent" (Hull, 2009; Cann, 2012; Gaur *et al*., 2016). Yet, this agent was still believed to be a toxin or microbe. It was not until 1898 that Martinus Beijerinck confirmed Ivanovsky's experiments and studied in detail the infectious agent characteristics (Bos, 1999; Hull, 2009; Cann, 2012). In particular, he noted that this agent multiplied inside the plants, refuting the toxin hypothesis. He concluded that the disease was due to a non-corpuscular entity which he named "*contagium vivum fluidum",* in opposition to "*contagium fixum"* which was used for microbes. And described it as an autonomous self-replicating entity dependent on an actively metabolizing host and fundamentally different from microorganisms known at the time (Bos, 1999; Villareal, 2005; Hull, 2009; Cann, 2012; Modrow *et al*., 2013; Gaur *et al*., 2016).

Soon after, other similar disease agents were described: between 1897 and 1898, Friedrich Loeffler and Paul Frosh described the causal agent of foot-and-mouth disease in cattle, considered the first report of an animal virus. In 1900, Walter Reed described the yellow fever pathogenic agent and in 1901 its transmission by mosquitoes. Thus, the first virus of humans and the first virus vector were reported (Hull, 2009; Cann, 2012; Modrow *et al*., 2013). In 1915, Frederick Twort described the first viruses infecting bacteria. And in 1917, Felix d'Herelle, having independently arrived at the same observations as Twort, coined the term bacteriophage (Villareal, 2005; Cann, 2012; Modrow *et al*., 2013).

It is interesting to note that the term "virus", Latin for poison, was initially used to describe all microbes. Hence, the term "filterable virus" was adopted to differentiate the newly described viruses from bacteria. But with the growing number of reports on these agents, the term "filterable" was dropped and the term "virus" was accepted as their sole descriptor (Hull, 2009; Gaur *et al*., 2016).

From this point onward, viruses, and bacteriophages in particular, were used as models to unravel, not only their own nature and characteristics, but many of the molecular mechanisms that govern cells, being fundamental to the development of the DNA model and of molecular biology (Villareal, 2005; Hull, 2009; Cann, 2012; Modrow *et al*., 2013). Virus understanding was, and still is, highly dependent on the

development and improvement of techniques for their detection, identification, quantification and isolation (Cann, 2012; Modrow *et al*., 2013). At first, the only mode of virus detection was by means of their peculiarities: filterability, lack of growth on culture media and non-observable under the light microscope, as well as by inoculation and symptom observation in hosts (Gaur *et al*., 2016).

The first breakthrough came in 1928, when Hugh and Mary Maitland developed a simple tissue culture method for vaccinia using hen's kidney cells. But it was not until 1949 that Thomas Weller, Frederick Robbins and John Franklin Enders managed to grow and maintain poliovirus in human tissue cultures. These tissue culture methods allowed the development of plaque tests for virus quantification (Cann, 2012). In 1929, Francis O. Holmes introduced the first indicator plant bioassays, showing that local lesions produced by mechanical inoculation in certain host plants could serve as a quantitative assay for some viruses (Hull, 2009; Modrow *et al*., 2013; Scholthof, 2014). In 1935, Wendell Stanley reported the crystallisation of TMV, allowing for the first time the study of viral molecular structure (Hull, 2009; Cann, 2012; Modrow *et al*., 2013). The electron microscope (EM) was developed in the early 1930s by Ernst Ruska and the first commercial microscope was produced in 1938 (Big *et al*., 1956). EMs had a far higher magnification and resolution power than light microscopes and supported the study of a whole new range of samples. In the field of virology, its use allowed the detailed study of virion structure and facilitated virus identification and classification (Hull, 2009; Cann, 2012; Modrow *et al*., 2013; Gentile and Gelderblom, 2014). It was in 1938 that the first electron pictures of viruses were published by Helmut Ruska (von Borries, Ruska and Ruska, 1938), followed the next year by an in-depth study on TMV using the EM (Kausche, Pfankuch and Ruska, 1939),

One year later, in 1939, Max Delbrünck and Emory Ellis devised and carried their "one-step growth" experiment, demonstrating the basic replication cycle of viruses (Villareal, 2005; Cann, 2012). Studies on lysogeny started in the 1920s and led to the study of the induction of lysogenic bacteriophages in *Bacillus megaterium* by André Lwoff, Louis Siminovitch and Niels Kjeldgaard, who coined the term prophage in 1950 (Lwoff, 1952). These studies demonstrated the existence of different life strategies of viruses: lytic or virulent viruses, which replicate inside host cells until these are lysed and virions are released, and lysogenic or temperate viruses, which are integrated into the host genome and transmitted during cell division, remaining in a latent state until certain factors induce the transition from this lysogenic cycle to the lytic cycle (Lwoff, 1952; Villareal, 2005; Cann, 2012).

Salvador Luria and Alfred Hershey demonstrated in 1945 that phages mutated and therefore, that the genetic mechanisms of viruses had to be similar to those of cells (Hull, 2009; Cann, 2012; Modrow *et al*., 2013). And some years later, in 1952, Alfred Hershey and Martha Chase proved that DNA is the genetic material in bacteriophage T4, obtaining the first evidence of DNA as the molecular basis of inheritance (Villareal, 2005; Hull, 2009; Cann, 2012; Modrow *et al*., 2013). The next year, 1953, James Watson and Francis Crick presented their double helix DNA model (Watson and Crick, 1953). Shortly after, 1955, both Gerhard Schramm and Heinz Fraenkel-Conrat demonstrated independently that TMV infection was caused by RNA (Villareal, 2005; Hull, 2009; Cann, 2012; Modrow *et al*., 2013;). And in 1970, Howard Temin and David Baltimore discovered, independently, the reverse transcriptase in retroviruses. This discovery established the path followed by genetic information from RNA to DNA, refuting molecular biology's "central dogma" (Villareal, 2005; Hull, 2009; Cann, 2012; Modrow *et al*., 2013).

Another major technical breakthrough came hand in hand with the studies on immunity. In the 60s, the radioimmunoassay (RIA) was developed by Rosalyn Sussman Yalow, marking the first serological assay to be used in virus detection and quantification (Cann, 2012; Modrow *et al*., 2013). While this assay was highly sensitive and specific, the use of radioactive material and the requirements for greatly specialized equipment and staff limited its application. Nonetheless, other serological assays were developed and improved during the 60s and 70s, focusing on gel-based detection and later, on quantification, with ELISA being described in 1971 (Cann, 2012; Modrow *et al*., 2013; Gaur *et al*., 2016; Shukla, 2016). These techniques were more accessible than RIA and were readily implemented by virologists, as they offered rapid, sensitive and highly specific alternatives for virus identification and quantification (Shukla, 2016).

Around this time, sequencing methods were under development, culminating in 1977 with the first complete genome sequence, that of bacteriophage ΦX174, published by Sanger *et al.* (Cann, 2012; Heather and Chain, 2016). Sequencing has allowed the study of genome composition and regulation, gene functions and phylogenetic relationships, and the development of new techniques dependent on sequence knowledge such as the polymerase chain reaction (PCR) (Villareal, 2005; Hull, 2009). The invention of the PCR in 1983 is attributed to Kary Mullis (Gaur *et al*., 2016). It is based on the amplification of a specific DNA or RNA sequence and presents higher sensitivity and specificity than serological methods (Shukla, 2016). The later

development of real-time PCR supported even higher sensitivity and the possibility of quantification, as well as being less time-consuming than conventional PCR (Shukla, 2016). Both are widely used nowadays and new techniques, aiming to maintain and improve these levels of specificity and sensitivity while allowing for minimal sample extraction and *in-situ* detection and quantification, have recently been developed (Modrow *et al*., 2013; Gaur *et al*., 2016; Shukla, 2016).

This collection of techniques enabled the discovery and following study of mimiviruses in 2003 (la Scola *et al*., 2003), the first described giant virus family. These viruses are bigger, both in particle and in genome size, than some bacteria (la Scola *et al*., 2003; Sun *et al*., 2010; Modrow *et al*., 2013; Bekliz *et al.*, 2016; Colson *et al*., 2017). Following this initial discovery, other giant viruses have been described and, interestingly, a new group of viruses named virophages has been discovered to infect these giant viruses (Sun *et al*., 2010; Modrow *et al*., 2013; Bekliz *et al.*, 2016; Krupovic *et al*, 2016; Colson *et al*., 2017).

Additionally, the beginning of the 21st century saw the development of next-generation sequencing (NGS) technologies. NGS is comprised of several different high-throughput sequencing technologies, which can sequence all DNA and RNA sequences present in a sample (Loebenstein, 2009; Gaur *et al*., 2016; Shukla, 2016). It is currently used as a powerful tool for the rapid identification and discovery of new viral sequences in plant samples. This has revealed the preponderance in wild populations, as well as in crop species, of plant viruses with a persistent lifestyle which are not obviously linked to disease symptoms, commonly referred to as cryptic or persistent viruses (Shukla, 2016).

1.3 Virus classification

Several virus classification systems have historically been used. The first ones were based on the disease symptoms caused by viruses. But, as different non-related viruses can produce similar or identical symptoms and some viruses can be responsible for different diseases or no disease, this classification was dropped. With the invention of the EM, classification according to virion's morphological characteristics was tentatively proposed. However, some viral species can be morphologically identical; therefore, this classification was unsuccessful (Villareal, 2005; Cann, 2012; Gaur *et al*., 2016).

Nowadays, a universal classification based on an ample range of criteria is used. It was created and is regularly updated by the International Committee on Taxonomy

of Viruses (ICTV). The advent of sequencing technologies has helped rationalize taxonomy, and the characteristics and comparison of viral sequences are some of the main criteria currently used for virus classification (Villareal, 2005; Cann, 2012; Gaur *et al*., 2016; ICTV, 2017).

1.3.1 Baltimore classification

This system of virus classification was developed by David Baltimore in 1971 and is still used as a complement to the ICTV's classification. This system is based on the virus' type of nucleic acid (DNA or RNA, double- or single-stranded) and its replication strategy, with the focus on mRNA synthesis, named (+) mRNA (Baltimore, 1971; Cann, 2012; Gaur *et al*., 2016; Villareal, 2005). Following these criteria, viruses can be grouped in seven classes (Figure 1.1):

- Class I: dsDNA viruses (double-stranded DNA viruses).
- Class II: ssDNA viruses (single-stranded DNA viruses).
- Class III: dsRNA viruses (double-stranded RNA viruses).
- Class IV: (+) ssRNA viruses (single-stranded positive-sense RNA viruses).
- Class V: (-) ssRNA viruses (single-stranded negative-sense RNA viruses).
- Class VI: (+) ssRNA-RT viruses (single-stranded positive-sense RNA viruses with reverse transcription intermediates).
- Class VII: dsDNA-RT viruses (double-stranded DNA viruses with reverse transcription intermediates).

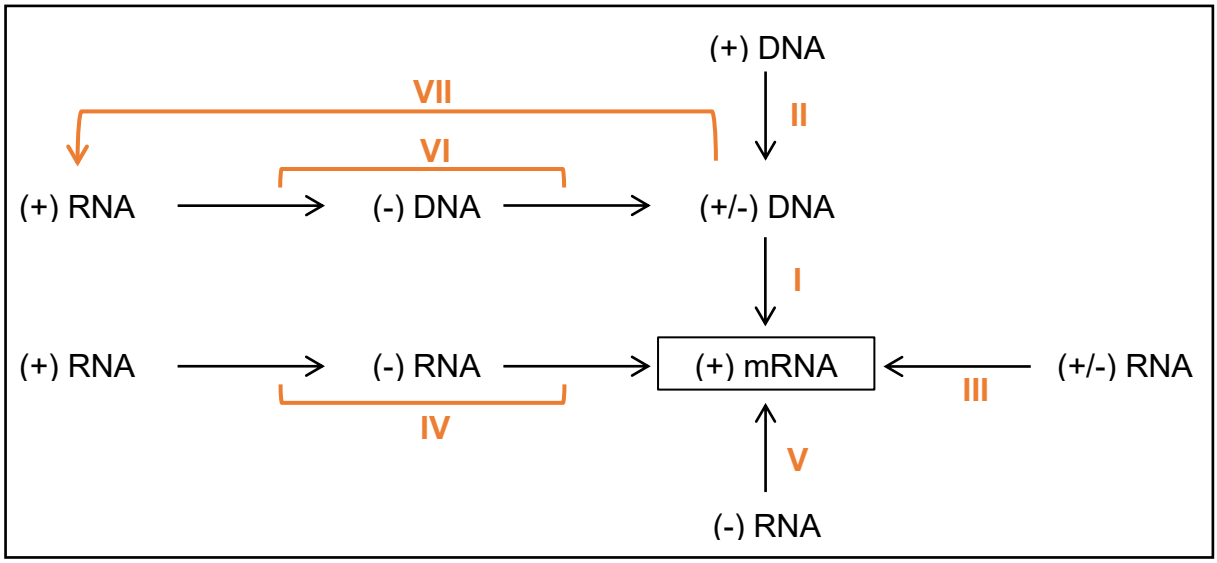

Figure 1.1: Schematic representation of the mRNA synthesis pathways followed by the viral groups defined in the Baltimore virus classification. "+/-" represents double stranded nucleic acid and "+" or "-" represent single stranded nucleic acid and its polarity, positive-sense and negative-sense respectively. Based on Figure 1 in Baltimore (1971).

1.3.2 ICTV classification

The ICTV was established in the 1966 International Congress for Microbiology with the aim to develop a uniform system of classification and nomenclature for viruses (Cann, 2012; Gaur *et al*., 2016; ICTV, 2017).

The current viral taxonomy follows a hierarchical system which includes the following levels: order, family, sub-family, genus and species. This system was introduced in the 7th ICTV Report, published in 1999, with the formalization of the concept of virus species. A virus species is defined by the ICTV (2017) as a "polythetic class of viruses that constitute a replicating lineage and occupy a particular ecological niche". A well characterised "type species" is chosen for each genus to serve as its model (Cann, 2012; Gaur *et al*., 2016; ICTV, 2017).

The main criteria used for classification are: virus biology (host range, epidemiology, and disease association), morphology, behaviour in cell culture, genome characteristics (genome organisation, replication strategies, etc.) and phylogenetic analyses (Modrow *et al*., 2013; Gaur *et al*., 2016; ICTV, 2017). The known virus taxa infecting plants are presented in Figure 1.2.

1.3.3 Viral life strategies

As noted before, viruses have different life strategies and they can be classified according to these.

Virulent viruses are viruses that produce acute infections, in which symptoms develop rapidly. Virulent viruses are linked to high levels of replication and production of viral progeny. In this case, the virus capacity to replicate is not maintained in an individual host, as it is limited by either the host's immune response or the host's death. Therefore, the virus must find a new host to continue its infection cycle. Transmission to other hosts is horizontal. They generally present no co-evolution with their host and high mutation rates, due to their high levels of replication, however the rate of mutational errors depends on the mode of replication, the nucleotide sequence context, and environmental factors. Usually, virulent viruses are able to infect different host species (Villareal, 2005; Hull, 2009; Cann, 2012).

Persistent viruses are characterised by their capacity to be maintained in an individual host while also maintaining their ability to be transmitted to new hosts. Transmission is vertical or through sexual contact. They are genetically stable and species specific (Villareal, 2005; Cann, 2012). We can distinguish three types of infection associated to these viruses: chronic, latent and persistent.

Chronic infection is similar to acute infections in that it causes disease, either chronic or recurrent, and is eventually cleared by either the host's immune system or the host's death. However, this type of infection persists for much longer time periods, usually for years. It is characterised by a continuous production of viral progeny. The *human immunodeficiency virus* (HIV) and the hepatitis B virus can both be considered examples of chronic infections (Cann, 2012).

In the case of latent infections, viral progeny production is episodic, presenting long time periods where the virus is virtually inactive, with no viral particles being created. Thus, disease symptoms caused by these viruses are also episodic. These infections persist for the entire life of the host (Bagasra *et al.*, 2012). *Herpes simplex virus* (HSV) in humans is a good example of a latent infection. Prophages can be considered to cause latent infections in bacteria. And in plants, the *banana streak virus* (BSV) is an example of a latent infection caused by the endogenization of the virus in the genome of banana, which can later be released as an active virus by different stresses (Roossinck, 2015c).

Persistent infection is characterised by an ongoing viral replication; generally, only a very small amount of viral progeny is produced, enough to ensure transmission to new hosts and avoid killing the host. Hence, most persistent infections are inapparent, causing no or very mild symptoms. As with latent infection, these viruses remain within a host for their entire lifetime (Cann, 2012). This is the type of infection associated to cryptic viruses in plants.

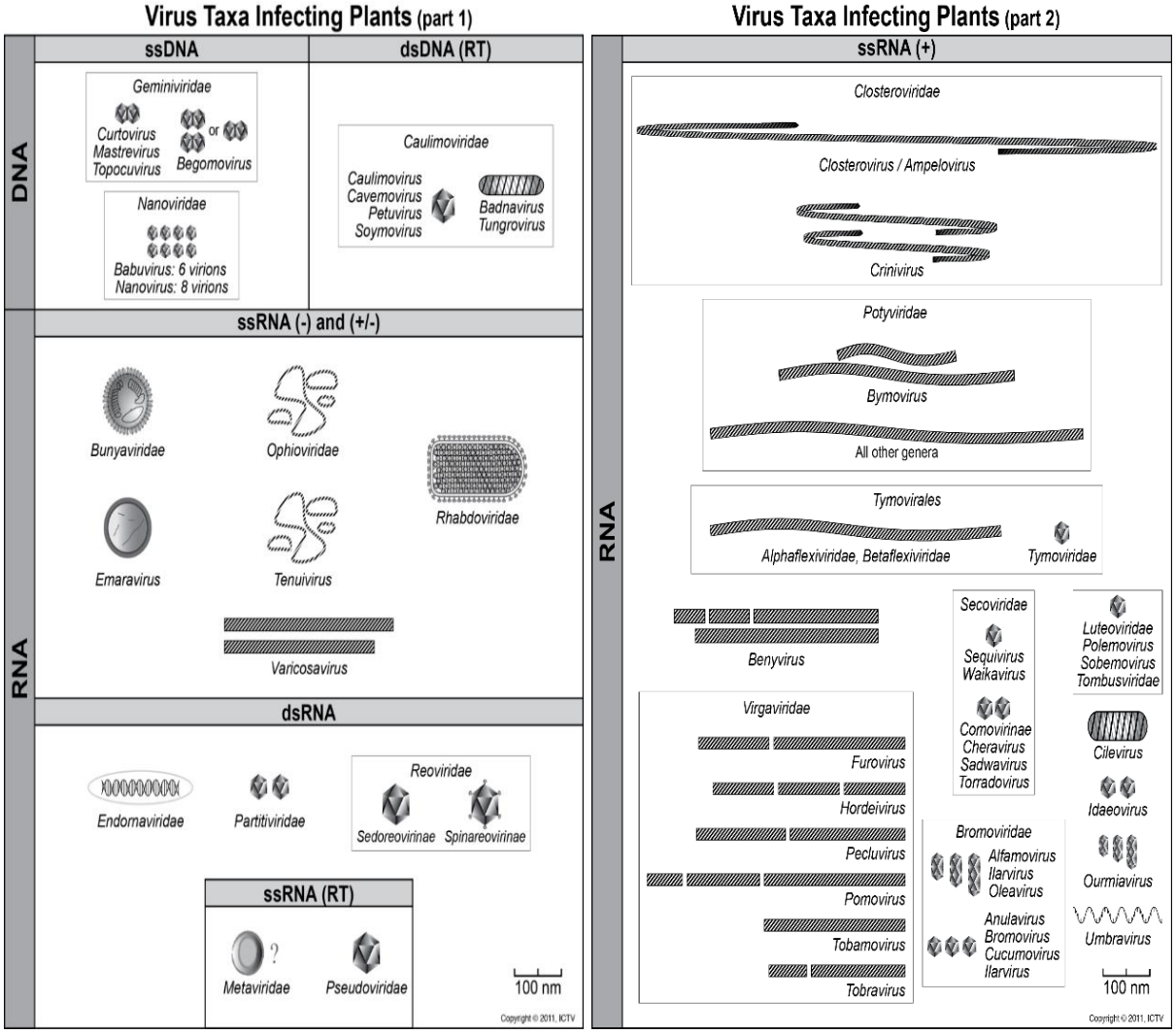

Figure 1.2: Viral families infecting plants. Taken from "The online (10th) report of the ICTV" (September 2017). *Copyright © 2017, International Committee on Taxonomy of Viruses (ICTV).* Creative Commons Attribution-ShareAlike 4.0 International License(ICTV's 10th report, 2017).

1.4 Detection and diagnostic tools in Virology

As mentioned before, the development of sensitive and specific virological techniques is fundamental to our understanding of viruses. Nowadays, there is an ample range of techniques based on different principles and suitable for different applications and research necessities.

1.4.1 Living host studies

The most basic form of viral study is the observation of symptoms on appropriate host species. In plants, these symptoms can be caused by a natural infection or by artificial inoculation on healthy hosts. Artificial inoculation can be mechanical, by extracting the virus from infected plant material in an appropriate buffer and rubbing this inoculum on the leaves of the host plant; by grafting of small infected stem pieces onto the host plant; if the viral species is transmitted by a vector (insect, fungus,

bacteria), this can be used to inoculate the virus in a controlled environment; if virus transmission is vertical, through pollen and seeds, breeding techniques can be used (Hull, 2009; Cann, 2012; Gaur *et al*., 2016).

Nevertheless, the information obtained is limited and often unreliable, as many viral species cause very similar or identical symptoms, co-infections cause different symptoms than the individual infections, and the observation of symptoms and their severity is subjective. In addition, while mechanical inoculation is generally a simple process, its efficiency greatly varies between plant and virus species and it takes around one to two weeks for hosts to start showing symptoms, proving to be one of the longest methods in viral studies. And most importantly, many viruses cannot be artificially inoculated, greatly limiting the use of this technique (Hull, 2009; Gaur *et al*., 2016; Lee and Vu, 2017).

1.4.2 In vitro methods

In vitro refers to the culture of cells, organs or organisms on artificial media under controlled environmental conditions. This technique is used for bacteria, fungus, animal cells and plants.

In plant virology, *in vitro* culture is mainly used for the study of virus characteristics, using a quicker and more manageable format than *in vivo* plants, and for the production of virus-free plants. The main virus elimination methods are: meristem culture, where, due to the high cell division activity in the meristem, meristematic cells are, in most cases, virus-free, and therefore, the plants derived from these cells are also virus-free; thermotherapy, where plants are subjected to high temperatures able to disrupt viral replication without killing the plant; chemotherapy, where plants are grown on media containing antiviral agents; and cryotherapy, where meristems are treated with liquid nitrogen to eliminate infected cells (Panattoni *et al.*, 2013).

1.4.3 Electron microscope

Electron microscopy (EM) takes advantage of the very short wavelength of accelerated electrons, which allows the EM to attain a resolution of up to 2 nm, compared to the 200 nm resolution of the light microscope. This high resolving power supports the visualization of extremely small particles, including viruses. Two main sample preparation methods are used: negative staining with heavy metals and the infiltration of organs, tissues or cells with resins and the preparation of ultrathin sections from them. EM is used mainly for virus identification and characterization but has also

proven useful for virus discovery (Hull, 2009; Cann, 2012; Gentile and Gelderblom, 2014).

However, virus identification is only possible up to the family level. And this technique requires highly specialized personnel as well as expensive equipment and infrastructure. Hence, electron microscopy has been slowly replaced by high-throughput serological and molecular methods (Hull, 2009; Cann, 2012; Gentile and Gelderblom, 2014).

1.4.4 Serological techniques

The underlying mechanism of this set of techniques is the recognition and binding of specific antibodies to antigens. In virology, the capsid proteins of viruses are usually used as antigens (Hull, 2009; Gaur *et al*., 2016; Shukla, 2016; Le and Vu, 2017).

The Enzyme-Linked Immunosorbent Assay (ELISA) is the most commonly used serological technique for detection and quantification of viruses. It is highly specific, sensitive and accurate. It is a simple technique that doesn't require specialized staff or equipment. It is generally considered a low-cost technique as the reagents used during the ELISA assay are cheap, but the production of the antibodies is a complex and expensive procedure (Hull, 2009; Modrow *et al*., 2013; Boonham *et al*., 2014; Shukla, 2016; Le and Vu, 2017). There are different types of ELISA (Figure 1.3) depending on the number of antibodies used and the order in which the antibodies and antigen are added. As a rule-of-thumb, the higher the number of antibodies used, the more sensitive and specific the assay is. But that also implies a higher risk of cross-reactivity between the antibodies and a longer and more expensive procedure (Boonham *et al*., 2014; Shukla, 2016; Le and Vu, 2017).

Other serological techniques are the Lateral flow immunoassay (LFIA), an immunochromatographic technique (Hull, 2009; Gaur *et al*., 2016; Shukla, 2016), and the Immunocapture Transmission Electron Microscopy (ICTEM), which combines serology and electron microscopy (Hull, 2009; Shukla, 2016).

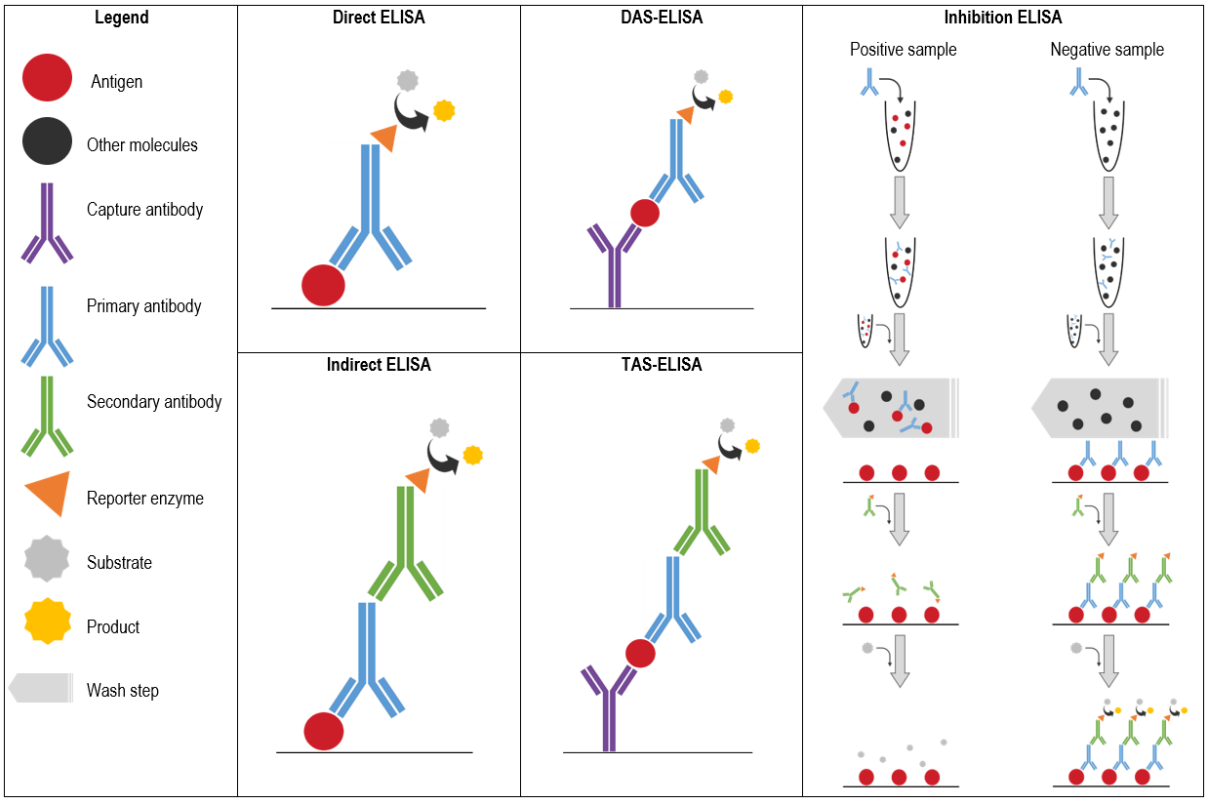

Figure 1.3: ELISA formats. From top to bottom: direct ELISA, indirect Elisa, double-antibody sandwich (DAS)-ELISA or direct sandwich ELISA, triple-antibody sandwich (TAS)-ELISA or indirect sandwich ELISA, and inhibition or competitive ELISA.

1.4.5 Nucleic acid amplification techniques

The basis for this set of techniques is the DNA strands complementarity. Nucleic acid amplification techniques provide very high specificity and sensitivity. Their main advantage over serological methods is the ease of design and production of primers compared to antibodies, as well as its lower cost. But, they are dependent on the availability of sequence information for the design of primers (Boonham *et al*., 2014; Gaur *et al*., 2016; Shukla, 2016; Le and Vu, 2017). We can distinguish two categories: thermal cycling methods and isothermal methods.

The PCR is the basic thermal cycling method. Primers, short DNA sequences, are designed to hybridize complementarily to specific regions of the DNA strands and serve as the initiation site for the polymerase. The polymerase elongates the primers, effectively creating a new copy of the target sequence. A series of denaturation, annealing and elongation cycles provide an exponential amplification of the target sequence, that is later identified by agarose gel electrophoresis and staining. Each of the cycles' steps requires a specific temperature that is given by the primers sequences and the polymerase used (Hull, 2009; Modrow *et al*., 2013; Shukla, 2016). Variants of the PCR are the Reverse Transcription (RT)-PCR, which allows the use of RNA

samples, and the Multiplex PCR, which allows the detection of several sequences in the same reaction (Hull, 2009; Modrow *et al*., 2013; Shukla, 2016; Le and Vu, 2017).

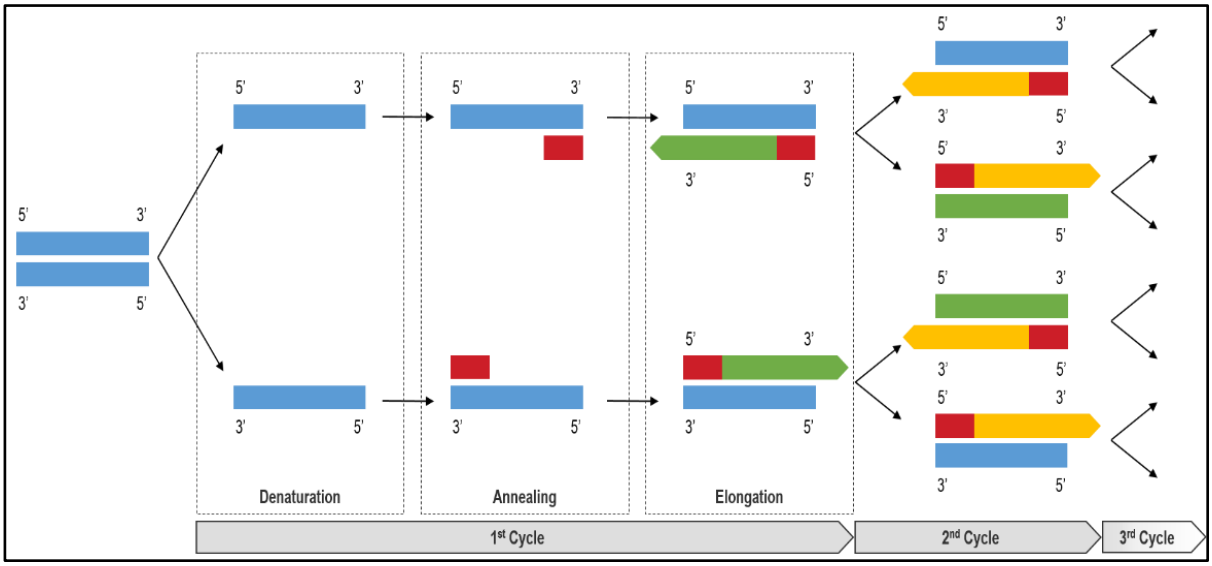

Figure 1.4: PCR process. Primers are represented in red, DNA/RNA sequence in blue and amplicons in green (1st cycle) and yellow (2nd cycle).

Real-time or Quantitative PCR (qPCR) can be considered the evolution of the conventional PCR. The basis of qPCR is that the time taken for a PCR reaction to enter the exponential phase is proportional to the quantity of target DNA present in the sample; thus, a sample with a high concentration of target DNA enters the exponential phase before a sample with a lower concentration. The only difference with the conventional PCR is the detection method. The most commonly used detection method consists of a DNA probe to which a fluorescent reporter and a quencher, which blocks the fluorescence, are attached. The probe hybridizes to the DNA strand and when the polymerase starts elongating the primer, it displaces and cleaves the probe, freeing the reporter from the quencher and allowing the fluorescence to be measured while the qPCR is still underway (Shukla, 2016). qPCR has a higher sensitivity than conventional PCR and allows both qualitative and quantitative assays. It is also faster and less labour intensive as there is no gel step. Variants have also been developed for qPCR: Reverse Transcription (RT)-qPCR and multiplex qPCR (Hull, 2009; Boonham *et al*., 2014; Shukla, 2016; Le and Vu, 2017).

Isothermal methods are designed to amplify nucleic acids at a constant temperature, which ranges from 35 °C to 65 °C depending on the method. These methods are faster, ranging between 15 min and 60 min, than thermal cycling ones, while also maintaining or improving their specificity and sensitivity levels. They are considered more cost-effective, as the lack of thermal cycling requires less power-

consuming equipment, which in turn facilitates the design of portable formats suitable for use in-field (Notomi *et al*., 2000; Gaur *et al*., 2016; Shukla, 2016; Le and Vu, 2017). Some examples of isothermal methods are the Loop-Mediated Isothermal Amplification (LAMP), which uses a polymerase with high strand-displacement activity and three pairs of primers (internal, external and loop primers) to generates single-stranded loop structures that allow primer annealing without the denaturation step (Notomi *et al*., 2000; Boonham *et al*., 2014; Gaur *et al*., 2016; Shukla, 2016; Le and Vu, 2017). The Rolling Circle Amplification (RCA), which is based on the rolling circle replication mechanism (Ali *et al*., 2014; Boonham *et al*., 2014; Gaur *et al*., 2016; Shukla, 2016), and the Nucleic Acid Sequence-Based Amplification (NASBA), which makes use of the T7 polymerase from bacteriophage T7 (Boonham *et al*., 2014; Shukla, 2016).

1.4.6 Sequencing

The Chain-termination sequencing method, also known as Sanger's sequencing, was developed in 1977 by Sanger *et al.* While not the first sequencing method, it quickly became the most used for over a decade due to its ease of use, accuracy and robustness. The first automated sequencers were based on this sequencing approach (Smith *et al.*, 1986). However, Sanger's sequencing is not always cost-effective, it is not a high-throughput technology and presents limited scalability, making it less than ideal for handling large genomes or a high number of samples (Heather and Chain, 2016).

NGS is the name given to several different sequencing platforms based on four main sequencing methods: sequencing by synthesis (SBS), sequencing by ligation (SBL), single-molecule sequencing (SMS) and nanopore sequencing. Developed around the mid-2000, they allow high throughput sequencing, generating in a single run up to gigabase pairs of data, which has heavily reduced sequencing costs. Being able to sequence all nucleic acid sequences present in a sample without any prior sequence knowledge, they have become a powerful tool for the discovery of new microorganisms, and especially, of viruses. They have also facilitated more complex studies than were previously possible with Sanger's sequencing: the study of viral genomes directly from samples, the study of viromes (all viruses present in a sample), the study of viral diversity, with a focus on quasi-species studies, as well as virus transmission and pathogenesis (Niedringhaus *et al.*, 2011; Boonham *et al*., 2014; Quiñones-Mateu *et al*., 2014; Gaur *et al*., 2016; Goodwin *et al.*, 2016; Heather and Chain, 2016; Shukla, 2016; Mardis, 2017).

1.5 Viruses and symbiosis

1.5.1 Definition of symbiosis

Symbiosis was defined by de Bary (1878) as “the living together of differently named organisms” (translated by Oulhen *et al.*, 2016). Nowadays, this definition is still valid and can be expressed as: the intimate biological interactions between different species, understanding by intimate the living in or on one another of the symbionts (the members of a symbiotic relationship). Symbiosis is considered to encompass all long-term relationships including the mutually beneficial or harmful, as well as the obligate, where a symbiont cannot survive on its own, or optional, where a symbiont can survive on its own (Villareal, 2005; Ryan, 2007; Roossinck, 2011; Oulhen *et al.*, 2016; Roossinck and Bazán, 2017).

The main symbiotic categories are antagonism: where one symbiont benefits while the others are harmed. Commensalism: where one symbiont benefits while the others are unaffected. Mutualism: where all symbionts benefit. Symbiogenesis, defined as the origin of new organisms by the fusion of different organisms living in symbiosis, is considered the extreme of mutualism (Ryan, 2007; Roossinck, 2011; Roossinck, 2015b; Roossinck and Bazán, 2017).

However, symbiotic relationships are not fixed and can vary across a wide spectrum, from antagonistic to mutualistic, following changes in their environment. These variations can be short-lived, termed conditional as they arise due to specific conditions and disappear with these, or permanent (Roossinck, 2011; Roossinck, 2015b; Oulhen *et al.*, 2016; Roossinck and Bazán, 2017).

1.5.2 Symbiotic viruses

Viruses have been historically regarded as pathogens, antagonistic symbionts, and most efforts have focussed on the study of the diseases they cause and their prevention. But, just as some decades ago bacteria were “promoted” from unwanted pathogens to essential components of the human holobiont with high ecological importance in all environments, this view of viruses is shifting. The notion of viruses as commensal or/and mutualistic symbionts has existed since the beginning of the 20th century, but it only took off and was developed around the 80s and 90s (Edson *et al.,* 1981; Ernst *et al.*,1983; Nesterova, 1988; Van den Heuvel *et al.*, 1994; Gu *et al.*, 1995; Kaunitz, 1995; Hayakawa and Yazaki, 1997; Filichkin *et al.*, 1997; Beckage, 1998; Griffiths, 1999), thanks to the growing knowledge on viruses and their interactions with other organisms. Examples of symbiotic relationships, spanning from antagonistic to symbiogenic, have been found for numerous viruses and their hosts (Villareal, 2005;

Ryan, 2007; Villareal, 2007; Roossinck, 2011; Roossinck, 2015a; Roossinck, 2015b; Roossinck, 2015c; Roossinck and Bazán, 2017). In the following paragraphs I present some of these examples.

As mentioned before, symbiotic relationships are influenced by environmental conditions and can adjust to these to ensure the survival of the organisms involved in them. An example of this is the case of several virulent plant viruses which have been found to confer tolerance to abiotic stresses to their hosts: *cucumber mosaic virus* (CMV), TMV, *tobacco rattle virus* (TRV) and *brome mosaic virus* (BMV) improve tolerance of host plants to drought stress. CMV also improves freezing tolerance in beet plants. Hence, in this case, antagonistic viruses can act as conditional mutualists under stress conditions, improving the chances of survival of their hosts and therefore, of themselves (Xu *et al*., 2008; Westwood *et al*., 2013).

Another example of viruses shifting between antagonistic and mutualistic are prophages. Prophages are one of the most prominent symbionts of bacteria. These viruses stay in a latent state and are transmitted vertically until they are induced to the lytic state, killing their host. Surprisingly, even though prophages could be considered ticking bombs, their bacterial hosts seem to actively conserve them. This appears to be mainly due to their use as protective components and as viral weapons against competing bacterial populations, benefits that seem to outweigh the danger of spontaneous lytic induction. Infection with a prophage protects the host bacteria against its lytic form and similar viral strains. This fact can be used on a population level to compete against other bacterial populations: lytic induction and lysis of a few individuals releases an important number of virions, which decimate susceptible populations while the prophage infected population remains unscathed. Thus, the prophage's host population competitive fitness is increased compared to non-host populations (Bossi *et al*., 2003; Brown *et al*., 2006).

Similarly, an important number of yeast species, known as killer yeasts, use toxins to compete against other yeast species. These toxins can be encoded by nuclear genes, linear dsDNA plasmids or cytoplasmically inherited dsRNA viruses. As before, killer yeasts are immune to their own toxin and therefore, show increased competitive fitness against susceptible yeasts. But in this case, these viruses are always considered mutualistic as they remain non-pathogenic to their host (Schmitt and Breinig, 2002; Schmitt and Breinig, 2006).

This protective component of some mutualistic viruses can also be found in higher organisms. In humans, hepatitis G virus C (GBV-C), a vertically transmitted virus

with no known disease pathology, has been associated to more positive outcomes in HIV-1 patients (Bagasra *et al.*, 2012).

A well-studied example of mutualistic viruses are the *Bracovirus* and *Ichnovirus* families infecting parasitoid wasps. Parasitoid wasps reproduce by injecting their eggs into insect larva, on which the wasp larva feeds once it has hatched. The replication genes of the viruses are integrated in the wasp genome. Viruses replicate in the female ovaries and are injected with the wasp's eggs into the insect host. The virions package viral genes that disable the defence response of the insect host, allowing the eggs to survive. These genes are believed to have an insect origin and to have been acquired horizontally by the viruses. Therefore, viral replication and transmission are preserved by the wasps, and wasp survival and reproduction are preserved by the viruses. Due to the integrated nature of the viral genes, it is considered that these viruses and their wasp hosts are in the process of becoming symbiogenic (Webb *et al*., 2006; Herniou *et al*., 2013).

One example of symbiogenesis between viruses and hosts are the syncytins, a family of endogenous retroviral envelope glycoproteins. Their most well studied function is cell-cell fusion, which seems to play an essential role in placental formation in, at least, higher primates, including humans, rodents, sheep, and lagomorphs. It is believed that the endogenization of the syncytin viral gene played an important role in the evolution of mammals, being in part responsible for the origin of the placenta (Pérot *et al.*, 2012).

Nonetheless, symbiotic relationships can involve more than two actors and are not always straightforward. *Cryphonectria parasitica*, native to East Asia, is the fungus responsible for the chestnut blight disease in *Castanea spp*. Chestnut blight is a serious threat, responsible for the destruction of the native chestnut forests in North America. However, in Europe, the disease is biologically controlled by Cryphonectria hypovirus 1 (CHV-1), an unencapsidated RNA virus that persistently infects *C. parasitica*. CHV-1 reduces the pathogenic potential of the fungus without killing it, which helps infected trees survive. Thus, while CHV-1 is an antagonist of its fungal host, it can be considered a mutualist of *Castanea spp* (Bryner *et al.*, 2012; Bryner *et al.*, 2014).

Another complex example of mutualism is the three-way symbiotic relationship between *Curvularia thermal tolerance virus* (CThTV), its fungal host, *Curvularia protuberata,* and the latter's host plant, *Dichantelium lanuginosum*. *D. lanuginosum* is a grass found growing at high soil temperatures in Yellowstone National Park (USA)

thanks to its mutualistic relationship with *C. protuberata*, neither of them being able to survive these temperatures on their own. Márquez *et al.* (2007) found that this heat tolerance is actually due to the presence of CThTV in the fungus, virus-free fungal strains not providing heat tolerance.

1.5.3 Cryptic viruses

Cryptic viruses have been known since the 1970s, but the fact that they pose no apparent economic threat to agriculture and the difficulties in detecting and working with them have hindered their study until recently. The development of new detection techniques, particularly NGS (section 1.4.6), has promoted the detection of new viruses in plants, many of which have been identified as persistent (Boccardo, 1987*;* Hull, 2009; Roossinck, 2011).

The first report of a cryptic virus was *Beet cryptic virus* (BCV), reported as a virus-like particle by Pullen in 1968 and 1969 and later purified and named by Kassanis *et al.* in 1977. BCV was found to infect up to 90% of plants in a cultivar at a concentration of 1 µg / g leaf tissue or less. It could not be eliminated with heat therapy nor transmitted mechanically; transmission was only possible through seeds. In the following decades, other viruses with similar properties were reported: *Alfalfa cryptic virus* (ACV)*, Beet cryptic virus* (BCV) *1* and *2, Carnation cryptic virus* (CarCV)*, Ryegrass cryptic virus* (RCV)*, Vicia cryptic virus* (VCV)*, White clover cryptic virus* (WCCV) *1, 2* and *3,* etc*.* (Boccardo, 1987). As these viruses shared many similarities with mycoviruses, doubts remained about the nature of their host until 1985, when two different reports (Boccardo *et al.*, 1985; Abou-Elnasr *et al.*, 1985) presented evidence that cryptic viruses were indeed plant viruses (Boccardo, 1987). In 1987, Boccardo *et al.* published one of the first comprehensive review of cryptic viruses, including information on their general characteristics, their particle structure and physical properties, their known transmission mechanisms, known symptoms, know detection methods and phylogenetic relationships. And proposed the creation of a taxonomic group.

The main characteristics of cryptic viruses are (Boccardo, 1987; Hull, 2009; Roossinck, 2010; Roossinck, 2011):

- Lack of disease symptoms.
- Very low virus titer.
- Lifelong persistence in individual hosts.
- Transmission to new hosts is vertical, through seeds, viral progeny being present in pollen and/or ovule. There is no proved mechanical, graft or vector transmission.

- There is no cell-to-cell movement, viruses are transmitted to new cells only during cell division.
- No successful virus elimination protocol has been described.
- They seem to not be affected by plant defence mechanisms such as RNA silencing.
- They are usually widespread in their host populations and species, resulting in an unavailability of negative samples for comparative studies.

These characteristics highlight a very close relations hip between cryptic viruses and their hosts. This would suggest that the viruses offer some kind of beneficial trade-off to their hosts, as their presence and replication entails a cost. Hence, the main hypothesis regarding cryptic viruses is that they act as mutualistic symbionts.

Unfortunately, the general lack of non-infected individuals heavily hinders the study of this hypothesis, as inferring the effect of the viruses on their host under these circumstances is difficult. To our knowledge, currently, in only two cases has a function been proposed for a cryptic virus.

Nakatsukasa-Akune *et al.* (2005) found that expression in transgenic *Lotus japonicus* plants (model legume) of the CP gene of *white clover cryptic virus 1* (WCCV1), a cryptic virus of white clover (*Trifolium repens*), suppressed nodulation. While the symbiosis between rhizobia and leguminous plants is generally beneficial, excessive nodulation can disrupt plant growth. In this instance, it would seem that white clover makes use of WCCV1 to ensure the correct regulation of nodulation, suppressing it when soil nitrogen levels are adequate. Nakatsukasa-Akune *et al.* (2005) found that the expression of the transgen containing the CP gene suppressed the emergence, length and branching of hairy roots, and reduced the number of root nodules per unit length of hairy root. This was associated to an increase in endogenous abscisic acid (ABA) concentration in the transgenic hairy roots. This association was confirmed with an abamine (inhibitor of ABA synthesis) treatment: ABA concentration in transgenic hairy roots was reduced to control levels and so was the number of root nodules. Additionally, several defence-response genes were up-regulated in the transgenic hairy roots, this seemed to be a response to the presence of a pathogen protein, as the CP gene is of viral origin. ABA is also linked to antiviral defence responses in plants (Alazem and Lin, 2017). Therefore, the CP gene of WCCV1 seemed to affect nodulation by suppressing rhizobial infection due to the activation of the plant's innate immune response. Nakatsukasa-Akune *et al*. (2005) also propose that white clover might use WCCV1 to maintain the constitutive defence response.

However, due to a lack of effective transformation methods for white clover, it was not possible to confirm these findings in the virus' host species, white clover.

Safari *et al*. (2019) found that *pepper cryptic virus 1* (PCV-1) influences the composition of secondary metabolites that discourage aphids from feeding on the host plant, helping reduce the incidence of aphid damage and the infection by pathogenic viruses transmitted by these. They studied the preference of the aphid *Myzus persicae* for volatile organic compounds (VOC) of plants infected with PCV-1 and/or CMV, known to change its host plant VOC profile to attract aphids that act as its vector. When comparing PCV-1 infected and virus-free plants, aphids showed preference for the virus-free plants. When comparing CMV infected plants to CMV non-infected plants, regardless of the PCV-1 infection status, aphids showed preference for CMV infected plants, implying that the effect of CMV on the plants' VOC profile is stronger than that of PCV-1. However, when comparing CMV infected/PCV-1 infected plants and CMV infected/PCV-1 non-infected plants, aphids showed preference for CMV infected/PCV-1 non-infected plants. Therefore, it would seem that PCV-1 influenced aphid preference. Additionally, Safari *et al*. (2019) studied the effect of PCV-1 on aphid reproduction. They observed a significant 2-fold reduction of aphid reproduction on PCV-1 infected plants compared to virus-free plants. Thus, the presence of PCV-1 deterred aphids and protected plants from the vector of acute viruses, as well as from the damage of aphid herbivory.

1.6 Importance and aim of this work

If cryptic viruses are indeed mutualistic to their hosts, it would mean that they could be exploited to provide improvements to crops, such as tolerance to biotic and abiotic stresses, that would provide sustainable crop benefits. This could be achieved through artificial viral transmission, if a method is found, in which case the virus would be introduced in its entirety into the host and allowed to function and replicate as normal. Or, through plant transformation, either using the whole viral genome or the specific regions that code for the beneficial effect, if these have been identified, thus obtaining the benefit while limiting possible negative effects of viral replication. Therefore, the aim of this work is to explore the role of cryptic viral infections in altering crop stress tolerance. We focus on three viruses recently identified in black-grass (*Alopecurus myosuroides*) populations associated with broad-ranging herbicide resistance (Sabbadin *et al*., 2017).

A. myosuroides is an annual grass native to Eurasia and an economically important weed species in temperate cereal crops. It has become one of the most damaging weeds of winter cereals in Western Europe due to changes in agricultural practices (reduced tillage systems, increased nitrogen fertilization, changes to sowing dates, etc.) and to its propensity to develop resistance to herbicides (Letouzé and Gasquez, 2001; Menchari *et al.*, 2007; Sabbadin *et al*., 2017; CABI, 2018). Two main resistance mechanisms have been detected in the United Kingdom: target site-based resistance (TSR), where point mutations render targeted proteins, mainly acetyl-coenzyme A carboxylase (ACCase) and acetolactate synthase (ALS), insensitive to herbicide inhibition; and non-target site resistance (NTSR), where resistance is due to the enhanced expression of multiple genes involved in detoxification. While TSR can be controlled by the use and alternation of herbicides with different modes of action and is inherited as a Mendelian trait, NTSR shows resistance to all types of herbicides, making its control more complicated, and behaves as a multigenic quantitative trait, its inheritance being affected by environmental factors (Letouzé and Gasquez, 2001; Menchari *et al.*, 2007; Sabbadin *et al*., 2017; CABI, 2018). Sabbadin *et al.* (2017) were interested in the potential role of microorganisms as an environmental driver of NTSR and studied the phytobiome of black-grass and rye-grass using a non-targeted next generation sequencing approach. They identified three previously undescribed persistent viruses widespread in the studied black-grass populations, with a marked infection incidence in the NTSR Peldon population.

Two of the viruses, *Alopecurus myosuroides* partitivirus 1 (AMPV1) and *Alopecurus myosuroides* partitivirus 2 (AMPV2), were identified as alphapartitiviruses (WCCV1 type species), family *Partitiviridae*. This family includes viruses that infect plants, fungi and protozoa. Partitiviruses are generally associated with persistent symptomless infections (ICTV's 10th report, *Partitiviridae* chapter, 2017). However, in recent years, some deleterious effects of fungal partitiviruses have been described, most of them causing hypovirulence in their hosts (Magae and Sunagawa, 2010; Bhatti *et al*., 2011; Xiao *et al*., 2014; Zheng *et al*., 2014). In protozoa, *Cryptosporidium parvum virus* (CPV) seems to improve fecundity in *Cryptosporidium parvum* (Jenkins *et al*., 2008). The only plant partitivirus with a known function is the previously mentioned WCCV1. *Alphapartitivirus* members infect plants and fungi, they have two dsRNA segments that encode the RNA-dependent RNA polymerase (RdRP) and the CP, AMPV1 and AMPV2 share this genome structure (Figure 1.5). These segments are individually encapsidated in separate particles, their genomes range from 3.6 to 3.9

kbp. Plant partitiviruses are transmitted vertically during cell division and through seeds, with no known mechanical transmission method or elimination method (Sabbadin *et al*., 2017; ICTV's 10th report, *Partitiviridae* chapter, 2017).

The third virus, *Alopecurus myosuroides varicosavirus 1* (AMVV1), was identified as a varicosavirus (*Lettuce big-vein associated virus* type species), family *Rhabdoviridae*. This family includes viruses that infect plants, animals, including mammals, birds, reptiles and fish, and arthropods, which are either the viruses' natural host or their vector (ICTV's 10th report, *Rhabdoviridae* chapter, 2017). Members of the genus *Varicosavirus* infect plants, they have bi-segmented genomes and non-enveloped virions. *Lettuce big-vein associated virus* is the only approved species in the genus by the ICTV, but AMVV1, *Red clover associated varicosavirus* (RCaVV) and *Tobacco stunt virus* (TStV), currently unclassified, are noted as related to this genus (ICTV's 10th report, *Rhabdoviridae* chapter, 2017). *Lettuce big vein associated virus* (LBVaV) is transmitted in soil by zoospores of *Olpidium virulentus,* an obligate root infecting fungal parasite. LBVaV is generally found together with *Mirafiori lettuce big-vein virus* (MLBVV, genus *Ophiovirus*), the causal agent of Lettuce big vein disease (LBVD), and is believed to be symptomless (Maccarone *et al*., 2010; Maccarone, 2013; Verbeek *et al*., 2013; Sabbadin *et al*., 2017; ICTV's 10th report, *Rhabdoviridae* chapter, 2017). However, Verbeek *et al.* (2013) found that LBVaV was responsible for necrotic symptoms in lettuce. AMVV1 has a bipartite genome, comprising an RNA1, coding for an RdRP and an RNA2, coding for (at least) one CP (Figure 1.5) (Sabbadin *et al.,* 2017).

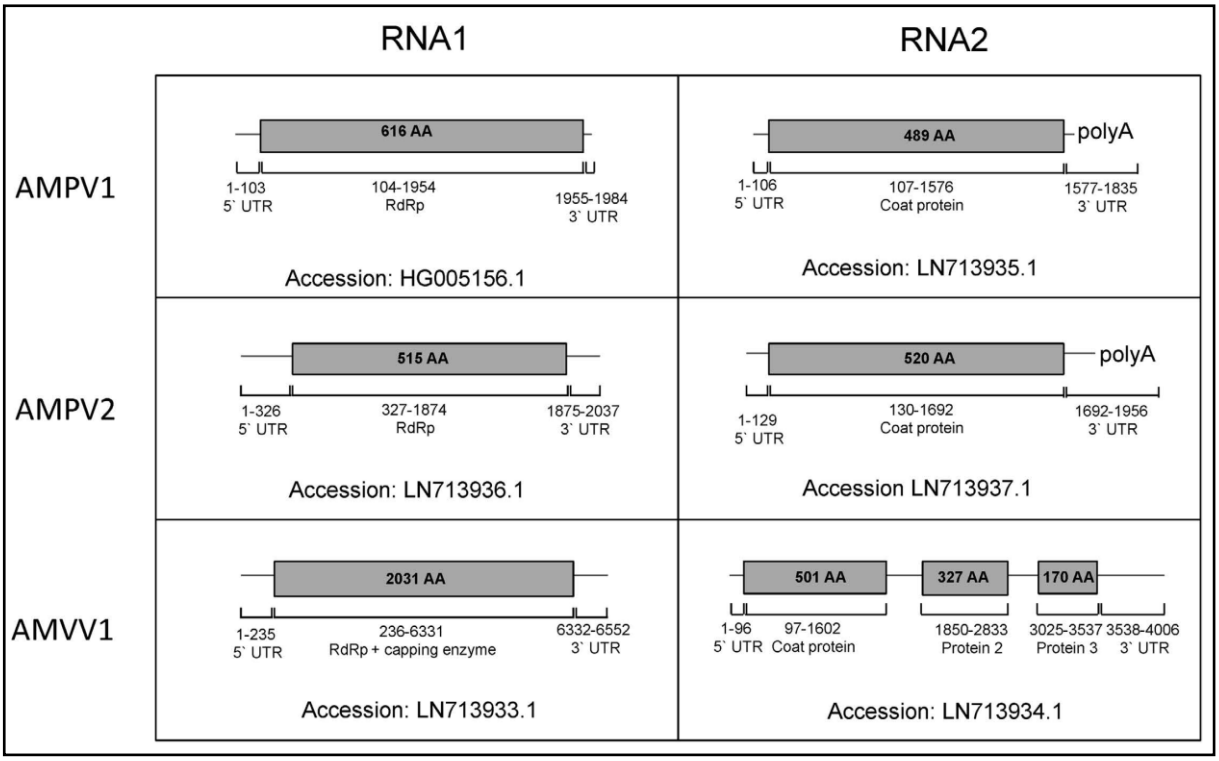

Figure 1.5: Schematic representation of the fully assembled genome sequences of AMPV1, AMPV2 and AMVV1 found in black-grass. Total nucleotide and protein length of the coded ORFs are indicated, together with the putative function and the accession number. Taken from Sabbadin *et al.* (2017).

The study of these viruses will allow us to better understand the relationship between cryptic viruses and their hosts, evaluating how much influence these viruses have on host phenotype and fitness. If a correlation is found between these viruses and stress tolerance, this knowledge could be used for the development of new black-grass management strategies, particularly if a relationship to herbicide resistance is found. And it would open the door to introducing the tolerance-induced effects of these viruses into crop species by means of virus transmission or plant transformation. I started by characterizing these viruses, focusing on incidence, infection pattern and transmission mechanism, as well as the effect of *in vitro* viral elimination methods. A drought stress assay was carried out to study the effect of the viruses on plant tolerance to abiotic stress.

Chapter 2: Development of simplex and multiplex RT-qPCR assays for the detection of AMVV1, AMPV1 and AMPV2.

2.1 Introduction

Cryptic viruses are a group of persistent plant viruses whose characteristics highlight a very close relationship with their hosts, suggesting that the latter actively preserve infection with these viruses. This would imply that the viruses offer some kind of beneficial trade-off to their hosts, as their presence and replication forcibly has an energy cost. Hence, the main hypothesis regarding cryptic viruses is that they act as mutualistic symbionts, see section 1.5.3 (Boccardo et al., 1987; Hull, 2009; Roossinck, 2010; Roossinck, 2011). This would mean that they could potentially be exploited to provide improvements to crops, such as tolerance to biotic and abiotic stresses.

Sabbadin et al. (2017) were interested in the potential role of microorganisms as an environmental driver of NTSR and studied the phytobiome of black-grass and rye-grass using a non-targeted next generation sequencing approach. They identified three previously undescribed persistent viruses: AMPV1, AMPV2 and AMVV1. See section 1.6.

Following on from Sabbadin et al's (2017) work, I aimed to study the effect of these viruses on the tolerance of black-grass to abiotic stresses. qPCR has higher sensitivity than conventional PCR and enables both qualitative and quantitative detection, supporting more in-depth studies of viral infection. A suite of Reverse Transcription (RT)-qPCR assays were developed for the black-grass viruses and compared to the existing conventional PCR assays. As viral quantification was an important element in the comparative experiments (Chapter 4), the effect of sample treatment prior to their extraction and analysis was studied and optimised.

2.2 Material and methods

2.2.1 Plant material

Black-grass and wheat (*Triticum aestivum* vr. Diego) were grown in a glasshouse cubicle at 20/18 °C under a light cycle of 16/8 h, this light cycle was ensured by the use of overhead artificial lighting, however, we recognised that at times (seasons with longer days) the photoperiod might have been longer due to natural light. Typically, leaf samples were tested. Black-grass material was taken from 6 different plants and

homogenised into one composite sample. This sample was divided into four: one quarter was dried at 65 °C for two days to obtain the Dry Weight (DW) material, a second quarter was freeze-dried for two days to obtain the Freeze-Dry Weight (FDW) material, while the other two quarters were frozen and kept as the Fresh Weight (FW) material. Similarly, material from about 6 young wheat plants was taken, homogenised into one sample and frozen.

For the specificity assay, 5 grass plants showing symptoms of viral infection (Table 2.2), and three *Poaceae* species were tested. Infected grass material was obtained from a grass virus collection kept at Fera (UK). Material had been collected in 2006, freeze-dried and stored in a cold-room. The infection status of the plants had been assessed visually and the following viruses were identified: *agropyron mosaic virus* (AgMV), *barley yellow dwarf virus* (BYDV), *ryegrass mosaic virus* (RgMV), *cocksfoot mottle virus* (CfCP) and *cocksfoot streak virus* (CSV). The tested *Poaceae* species were wheat (*Triticum aestivum*), barley (*Hordeum vulgare*) and oat (*Avena sativa*).

2.2.2 RNA extraction method

For the drying treatment comparison, the DW, FDW and one of the FW samples were divided into two 0.3 g samples. Extraction buffer (6 mL) (Mumford, 2002) was added to the DW and FDW samples and 4 mL to the FW samples. A healthy control (not virus-infected) and an extraction control were prepared with wheat and extraction buffer respectively. Samples were ground and centrifuged at 20 000 x g for 5 min. Total RNA was extracted using the Kingfisher®mL system (Thermo labsystems) method according to Mumford (2002). DW, FDW and FW extraction processes were carried out in parallel to ensure all samples were under the same conditions. Samples were eluted in 200 µL molecular grade water and stored at -20°C.

For the Sensitivity analysis, the remaining FW sample and wheat samples were used. They were divided into 0.3 g samples and 4 mL extraction buffer (Mumford, 2002) was added. Samples were ground and centrifuged at 20 000 x g for 5 min. A dilution series, 1 to 10, was prepared by diluting the black-grass supernatant in the wheat supernatant at a factor of 1.76. Dilution 0 and the healthy control were prepared with pure black-grass and wheat supernatant respectively. An extraction control was prepared with extraction buffer. Total RNA was extracted using the Kingfisher®mL system and samples were eluted in 200 µL molecular grade water (Severn Biotech LTD, UK) and stored at -20°C. RNA content was tested using the Nanodrop for dilutions 0 and 1 (Table 2.1).

For the specificity assay, extraction buffer (4 mL) (Mumford, 2002) was added to the samples. An extraction control was prepared with extraction buffer. Samples were homogenised and centrifuged at 20 000 x g for 5 min. Total RNA was extracted using the Kingfisher®mL system (Thermo labsystems) method according to Mumford (2002). Samples were eluted in 200 µL molecular grade water (Severn Biotech LTD, UK) and stored at -20°C.

Table 2.1: RNA content in ng in the dilution series used in the sensitivity assay. Dilutions were prepared with wheat supernatant at a factor of 1.76.

Dilution	ng RNA
0	24.89
1	14.13
2	8.03
3	4.56
4	2.59
5	1.47
6	0.84
7	0.48
8	0.27
9	0.16
10	0.09

Table 2.2: References for the primer sequences used to confirm the viral infection of the grass material in the specificity assay.

Virus	Reference
Agropyron mosaic virus	Hodge and Paul, 2018
Barley yellow dwarf virus	Malmstrom and Shu, 2004
Ryegrass mosaic virus	Webster *et al.*, 1996
Cocksfoot mottle virus	Alderman *et al.*, 2016
Cocksfoot streak virus	Pallett *et al.*, 2009

2.2.3 Primer design

Primers for the simplex and multiplex RT-qPCR assays were designed using MEGA version 6 (Tamura *et al.*, 2013), to select potential amplicon sequences and appropriate primer and probe sequences for these, and Blast® (NCBI), to check amplicon identity to wanted (our virus of interest) and unwanted (any other virus, plant or other) species, and checked for self-complementarity using Oligo Calc (Kibbe, 2007). Primers were targeted at the CP genes located, in all cases, in the RNA 2 segment. Primer sequences can be found in Table 2.3. For the multiplex assay, we used the Spectral overlay tool for multiplexed qPCR (Biosearch™ technologies) to choose the probes' fluorophore, making sure the absorption and emission peaks for each probe were clearly differentiated (Figure 2.1).

For the RT-PCR assay, primer sequences were taken from Sabbadin *et al*. (2017). To confirm the viral infection of the grass material used in the specificity assay, conventional PCR primer sequences were taken from the literature (Table 2.2).

Table 2.3: Primer sequences. For all assays, the target gene is the CP, located in the RNA 2 fragment. Accession numbers are: LN713934.1 for AMVV1, LN713935.1 for AMPV1, LN713937.1 for AMPV2.The multiplex RT-qPCR assay uses the same sequences as the simplex RT-qPCR assay except for AMPV2's forward and reverse primers. The multiplex probes use the following combinations of reporters and quenchers: FAM-NFQ for AMVV1, VIC-NFQ for AMPV1 and TAMRA-NFQ for AMPV2. F indicates forward primer; R, reverse primer and P, probe. The melting temperature (Tm) is given in C°.

Target		Name	Sequence (5' to 3')	Tm (C°)	Amplicon size (bp)
RT-PCR	AMVV1	AMVV1-C-F	CAAAGGAGCCAGGAGACAATGATGAAGAAG	70.8	968
		AMVV1-C-R	TAGTCCTGTGCCAGAGTCCACTGCTTAGTTC	73.8	
	AMPV1	AMPV1-C-F	ACGCCACTGAACAATTCACTGGCTC	67.4	728
		AMPV1-C-R	TTGAGCCGACGAAGAAGCGACTGTAC	69.5	
	AMPV2	AMPV2-C-F	TCACCCGCTTTGGATACTATTGGGTTGC	70.1	248
		AMPV2-C-R	ATCAAAGCCTATGATGGGGCTCTGTGACTCTAG	73.7	
RT-qPCR	AMVV1	AMVV1-Q-F	CGAAACTGTCTCAGCAGAACTTGA	63.6	123
		AMVV1-Q-R	CATTCATGTCGAAGCCCATTC	59.5	
		AMVV1-Q-P	ATAGCCAATGCAAGGGCGGTTGG	66.6	
	AMPV1	AMPV1-Q-F	ACAACTACCAACAGGTGATCG	59.5	113
		AMPV1-Q-R	GTCTGCTATATGCAGTTGGGT	59.5	
		AMPV1-Q-P	TCCAAGTACCACCGTTACAGCCCAATTTAAG	70.9	
	AMPV2	AMPV2-Q-F	TCGCCACCATGAACAATAAGG	59.5	105
		AMPV2-Q-R	CCAAGACGGCGAACACAATGT	61.2	
		AMPV2-Q-P	ACCATGCGCCCTTTCGGTGAATCCG	70.7	
Multiplex	AMPV2	AMPV2-M-F	TCACAACCTCGCCACCATG	59.5	118
		AMPV2-M-R	CAATAGCCAAGACGGCGAA	57.5	

2.2.4 RT-PCR assay

Primer sequences were taken from Sabbadin *et al*. (2017). The PCR reaction mix was prepared using Thermo Scientific's Verso 1-step RT-PCR Hot-Start kit; 1 µL of sample was added to a reaction mixture containing the Verso kit's mastermix and reverse transcriptase and 200 nM of each primer, to make up a final volume of 15 µL. Cycling conditions were as follows: 48°C for 30 mins for the reverse transcription step, 95°C for 15 mins for the Hot-Start activation step, followed by 35 cycles of 98°C for 10 s, 63°C for 30 s and 72°C for 1 min and a final step of 72°C for 5 min. Samples were run on a C1000^{TM} Thermal Cycler (Biorad). Amplified products were separated on a 1.2% agarose gel stained with ethidium bromide using Thermo Scientific's GeneRuler 100 bp DNA Ladder, and visualised using a UV transilluminator.

2.2.5 Simplex RT-qPCR assay

The PCR reaction mix was prepared using Biorad's iTaq^{TM} Universal Probes One-Step kit; 1 µL of sample was added to a reaction mixture containing the kit's mastermix and reverse transcriptase, 375 nM of each primer and 125 nM of the probe, to make up a final volume of 12 µL. When running this assay together with the multiplex assay, 2 µL of sample were added and the final volume adjusted to 20 µL. Cycling conditions were as follows: 50°C for 10 mins for the reverse transcription step, 95°C for 2 mins for the initial denaturation step, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Samples were run on a QuantStudio 6 Flex (Applied biosystems). To standardize results between different assays and machines, the threshold was set at 0.2 in all cases.

2.2.6 Multiplex RT-qPCR assay

The PCR reaction mix was prepared using Biorad's iTaq^{TM} Universal Probes One-Step kit; 2 µL of sample was added to a reaction mixture containing the kit's mastermix and reverse transcriptase. For AMVV1 and AMPV1, 375 nM of each primer and 125 nM of the probe were added; and for AMPV2, 260 nM of each primer and 125 nM of the probe were added. The final volume was 20 µL. Cycling conditions were as follows: 50°C for 10 mins for the reverse transcription step, 95°C for 2 mins for the initial denaturation step, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Samples were run on a QuantStudio 6 Flex (Applied Biosystems). To standardize results between different assays and machines, the threshold was set at 0.2 in all cases.

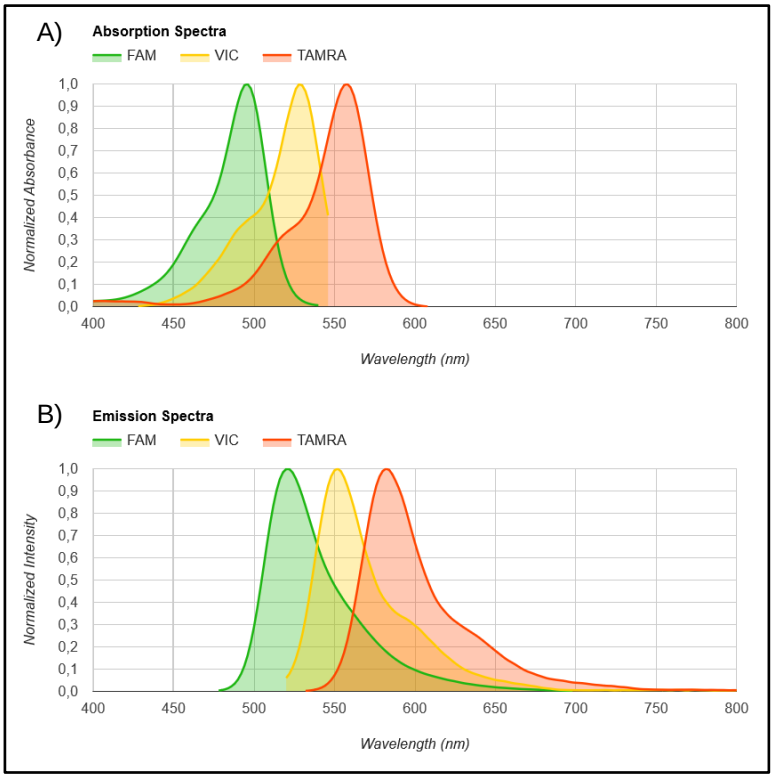

Figure 2.1: Spectral overlay charts for our chosen multiplex probe's fluorophores. A) shows the absorption spectra and B) the emission spectra. FAM, VIC and TAMRA were chosen as they have clearly differentiated absorption and emission peaks, which ensures differentiated detection during the PCR process. Obtained using Biosearch™ technologies' spectral overlay tool for multiplexed qPCR. https://www.biosearchtech.com/qpcr-multiplex-spectral-overlay-tool

2.3 Results

2.3.1 Primer specificity

The first specificity check was done by investigating any sequence identity between the amplicon and primer/probe sequences and other sequences present on Genbank using a BLAST search (Blast®, NCBI). No matches were found, except for the viral genomes uploaded by Sabaddin *et al.* (2017).

The second specificity check was done by running the RT-qPCR assays against samples of viral and plant species that could show cross-reactivity due to, respectively, their similarity to the viral genomes or to their similarity to black-grass' genome. To check for potential cross-reactivity with the viral genomes, grass material infected with different viruses was used. There was no amplification. However, these samples were also tested (with RT-PCR) for the viruses believed to be infecting them (Table 2.2), and only BYDV gave a positive amplification band. To check for potential cross-reactivity with the black-grass genome, other *Poaceae* species (wheat, barley and oat) were used. There was no amplification.

All samples were tested for WPal (wheat phenylalanine ammonia-lyase), used as an extraction control for monocotyledons. All samples, except the extraction and negative controls, showed amplification.

2.3.2 Assay sensitivity

The RT-PCR assay detection limits were established at dilution 0 for AMVV1, dilution 1 for AMPV1 and dilution 2 for AMPV2 (Figure 2.2).

The RT-qPCR simplex assay detection limits were established at dilution 3 for AMVV1, dilution 8 for AMPV1 and dilution 8 for AMPV2 (Figure 2.3).

The RT-qPCR multiplex assay detection limits were established at dilution 3 for AMVV1, dilution 7 for AMPV1 and dilution 7 for AMPV2 (Figure 2.4).

2.3.3 Drying treatment comparison

There was a reduction in the sensitivity of detection of AMVV1 due to the treatment of the samples (Table 2.4 and Figure 2.6). The reduction was 22% for the freeze-dried or low-temperature treatment and 97% for the heat or high-temperature treatment. In the case of the multiplex assay, the reduction was very similar: 26% for the low-temperature treatment and 97% for the high-temperature treatment.

There was an increase in the sensitivity of detection of AMPV1 due to the treatment of the samples (Table 2.4 and Figure 2.6). The increase was 92% for the low-temperature treatment and 90% for the high-temperature treatment. In the case of the multiplex assay, the increase was: 50% for the low-temperature treatment and 80% for the high-temperature treatment.

There was an increase in the sensitivity of detection of AMPV2 due to the treatment of the samples (Table 2.4 and Figure 2.6). The increase was 306% for the low-temperature treatment and 50% for the high-temperature treatment. In the case of the multiplex assay, the increase was: 297% for the low-temperature treatment and 64% for the high-temperature treatment.

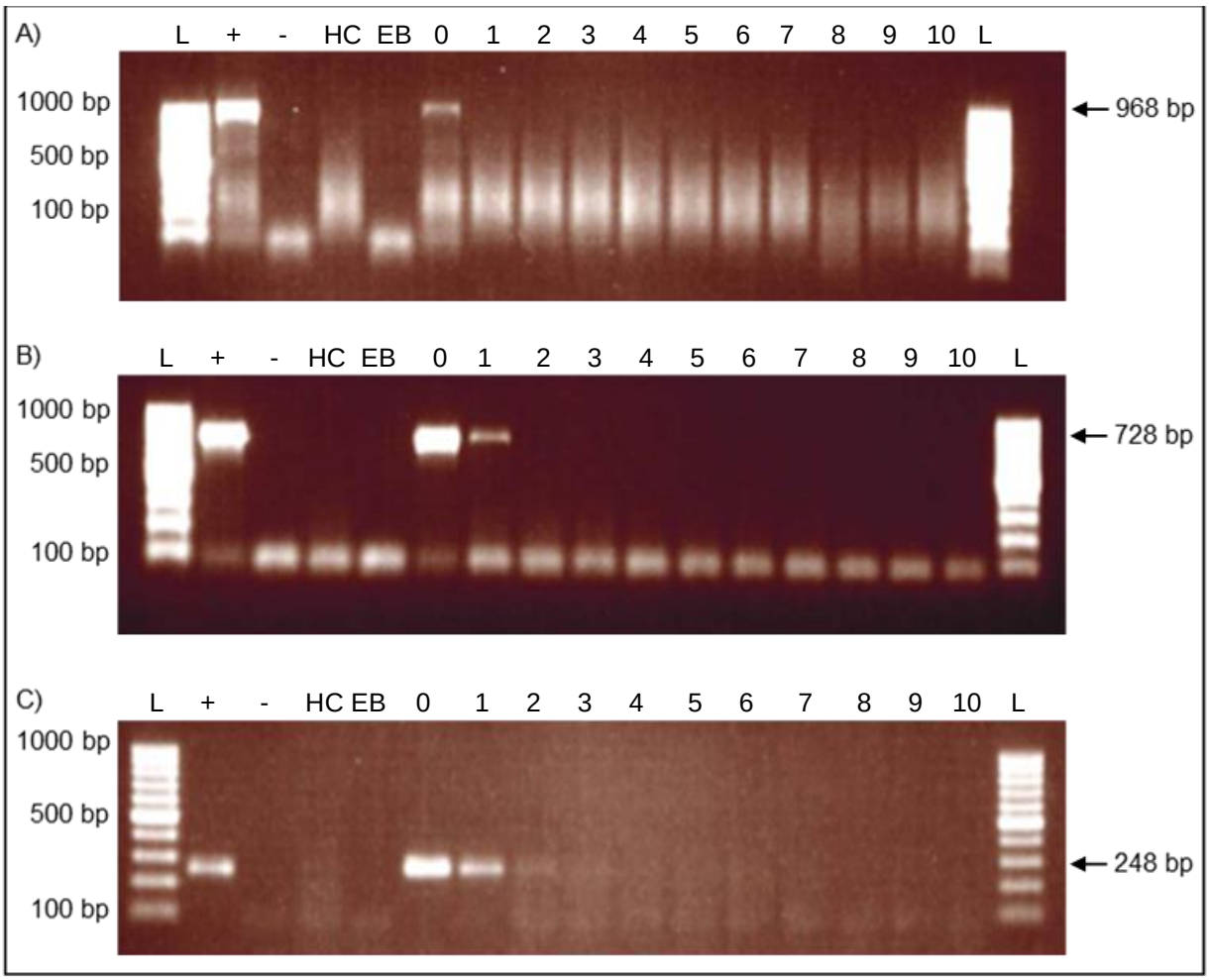

Figure 2.2: Sensitivity of the AMVV1 (A), AMPV1 (B) and AMPV2 (C) RT-PCR assays. Dilution 0 has approximately 24.89 ng total RNA, 1 to 10 are serial dilutions at a factor of 1.76 from dilution 0. Amplicon sizes are: 968 bp for AMVV1, 728 bp for AMPV1 and 248 bp for AMPV2. Code: L for ladder (Thermo Scientific's GeneRuler 100 bp DNA Ladder), + for the positive control, - for the negative control (sterile water), HC for healthy control (not virus-infected wheat), EB for extraction blank.

Table 2.4: Results following the drying treatments of the black-grass samples. “Viral concentration” values (ng/µL) correspond to the average of two samples with three replicates each. The reduction percentage of the detection sensitivity for the simplex FDW and DW samples compares these sample to the simplex FW sample. The reduction percentage of the detection sensitivity for the multiplex FDW and DW samples compares these sample to the multiplex FW sample.

Virus	**Sample**	**Simplex assay**		**Multiplex assay**	
		Viral concentration (ng RNA/µL)	*Reduction (%)*	*Viral concentration (ng RNA/µL)*	*Reduction (%)*
AMVV1	FW	143,25	/	78,97	/
	FDW	111,77	22	58,06	26
	DW	3,66	97	2,29	97
AMPV1	FW	35,37	/	28,68	/
	FDW	67,86	- 92	43,05	- 50
	DW	67,23	- 90	51,58	- 80
AMPV2	FW	52,24	/	31,67	/
	FDW	211,84	- 306	125,66	- 297
	DW	78,09	- 50	51,84	- 64

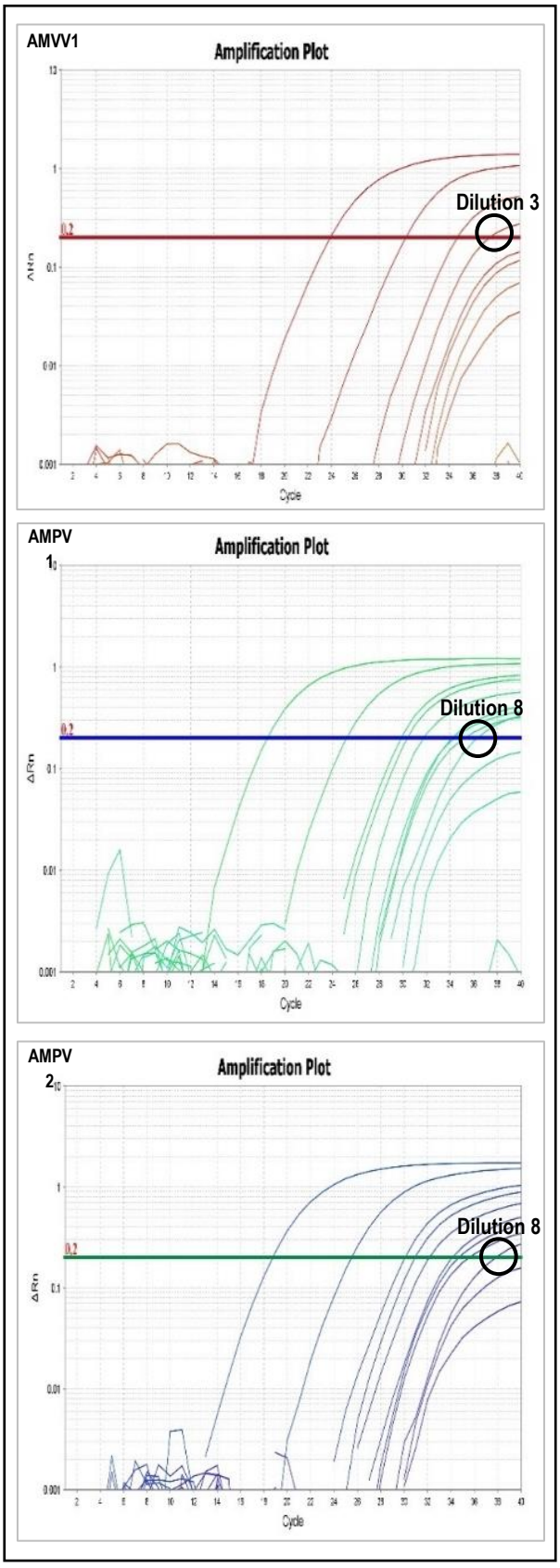

Figure 2.3: Sensitivity of the AMVV1, AMPV1 and AMPV2 simplex RT-qPCR assays. Serial dilution from 0 (24.89 ng RNA) to 10 at a factor of 1.76. Last detected dilution for AMVV1 was dilution 3, for AMPV1 and AMPV2 was dilution 8.

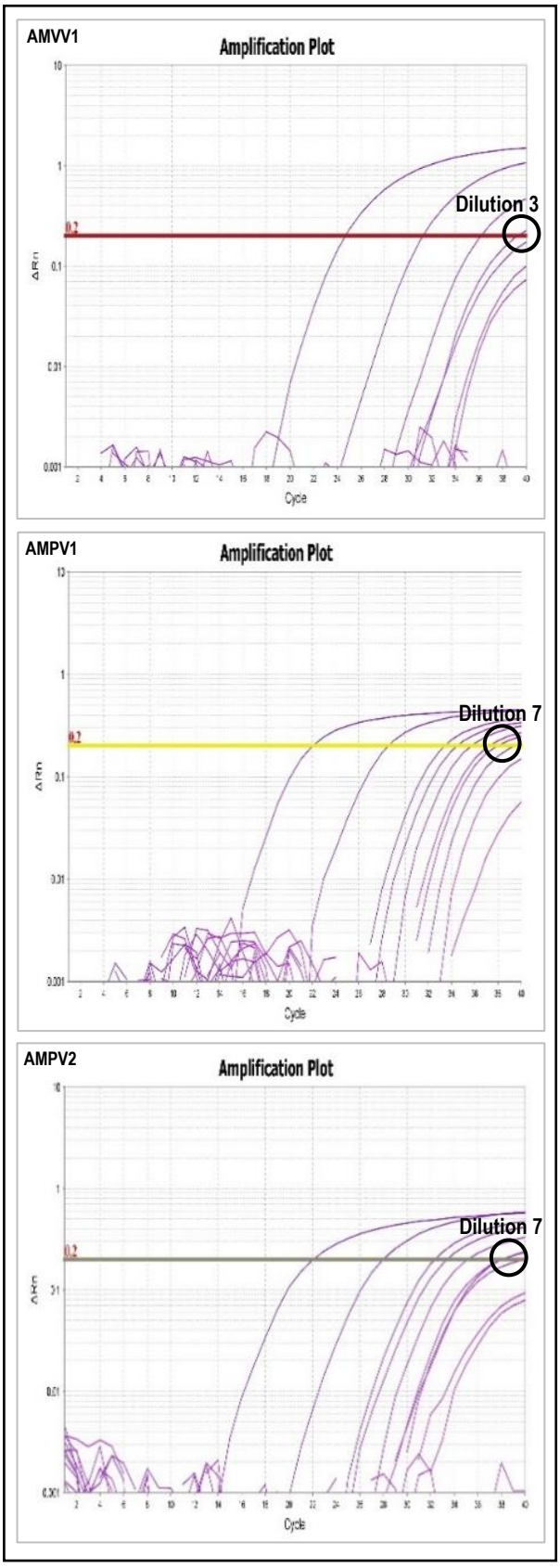

Figure 2.4: Sensitivity of the AMVV1, AMPV1 and AMPV2 multiplex RT-qPCR assay. Serial dilution from 0 (24.89 ng RNA) to 10 at a factor of 1.76. Last detected dilution for AMVV1 was dilution 3, for AMPV1 and AMPV2 was dilution 7.

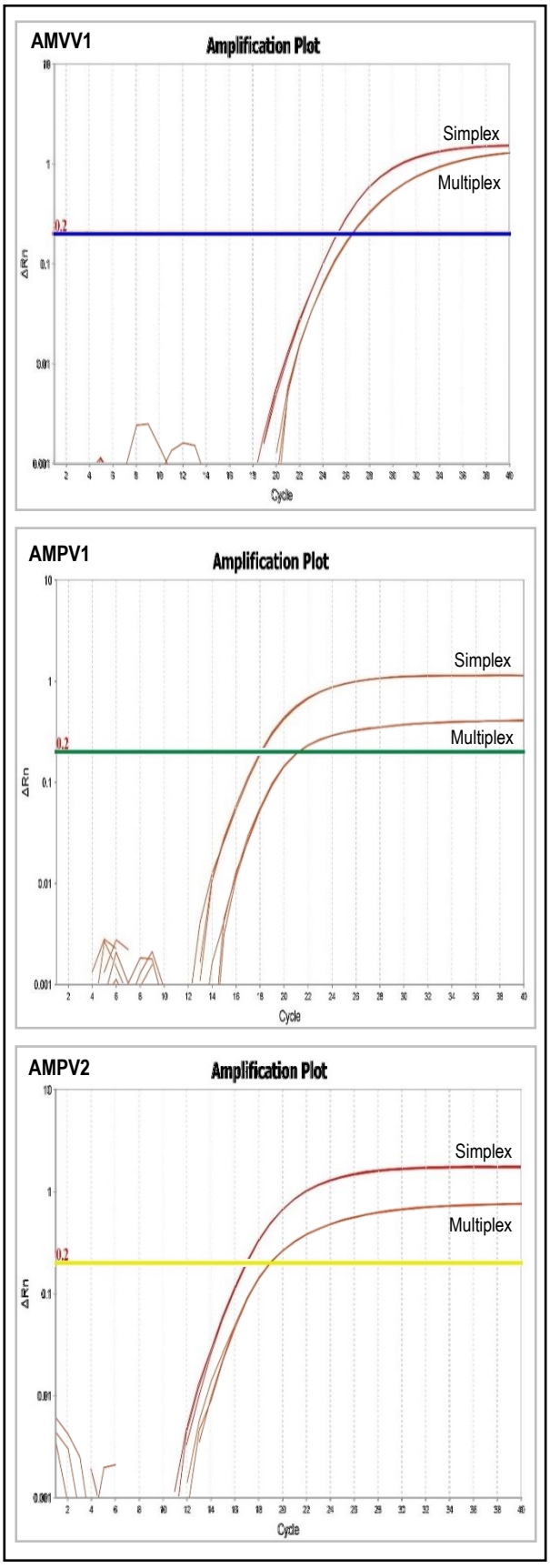

Figure 2.5: Comparison of the simplex RT-qPCR assays and the multiplex RT-qPCR assay for AMVV1, AMPV1 and AMPV2. The FW dilution 0 (24.89 ng RNA) was used. The detection sensitivity is slightly reduced in the multiplex assay.

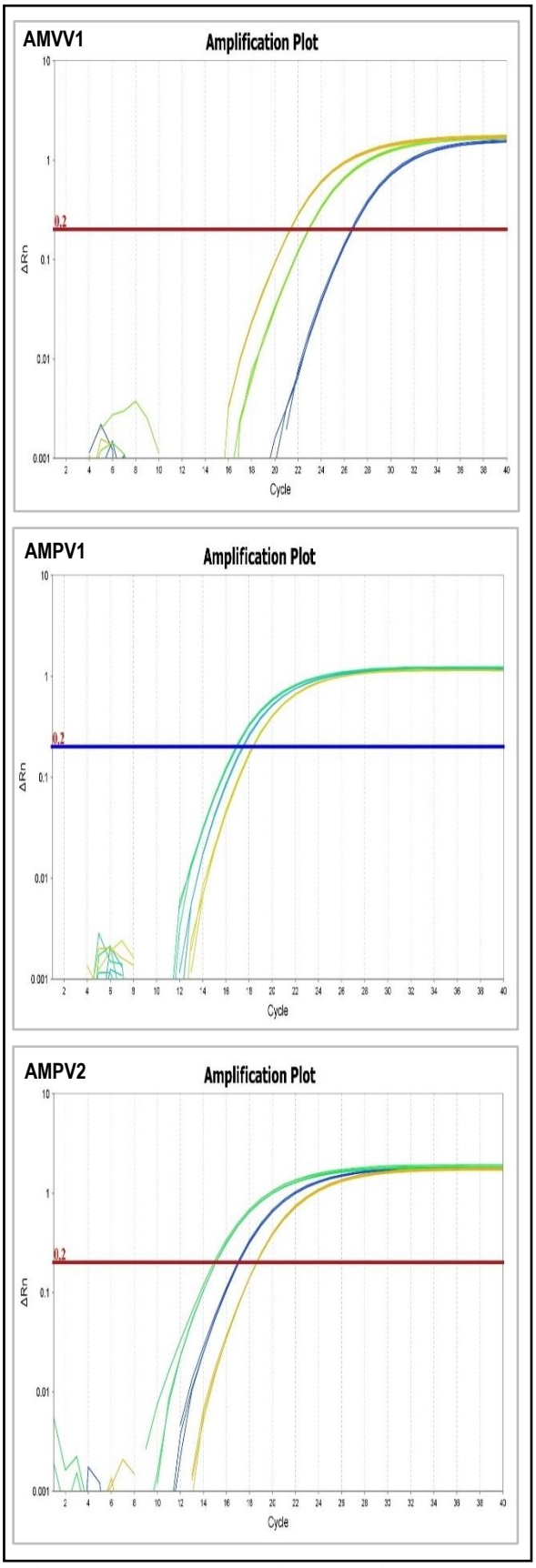

Figure 2.6: Comparison of the effect of the drying treatments on the detection sensitivity of the simplex RT-qPCR assays for AMVV1, AMPV1 and AMPV2. Yellow for FW samples, green for FDW samples and blue for DW samples.

2.4 Discussion

2.4.1 Primer specificity

The BLAST results indicated that the amplicon sequences used for our PCR assays were found in the genomes of the viruses under study and did not have a significant level of identity to any other known and uploaded genome on the NCBI database.

Our assays were tested against different *Poaceae* species: wheat, barley and oat. Ideally, this would have also been checked against non-infected black-grass material, but the lack of plants not infected with the partitiviruses made this impossible for AMPV1 and AMPV2 assays. The aim of this check was to ensure that no plant-derived sequences were being amplified in place of the virus sequences of interest. Other *Poaceae* species (wheat, barley and oat) were chosen as black-grass also belongs to this family, and thus, they are more genetically similar than other plant families. No amplification of these *Poaceae* species was observed for any of our assays. Additionally, in the case of the AMVV1 assays, non-infected plants showed no amplification, hence demonstrating that AMVV1 assays dd not amplify black-grass genetic material.

A specificity check against other grass infecting viruses was attempted. However, confirmation of the infection status of these samples only yielded one positive result (BYDV). These results could have been due to several reasons: an erroneous diagnosis of the viral infection, a loss of viral particles/RNA during sample storage, a viral titer below the limit of detection (LOD) of the used assays or a problem during extraction. Extraction was checked against WPal, which was positive for all samples, confirming that plant material was successfully extracted. However, it does not directly confirm the extraction of viral RNA, and therefore, extraction might not have been successful for all tested viruses. Regardless, the grass samples did show symptoms of viral infection (mosaic, leaf discolouration…), and the lack of any amplification does at least confirm that our assays did not detect neither the grass host nor the infecting viruses.

An additional specificity check could have been done by sequencing the amplicons to confirm the amplified sequence corresponded to the viral sequences, but given the results of the previous checks and the lack of any false-negative and false-positive results when repeating RT-qPCR tests across this project, we deemed that we had enough evidence for the specificity of our assays.

Both the simplex and multiplex RT-qPCR assays were specific to the selected AMVV1, AMPV1 and AMPV2 sequences. This is particularly important in the context of my study, as we were trying to differentiate three viruses present in the same samples, said samples also containing black-grass', and potentially other inhabitants' (pathogens, beneficial symbionts or/and saprophytes) genetic material.

2.4.2 Assay sensitivity

The dilution series was prepared with wheat extract to ensure the dilution background was as similar as possible to the non-diluted black-grass samples. Ideally, this would have been made by using non-infected black-grass but, as no partitivirus-negative samples have been found, wheat, family *Poaceae*, was used instead. This ensures a similar level of inhibitor presence to test samples and therefore, more realistic estimates of LOD than those obtained with dilutions performed in water or buffer.

The RT-PCR assays had a much higher detection limit than the RT-qPCR assays (Figures 2.2 to 2.4), particularly in the case of AMVV1 where only the non-diluted sample had a visible band, establishing the detection limit for AMVV1 at around 24.89 ng RNA. AMPV1 detection limit was established at around 14.13 ng RNA for dilution 1 which was the lowest dilution with a clearly visible band of the correct size. AMPV2 detection limit was established at around 8.03 ng RNA for dilution 2 which was the lowest dilution with a clearly visible (though faint) band. The low detection limit was a limiting factor at the start of this project, where frequently, plant samples gave negative or inconsistent results (different result when retesting), only to be found infected at a later date, indicating the need to design a more sensitive assay.

qPCR has been demonstrated to have a higher sensitivity than conventional PCR and allows for both qualitative and quantitative assessment. It is also faster and less labour intensive, as there is no requirement for gel electrophoresis to visualise the amplification products (Boonham *et al.*, 2014; Shukla *et al.,* 2016). We designed simplex RT-qPCR assays for the viruses under study. These assays were markedly more sensitive than conventional RT-PCR (Figures 2.2 and 2.3): AMVV1 detection limit was established at around 4.56 ng RNA for dilution 3, AMPV1 and AMPV2 detection limits were established at around 0.27 ng RNA for dilution 8.

It was also desirable to design a multiplex assay capable of detecting and quantifying the three viruses at the same time, reducing time and cost of testing (Boonham *et al.*, 2014; Shukla *et al.,* 2016). This assay was found to be slightly less sensitive than the simplex assays (Figures 2.3 and 2.4). The detection limit for AMVV1

was established at around 4.56 ng RNA for dilution 3, the detection limits for AMPV1 and AMPV2 were established at around 0.48 ng RNA for dilution 7. Multiplex assays have limited resources for amplification, and these are in competition between the different sequences amplified in the reaction, thus, a reduction in detection capability is expected. The effect of this reduction can be clearly seen in Figure 5. Adjusting the concentration of primers and probes, and in some cases transcriptase, is commonly recommended to balance the amplification of all sequences of interest. As different sequences can be present in a sample in very different numbers, if the concentration of each element (primers/probes) of each assay present is the same, the more frequent sequences will be more easily amplified at the beginning of the reaction, thus using up more of the resources, limiting the amplification of the less frequent sequences. The outcome of the competition is that the assay for the less frequent sequences will yield inaccurate results, in some cases even resulting in false negatives. By reducing the concentration of primers and probes involved in the amplification of the more frequent sequences or/and increasing the concentration of those involved in the amplification of the less frequent sequences, we compensate for the differences in frequency, resulting in a more even amplification of the different sequences (Boonham *et al.*, 2014; Lifetechnologies™, 2014; Shukla *et al.,* 2016). In our case, AMPV2 had the broadest range of titer values, meaning that its sequence can reach a much higher frequency in the samples than the sequences for AMPV1 and AMVV1, followed by AMPV1 and then AMVV1. Therefore, we trialled different combinations of primers and probe concentrations, focusing on the reduction of AMPV2 and AMPV1 primers concentration, to obtain a detection capability for the multiplex assay as similar as possible to the simplex assays. No important changes were observed (Table 2.5) and, consistently across all tested samples, combination B's results were the most similar to the simplex results. Thus, the combination reported in this work (concentration B), with a reduced concentration of AMPV2's primers (from 0.375 µM to 0.260 µM), was identified as being the most effective. This primer limitation optimisation resulted in more even amplification of the viruses present in mixed infections.

The qPCR assays were far more sensitive than the conventional assays, as well as less labour-intensive and time-consuming, making them a better choice for the detection of AMVV1, AMPV1 and AMPV2. However, running the assays in simplex or multiplex formats had different advantages, and using one or the other depends on the circumstances and the objectives of testing. If a highly sensitive detection and/or quantification are required, using the simplex assays is recommended. But, if all

viruses need to be detected and/or quantified at the same time, and a loss of sensitivity is acceptable, the multiplex assay allows for an important reduction in both cost and time, facilitating the processing of large numbers of samples. Nevertheless, focusing on the detection aspect, it is possible to use the multiplex assay for a faster diagnostic analysis and use the simplex assays for the confirmation of contentious results, overall reducing the time and cost of the analysis.

Table 2.5: Example of results from the combination trials for the multiplex assay. Results correspond to the average of two repeats of a seed sample, values are given in Cts. Three positive samples and a AMVV1-negative sample were trialled in the original experiment. Concentration combinations are as follow: A corresponds to a 0.375 µM primer concentration and a 0.125 µM probe concentration for all viral sequences; B corresponds to a 0.375 µM primer concentration and a 0.125 µM probe concentration for AMVV1 and AMPV1 sequences, and a 0.260 µM primer concentration and a 0.125 µM probe concentration for AMPV2 sequence; C corresponds to a 0.375 µM primer concentration and a 0.125 µM probe concentration for AMVV1 sequence, a 0.260 µM primer concentration and a 0.125 µM probe concentration for AMPV1 sequence, and a 0.150 µM primer concentration and a 0.125 µM probe concentration for AMPV2 sequence.

		Ct values		
		AMVV1	**AMPV1**	**AMPV2**
Simplex assay	A	19.25	17.53	12.06
Multiplex assay	A	19.42	17.81	11.99
	B	19.04	17.57	11.91
	C	19.10	17.49	12.14

2.4.3 Drying treatment comparison

Given the phenotypically heterogenous nature of black-grass populations, we decided to standardise the weight of the samples being used for extraction prior to viral quantification. Water content can differ importantly from plant to plant and has an important effect on sampling, with high-water-content samples containing less tissue material and cell components, due to dilution, than low-water-content samples for the same weight. These differences lead to differential extraction and analysis of elements of interest, producing misleading results. Eliminating the cell water helps standardize the samples, ensuring the measured weight corresponds only to tissue and cell components, thus, allowing for a uniform extraction and analysis across different samples. We tested a high-temperature and a cold-temperature drying treatment and compared the effect of treatment on detection and quantification of our viruses against non-treated samples.

Looking at the results for the individual viruses (Table 2.4 and Figure 2.6), we observed that AMVV1 was very susceptible to the high-temperature treatment with a 97% reduction in detection. Many viruses are susceptible to heat, this being a common treatment when trying to eliminate viral infection in plants. Both proteins and nucleic acids suffer degradation under high temperatures, leading to the degradation of viral particles and the reduction in viral replication. There was also a reduction (22%) in the freeze-dried samples, which implied that AMVV1 might be susceptible to the elimination of cell water; as we were comparing these samples to frozen samples, we can eliminate the possibility of this reduction being due to cold temperatures. As such, we recommend using the low-temperature treatment for the quantification of AMVV1, detection being preferable with non-treated samples as there is no loss, or a smaller loss, of viral particles. Using the multiplex assay did not seem to have an additive effect with the treatments, the observed reduction being very similar to that of the simplex assay.

Contrary to AMVV1, AMPV1´s detection and quantification benefited from the treatment of the samples, with an increase in detection capability of 90% for the high-temperature treatment and a 92% increase for the low-temperature treatment. This was most probably due to the concentration of the viral particles in the plant tissues after the elimination of cell water, resulting in a higher quantity of viral particles per g of plant material than in the non-treated samples. As such, detection was improved, reducing the risk of false-negatives. Additionally, AMPV1 was not affected by high temperatures with the effect of the high- and low-temperature treatment being almost identical, 90% and 92% increase in detection respectively. Given these results, we highly recommend the treatment of the samples for both detection and quantification of AMPV1. In the case of the multiplex assay, there seemed to be an additive effect with the treatments, the increase in detection being lower than for the simplex assay, particularly for the low-temperature samples.

Similarly, the detection of AMPV2 also benefited from the treatments, with a 306% increase in detection for the low-temperature treatment and a 50% increase for the high-temperature treatment. This implied that AMPV2 was also susceptible to high temperatures, but the concentration effect remained beneficial. As for AMPV1, we recommend the treatment of the samples for both detection and quantification of AMPV2, preferably using a low-temperature treatment. Again, the multiplex assay seemed to have an additive effect, with a reduction in the detection improvements of the treatments.

For this work, viral detection was done with non-treated samples using the simplex assay and, after its design and test, the multiplex assay. Since non-treated samples allowed for a faster analysis and there was no loss of AMVV1 particles. Given the high number of samples analysed, multiplex use, being faster and cheaper, was favoured regardless of its limitations. Viral quantification, on the other hand, was done with high-temperature treated samples using only the simplex assays. Firstly, the normalisation of samples´ weight results in more reliable quantification results, and the high variation in water content inside our populations made standardization necessary. Secondly, the use of an oven allowed for a high number of samples to be treated at the same time, unlike the freeze-drier which had more limited capacity, ensuring all samples in a replicate were treated under the exact same conditions. And thirdly, quantification was used in the comparative study of the effect of the viruses. Due to the lack of non-infected AMPV1 and AMPV2 plants, this study relied on the comparison of samples with different viral titers, instead of the usual presence/absence of virus. Therefore, if the loss of viral particles was uniform across all samples, which we assumed it was, the comparison factor (the difference in titer) remained the same before and after the treatment.

2.5 Conclusion

We developed three simplex and one multiplex RT-qPCR assays for the detection of AMVV1, AMPV1 and AMPV2. All assays had a high specificity and their sensitivity was increased compared to the already existing conventional RT-PCR assays. The simplex assays were slightly more sensitive than the multiplex assay, but the reduction in cost and time of analysis are important advantages of the latter. Use of the simplex or multiplex assay should be decided according to the circumstances and objectives of the study.

Drying treatments, high- and low-temperature, for sample normalisation were also tested. AMVV1 detection was reduced by both drying treatments, but particularly by the high-temperature one, strongly implying that AMVV1 is susceptible to heat. AMPV1 and AMPV2 detection was improved by the drying treatments, possibly due to a concentration of the viral particles in plant tissues following water elimination. However, AMPV2 seemed to also be susceptible to heat. As such, we recommend using non-treated samples for viral detection; unless only the partitiviruses are of interest, in which case, treatment of the samples should increase the assays efficiency. For viral quantification, assuming weight standardization is necessary, we recommend

using low-temperature treatments, to limit the deleterious effects on AMVV1 and AMPV2. However, in this work, we used a high-temperature treatment, as it allowed more efficient sample processing.

Chapter 3: Biological characterization of AMVV1, AMPV1 and AMPV2: incidence, infection pattern and transmission mechanism.

3.1 Introduction

While there is a focus on the molecular and genetic aspects of viruses for their characterization, knowledge of the symptoms they cause, their infection pattern, transmission mechanisms and spread in host populations remains essential for their classification, study and management (Cann, 2012; Gaur et al., 2016).

Viral epidemiology is defined by Hull (2009) as: “the study of the determinants, dynamics and distribution of viral diseases in host populations”; thus, two important concepts in epidemiology are incidence and infection. The oxford dictionary defines incidence as: “the occurrence, rate, or frequency of a disease, crime, or other undesirable things”, and infection as: “the process of infecting or the state of being infected”. In this chapter, we use incidence as the frequency of infection in a population, using infection as a proxy for disease, since we do not have any obvious symptoms. And infection as the state of being infected, both at a general plant level and at smaller levels such as individual tillers and tiller sections.

Both incidence and infection are important as they help establish the status and evolution of diseases, develop managing strategies for them and can help elucidate transmission and tolerance/resistance mechanisms of hosts. For newly discovered viruses, as is our case, knowing their incidence and infection pattern is essential to designing effective assays to study them further.

Viruses are obligate intracellular parasites, and as such, their survival depends on the ability to find and infect a new host before the current one dies. The passage from one host to another is known as viral transmission. In the case of plant viruses, transmission is limited by the plant cell wall, a hard, sometimes rigid, cellulose structure that protects cells, mediates the interactions between these and outside elements and forms the structural support of the plant (Hébrard et al., 1999; Hull, 2009; Cann, 2012; Gaur et al., 2016).

Viruses have developed two main strategies to pierce through this wall. The first one is horizontal transmission which relies on mechanical transmission and the use of vectors. These strategies directly penetrate the cell wall. Mechanical inoculation aims to introduce infective viral material into a plant host through light wounding of its

surface. This transmission mechanism happens naturally for some viruses such as TMV and *potato virus X* (PVX). Artificial mechanical inoculation is used routinely in viral diagnosis and characterization, as it is a simple and, in some cases, very specific technique. However, it is also time-consuming, results usually only being available after at least two weeks. Not all viral species can be transmitted mechanically, and in some cases, transmission is only possible to specific hosts. Resulting infection might be temporary, and the transmission efficiency can be low (Hébrard et al., 1999; Hull, 2009; Cann, 2012; Gaur et al., 2016).

Vector transmission is one of the main viral transmission mechanisms, especially in the case of plant viruses, which have to overcome the lack of movement of their hosts. Viruses infect the vector when it comes into contact with their host and remain inside the vector until it comes into contact with a new uninfected plant, which they infect through the channel opened by the vector in the cell wall. Plant vectors are usually characterised by being autonomous and highly mobile, and their damage to the plant is not enough to cause instant death. They include fungi, nematodes, arachnids, such as mites and ticks, and invertebrates, notably sap-sucking insects. Vector transmission can be classified according to the movement of the virus inside the vector: circulative, when the virus reaches the intestine and from there transfers to the salivary glands, and non-circulative, when the virus stays in the stylus. And the replication activity of the virus inside the vector: propagative, when there is viral replication in the vector, and non-propagative, when there is no replication (Hébrard et al., 1999; Hull, 2009; Cann, 2012; Gaur et al., 2016).

The second strategy is vertical transmission, which eliminates the need to traverse the cell wall as viruses are transmitted through reproduction. Plants can use two modes of reproduction: asexual and sexual. In asexual reproduction, there is no formation of gametes. Reproduction is either by means of vegetative propagation, new plants are formed from somatic tissues (stolons, rhizomes, tubers and bulbs) of the parent plant, or apomixis, seeds are developed from the maternal tissues without meiosis or fecundation. Thus, new individuals are clones of the parent plant. Asexual reproduction is faster than sexual reproduction but very few species rely solely on it as it does not introduce variability in the populations. In this case, viral transmission happens during the formation of the new plant, by cell division or cell-to-cell movement (Hébrard et al., 1999; Hull, 2009; Cann, 2012; Gaur et al., 2016).

In sexual reproduction, two haploid gametes, sperm cells formed in the pollen and egg cells formed in the ovule, fuse to produce a diploid zygote which will develop

into an embryo and later an adult plant. This type of reproduction increases variability in a population, making it the most common type in plants. However, many plants are hermaphrodite, meaning they can fertilize themselves, reducing the genetic variability of their offspring, an undesirable circumstance in many species. Thus, several self-incompatibility strategies have been developed by plants to avoid this (Owens, 1992; Seguí Simarro, 2010; Linhart, 2014). In this case, viruses are transmitted through seeds: pollen or/and ovules are infected and the virus is transmitted to the embryos (Hébrard et al., 1999; Hull, 2009; Cann, 2012; Gaur et al., 2016). Pollen is the male gametophyte; it produces the male gametes or sperm cells after, usually, germination on the female stigma. Pollen can be transmitted by wind, pollinator insects and animals and, in some cases, water. Therefore, it is very resistant to abiotic factors and is able to survive for long periods of time, being able to travel great distances before pollinating a new plant (Owens, 1992; Seguí Simarro, 2010; Linhart, 2014).

Sexual reproduction has been used by humans for the development of new plant varieties, including hybrids, the commercial production of crops and the study of phenotypic and genetic traits. This is mainly done by artificial breeding. A basic breeding assay consists of protecting the female parent plant from non-controlled pollination, usually by covering the flowers or the whole plant. If self-pollination is possible, the stamens are eliminated. The male parent plant is used to pollinate the female parent, either by placing them together and allowing natural transmission of the pollen, or by collecting the pollen and dusting the female parent´s stigmas with it. This process is time consuming and, in some cases (manual elimination of stamens or pollination of stigmas), very labour intensive. However, it remains an important tool in research and commercial production (Owens, 1992; Seguí Simarro, 2010; Linhart, 2014).

Knowledge of a virus' transmission pathway is an important part of their study as it helps understand its biological cycle, is used in virus classification and is an essential part of developing management strategies. It also allows for greater control when designing and carrying bioassays.

In this chapter, I studied the incidence, infection pattern and transmission mechanism of AMVV1, AMPV1 and AMPV2. In the case of the mechanical transmission, I used three different approaches. First, mechanical inoculation. We focused on mechanical transmission of AMVV1 for a number of reasons. Firstly, there were reports of mechanical transmission of LBVaV, the type-species of the genus *Varicosavirus*, which suggests that it might be possible to transmit AMVV1 through this

method. Secondly, as part of basic virus characterization, the virus being mechanically transmitted can hint to how the virus might move between cells, how it interacts with cells, etc. Thirdly, mechanical transmission would allow us to more easily study the virus' host range. And finally, it would allow us to potentially create an homogenous population which would not have the working constraints of our original population, facilitating the study of the effect of AMVV1 on its host.

Second, vector transmission. *Olpidium virulentus* is an obligate root fungus linked to the LBVD. LBVD is found worldwide in lettuce production areas and decreases yield, it´s symptoms are chlorophyll clearing, which gives the disease its name, crinkling of leaves and reduction of head size, which can also show abnormal shapes. Two viruses are associated to LBVD: MLBVV, the causal agent, and LBVaV, believed to be asymptomatic, but Verbeek et al. found that it causes necrotic symptoms in lettuce. Both viruses are transmitted by O. virulentus zoospores. LBVaV is currently the only accepted varicosavirus by the ICTV, therefore, we hypothesised that AMVV1 could also be vector transmitted by O. virulentus. Zoospores of this fungus are mobile in liquid and are released in large numbers under flood conditions. Based on this, we set-up two different assays to study the transmission of AMVV1 by O. virulentus (Maccarone, 2013; Lot et al., 2002).

Third, pollen transmission. We hypothesised that our viruses might be pollen transmitted, since this has previously been demonstrated for some partitiviruses. In addition, we had observed some cases of AMVV1 contamination in healthy control samples that could be explained if the virus was present in the pollen.

3.2 Material and methods

3.2.1 Plant material

Seeds previously collected from five different English black-grass populations: Peldon (Peldon, Essex), Roth (Rothamsted, Hertfordshire), Nott (Nottingham, Nottinghamshire), Camb (Huntington, Cambridgeshire) and Mart (Marton, North Yorkshire), conserved at 4 °C, were used. County origin of these populations can be found in Figure 3.1. Seeds from these populations were sown and grown for at least one and a half months before sampling.

Initially, cereal species were used to test mechanical transmission: wheat (Triticum aestivum) var. Diego (n = 46), barley (Hordeum vulgare) var. Venture (n = 16) and oat (Avena sativa) var. Coast black (n = 16). But, after finding AMVV1-negative plants in some of the populations, black-grass plants from the following UK populations

were used: Roth (n = 17), Nott (n = 21), Camb (n = 18) and Mart (n = 20). Similarly, for the inoculum method, initially, cereal species were used: wheat (n = 5), subsequently, AMVV1-negative black-grass plants were used: Roth (n = 17), Nott (n = 21), Camb (n = 20) and Mart (n = 20). And for the vessel method, initially, cereal species were used: wheat (n = 7), barley (n = 4) and oat (n = 8), subsequently, AMVV1-negative black-grass plants were used: Roth (n = 21), Nott (n = 20). All AMVV1- positive plants used belonged to the Peldon population.

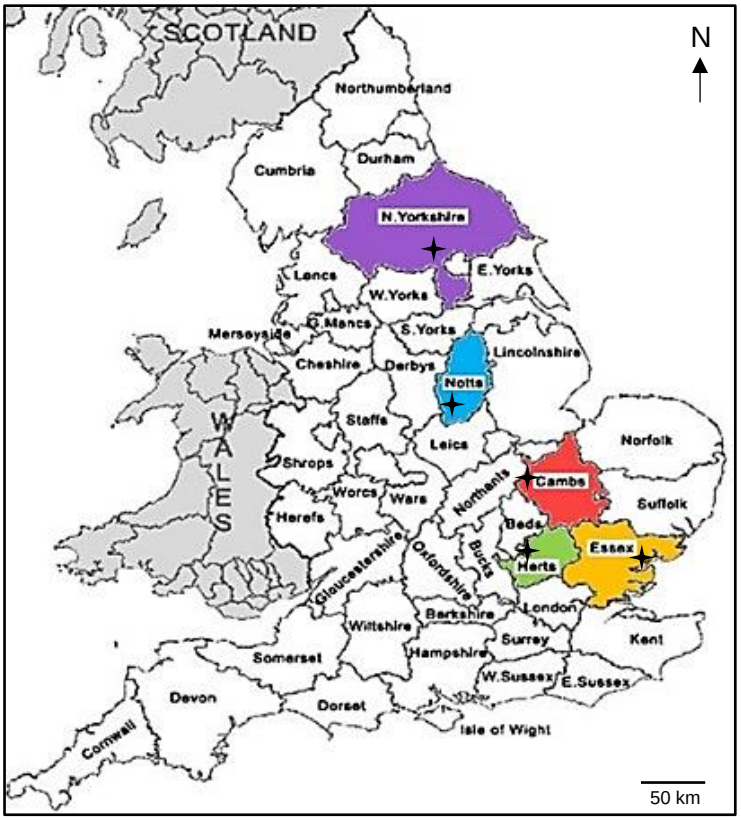

Figure 3.1: Map of England showing the geographical origins of the black-grass populations. Colour code: Peldon in yellow, Roth in green, Nott in blue, Camb in red and Mart in purple.✦ gives an approximation of the original location within the counties.

3.2.2 Extraction methods

Viral RNA was extracted from leaf samples as follows: 0.3 g of tissue was homogenised in 4 mL extraction buffer (Mumford, 2002) and centrifuged at 20 000 x g for 2 min. Total RNA was extracted using the Kingfisher®mL system (Thermo labsystems) method according to Mumford (2002). Samples were eluted in 200 µL molecular grade water and stored at -20°C.

O. virulentus extraction was done following a modified version of Qiagen's DNeasy® Plant Mini Kit protocol: root samples were extracted using Qiagen's DNeasy® Plant Mini Kit. A 1.5 mL tube is prepared with a mixture of 0.5 mm and 1 mm

glass and zirconia/silica beads (Thistle Scientific) and 400 µL of buffer AP1. Samples are grinded with liquid nitrogen using a pestle and mortar and added to the tube. 4 µL of RNase A are added and mixed by tube inversion. Cell disruption is ensured by using a Precellys 24 (Bertin technologies) at 6800 rpm for two 30 s cycle. Protocol is followed normally from step 3. Samples were stored at -20°C.

3.2.3 Detection methods

Viral detection was done as follows: the RT-qPCR reaction mix was prepared using Biorad's iTaqTM Universal Probes One-Step kit; 1 µL of sample was added to a reaction mixture containing the kit's mastermix and RNA reverse transcriptase, 375 nM of each primer and 125 nM of the probe, to make up a final volume of 12 µL. Cycling conditions were as follows: 50°C for 10 mins for the reverse transcription step, 95°C for 2 mins for the initial denaturation step, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Primer and probe sequences can be found in Table 2.3.

The detection method for O. virulentus is as follows: primer sequences were taken from Herrera-Vásquez et al. (2009). The PCR reaction mix was prepared using Thermofisher's Hot Start PCR 2X Master Mix; 1 µL of sample was added to a reaction mixture containing the mastermix and 200 nM of each primer, to make up a final volume of 15 µL. Cycling conditions were as follows: 94°C for 5 mins, followed by 35 cycles of 94°C for 45 s, 55°C for 60 s and 72°C for 1 min and a final step of 72°C for 10 min. Samples were run on a C1000TM Thermal Cycler (Biorad). Amplified products were separated on a 1.2% agarose gel stained with ethidium bromide and visualised using a UV transilluminator.

3.2.4 AMVV1, AMPV1 and AMPV2 incidence

A total of 134 plants were tested for the AMVV1, AMPV1 and AMPV2. 62 plants from the Peldon population and 18 plants from each of the remaining populations. Peldon being the main experimental population for this project, it has a higher number of screened plants than the other populations.

3.2.5 *AMVV1, AMPV1 and AMPV2 i*nfection pattern

To study the infection pattern at the intra-tiller level, four flowering tillers from four different plants were taken and divided into the following sections: flower, leaves, stem and roots (Figure 3.2). To study the infection pattern at the inter-tiller level, four plants were divided into all their tillers (Figure 3.3), for a total of 115 samples, each plant having between 20 and 30 tillers. These were extracted with no weight standardization due to limitations in the amount of material.

Figure 3.2: Structure of black-grass tillers. Vegetative stage on the left and flowering stage on the right.

Figure 3.3: Tiller structure of a black-grass plant.

Additionally, a total of 489 plants from the five populations were tested by RT-qPCR across this project and their Cts compared. The Ct (threshold cycle) is the PCR cycle number at which the fluorescent signal of the reaction crosses the threshold, the Ct is used to calculate the initial DNA copy number, because the Ct value is inversely related to the starting amount of target (Lifetechnologies™ ,2014). Virus titers in 50 plants from the population Peldon, were quantified as follows: an extracted sample found positive for the three viruses, and showing low Ct values for them, was selected to create a 10-fold serial dilution. The sample was nanodropped to know its RNA content. The RT-qPCR was run with the samples we wanted to quantify and the dilution series. The dilution series results were used to create a standard curve (Figure 3.4), by plotting the obtained Ct values against the log of the RNA concentration in each dilution. The resulting linear equation was used to infer the RNA concentration of the samples. This process was done for each individual PCR plate. The extraction and RT-qPCR analysis were carried out as before, using leaf samples. The samples used were dried using the high-temperature drying treatment (Chapter 2, section 2.2.1) to standardize their weight. We did not use a standard reference gene as we do not have enough information on the black-grass genome and transcriptome: there is no

reference gene for this species, and while a reference gene for wheat or other Poaceae species could have been used, we also had no information on whether it would work the same way in black-grass. Therefore, we chose to use the above method for viral quantification.

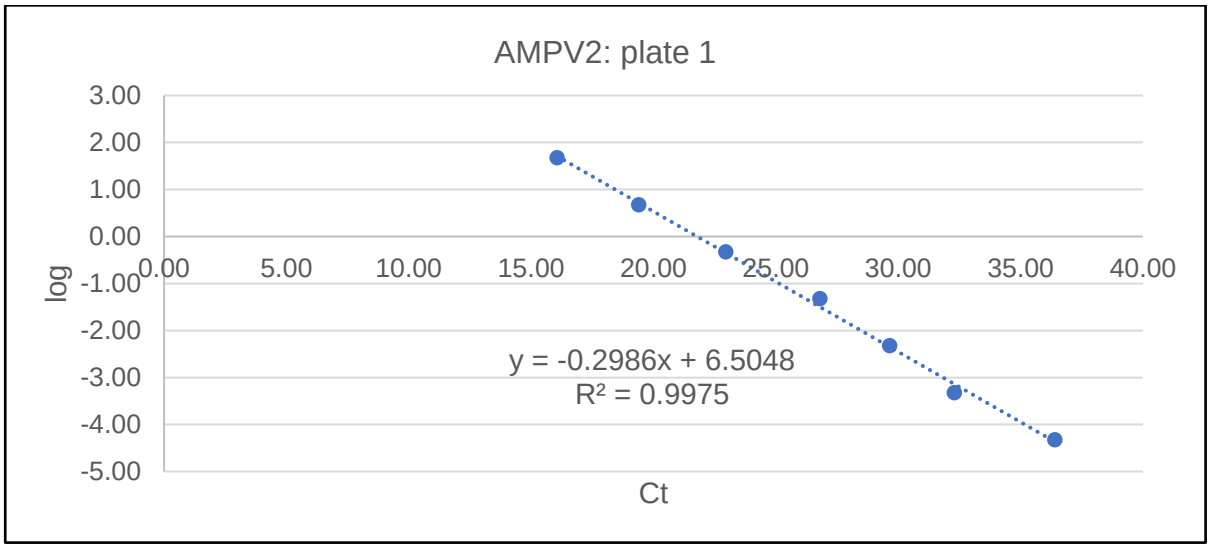

Figure 3.4: Example of a standard curve used in the quantification assays. A 7-point dilution series was used. Ct values (PCR cycle number at which the fluorescent signal of the reaction crosses the threshold) are placed on the x-axis and the log of the RNA concentrations (ng RNA / µL extract) are on the y-axis. The graph, linear equation and correlation coefficient (R^2) were created with Windows Office's Excel.

3.2.6 Mechanical transmission

Following a modified version of Huijberts et al. (1990), AMVV1-positive leaves were homogenised in the inoculation buffer, adding celite instead of activated charcoal. This inoculum was rubbed onto the leaves of three-week-old AMVV1-negative plants. Mock-inoculated plants were inoculated with AMVV1-negative leaves. Control plants were mock inoculated with buffer. Plants were left to grow for four weeks, sampled and analysed for AMVV1 using RT-qPCR. The assay was repeated once for barley and oat, twice for wheat and three times for non-infected black-grass.

3.2.7 Olpidium virulentus transmission

Two methods were used to test O. virulentus transmission of AMVV1: the inoculum method and the vessel method.

The inoculum method was done following Lot et al. (2002): roots infected with AMVV1 and O. virulentus were incubated in distilled autoclaved water for three hours. AMVV1-negative plants were watered with 5 mL of the inoculum. The inoculum for Mock-inoculated plants was prepared with AMVV1-negative roots infected with O. virulentus. Control plants were inoculated with clean distilled autoclaved water. Plants

were left to grow for four weeks, sampled and analysed for AMVV1 using RT-qPCR. The transmission experiment was repeated once for wheat and three times for non-infected black-grass.

The vessel method was based on the principle behind Lot et al. (2002) method and a variation on the method used for the soil isolation method in PM 7/66 (1) (EPPO, 2006), vessels were filled with liquid Petri solutions and AMVV1-positive and O. virulentus infected roots were submerged in the solution (Figure 3.5). AMVV1-negative tillers were left to grow with their roots submerged in the solution. If necessary, Petri solution was topped-up. The transmission experiments were repeated once for barley, oat and wheat and three times for non-infected black-grass.

Figure 3.5: Example of the Vessel assay using a black-grass tiller.

3.2.8 Pollen transmission

To test pollen transmission, we started by checking if our viruses are present in pollen and seeds. Two samples of approximately 0.1 g of seeds from each population were extracted and analysed by RT-qPCR as before. Pollen was collected from plants. Two samples of about 0.03 g of pollen from AMVV1-negative plants and two samples from AMVV1-positive plants were extracted as before. Another four samples, two AMVV1-positive and two AMVV1-negative, were mixed with 0.3 g of fresh wheat leaves and extracted as before. This was done on the one hand, to check if infected pollen was responsible for the contamination of wheat control samples, and on the other hand, to facilitate extraction in case the pollen samples without leaves were not homogenised effectively. All samples were analysed by RT-qPCR.

A series of breeding assays, focused on AMVV1 as no non-infected plants were found for AMPV1 and AMPV2, were established. Positive plants were taken from the Peldon population and negative plants were taken from all other populations. A first breeding assay was performed as follows: AMVV1-negative flowers were covered with paper envelopes before reaching maturity. AMVV1-positive flowers with mature stamens were cut and introduced into the envelopes, making sure to dust the pollen on the flowers of the virus-free plants. Controls of virus-positive and -negative plants were set-up by covering flowers with envelopes. Flowers were left to mature until seeds were harvested (between 3 and 4 months). After harvesting, seeds were sown and germinated F1 plants were tested by RT-qPCR. However, the method negatively affected the covered flowers: the whole stem dried and flowers did not mature correctly. Thus, most of the seeds were not viable.

In the second assay we substituted the paper envelopes with pollination bags (PBS international), which covered the entire plant (Figure 3.6). Pairs of AMVV1-positive, from the Peldon population, and -negative plants were covered with the bags and left to grow, flower and develop seeds. Control plants were covered alone. Seeds were harvested and sown. However, there was no seed germination, therefore, we tested the remaining seeds using RT-qPCR.

Figure 3.6: Breeding assays using PBS international pollination bags.

In the third assay, we repeated the above experiment. After harvesting, approximately 0.1 g of seed from each cross and control was extracted and tested using RT-qPCR as before. The remaining seeds were kept in a fridge at 4 °C for two weeks to imitate a short vernalization process, after which they were sown.

3.3 Results

3.3.1 AMVV1, AMPV1 and AMPV2 incidence

Results are presented in Table 3.1. AMPV1 and AMPV2 had a 100% incidence in all populations. AMVV1 had a 44.03% incidence across the five populations. In more detail, AMVV1's incidence in each population is as follows: 79.03% in Peldon, 0% in Roth, 16.67% in Nott, 11.11% in Camb and 27.78% in Mart.

Table 3.1: Incidence of AMVV1, AMP1 and AMPV2 in 5 English black-grass populations. Number of positive plants is given against the total plants screened.

	AMVV1	AMPV1	AMPV2
Peldon	49 / 62	49 / 62	49 / 62
Roth	0 / 18	18 / 18	18 / 18
Nott	3 / 18	18 / 18	18 / 18
Camb	2 / 18	18 / 18	18 / 18
Marb	5 / 18	18 / 18	18 / 18

3.3.2 AMVV1, AMPV1 and AMPV2 infection pattern

At the intra-tiller level, we saw that all sections from the same tiller showed the same infection pattern. However, titer varied between sections: high titers of AMVV1 and AMPV2 were mostly found in flowers and leaves, while high titers of AMPV1 were mostly found in leaves and stems.

At the inter-tiller level, we saw that all tillers from the same plant showed the same infection pattern. However, titer varied between tillers.

Overall, we observed a wide range of variation in viral titer across all populations for all three viruses, Cts ranging from around 15 to 40. The results (Table 3.2) show that the variation between plants of the same population can be up to 224 pg RNA for AMVV1, 3728 pg for AMPV1 and 8061 pg for AMPV2. We also note that AMVV1 seems to have a narrower titer range, the highest values found being in the hundreds of pg, while AMPV1 and AMPV2 go up to the thousands, with AMPV2 showing the highest values. No obvious correlation between the three viruses was observed; a high or low titer of one virus does not affect the titer of the other viruses. We observed plants with similar relative titer values for all the viruses, titers being in the low, middle or high end of the titer range for each virus; and plants with completely different relative titer values, titers being in the low, middle and high end of the titer range for each virus. None of these combinations seemed to have a distinct effect on the host visual phenotype (size, number of tillers, number of leaves, days to flowering, etc.).

Table 3.2: Quantification results from 50 Peldon plants. Dry weight material was used following the high-temperature treatment presented in chapter 2 (section 2.2.1). Results are given in pg RNA/µg plant. Colour scale classification (done independently for each virus): lowest value in green, 50th percentile in yellow and highest value in red. Note that in the case of AMPV1 and AMPV2, a value of 0.00 does not indicate a lack of infection but simply that the titer value was low enough that 0.00 is the resulting value when reducing the number of decimals for visual ease.

Plant sample	pg RNA/ µg plant material8		
	AMVV1	AMPV1	AMPV2
1	87,62	41,07	431,40
2	76,02	266,23	596,99
3	157,25	228,61	0,04
4	128,18	0,01	0,02
5	0,00	0,01	1647,76
6	78,16	0,00	0,03
7	135,99	0,00	278,06
8	224,31	394,76	702,30
9	99,28	369,21	336,21
10	0,00	0,01	0,01
11	0,00	0,01	0,24
12	68,01	92,68	429,66
13	0,00	694,33	0,02
14	11,90	0,02	371,49
15	144,55	0,01	0,01
16	0,00	0,01	467,63
17	46,65	264,85	0,02
18	0,00	34,47	486,96
19	21,78	0,00	1,01
20	158,75	0,00	0,01
21	0,00	0,02	0,03
22	0,00	21,67	161,01
23	0,00	72,40	55,51
24	0,00	0,00	0,02
25	0,00	0,01	292,86
26	91,86	0,00	0,00
27	0,00	16,22	36,90
28	0,00	0,01	0,02
29	67,83	0,00	0,00
30	92,89	0,00	373,28
31	0,00	0,07	0,01
32	20,44	0,03	0,01
33	25,93	0,02	0,01
34	34,98	0,58	546,25
35	0,00	0,03	0,04
36	4,54	19,42	7496,53
37	11,53	86,97	3254,90
38	0,01	0,55	8062,00
39	7,90	41,64	509,06
40	9,79	683,71	222,23
41	0,00	4,86	0,18
42	0,00	1,41	0,05
43	2,69	1738,04	494,60
44	0,01	3728,17	0,37
45	0,03	2,12	316,12
46	12,85	1,33	0,90
47	9,81	0,20	0,43
48	0,09	5,06	16,46
49	0,00	0,02	0,05
50	0,00	0,01	0,08

3.3.3 Mechanical transmission

All control, mock-inoculated and inoculated plants, of all species and populations, were found to be negative for AMVV1.

3.3.4 Olpidium virulentus transmission

Presence of *O. virulentus* was tested in root and inoculum samples by conventional PCR to confirm its transmission through these methods. It was present in both types of samples. For both methods, inoculum and vessel, all Control, Mock-inoculated and Inoculated plants, including all species and populations, were found negative for AMVV1.

3.3.5 Pollen transmission

Seed and pollen had the same infection pattern as the populations and plants, respectively, they originated from (Table 3.3). All pollen samples were infected with AMPV1 and AMPV2. AMVV1-positive plants had AMVV1-positive pollen and vice-versa. The same results were obtained when including wheat leaves in the samples, which confirmed that the extraction method was effective and that the previous contamination issues could have been due to the presence of infected pollen. All seeds were infected with AMPV1 and AMPV2. Only seed from the Roth population was not infected with AMVV1.

Table 3.3: Pollen and seed test. "+" indicates the sample was infected, "-" indicates the sample was not infected. "- pollen" stands for the samples made of pollen from AMVV1-negative plants, "+ pollen" stands for the samples made of pollen from AMVV1-positive plants, "- pollen/wheat" stands for the samples made of pollen from AMVV1-negative plants and mixed with fresh wheat leaves, "+ pollen/wheat" stands for the samples made of pollen from AMVV1-positive plants and mixed with fresh wheat leaves.

	Pollen				**Seed**			
	- pollen	+ pollen	- pollen /wheat	+ pollen /wheat	Peldon	Roth	Nott	Mart
AMVV1	-	+	-	+	+	-	+	+
AMPV1	+	+	+	+	+	+	+	+
AMPV2	+	+	+	+	+	+	+	+

In the first breeding assay, only one of the crosses produced viable seeds resulting in 5 plants. Two plants were shown to be positive for AMVV1, with Ct values (22.82 and 23.46) very similar to that of the father plant (22.52). The remaining three plants were negative (Table 3.4).

Table 3.4: Breeding assay 1. Infection results for the 5 F1 plants, from the same cross, that germinated in this assay. All seeds were sown and therefore could not be tested. "+" indicates the sample was infected, "-" indicates the sample was not infected.

	F1 plant				
	1	2	3	4	5
AMVV1	-	-	+	-	+

In the second breeding assay, there was no seed germination, therefore, we tested the remaining seeds using RT-qPCR. The following crosses were tested: Peldon 10 x Roth, Peldon 9 x Roth, Peldon 2 x Camb and Roth control. The results showed that all the crosses produced infected seeds. Ct values were slightly higher for Peldon 10 x Roth (33.47) and Peldon 2 x Camb (34.33) than the values found for their Peldon parents (27.19 and 27.33 respectively) and slightly lower for Peldon 9 x Roth (25.92 against 27.32). The negative control for the Roth population produced negative seeds (Table 3.5).

Table 3.5: Breeding assay 2. Infection results for the F1 seeds, no plants germinated in this assay. "+" indicates the sample was infected, "-" indicates the sample was not infected.

	F1 Seeds			
	Roth control	Roth x 10	Roth x 9	Camb x 2
AMVV1	-	+	+	+

In the third assay, analysis of the seeds showed differences between the crosses. Two lines of clonal plants were used as the AMVV1-positive parents: 10 and 7. All crosses with line 10 gave a positive result, with Cts ranging between 26.71 and 28.07. However, when using line 7, only one cross was positive, Camb x 7, with a Ct of 32.75. Controls for Roth, Mart, Nott and Camb were negative. Controls for line 10 and 7 were positive, with Ct values of 22.81 and 26.01 respectively. The remaining seeds were kept in a fridge at 4 °C for two weeks to imitate a short vernalization process, after which they were sown. Three plants germinated, corresponding to crosses Nott x 10, Camb x 10 and the Roth control, and they were tested by RT-qPCR. Results were as follow: 23.59 for Camb x 10 and Nott x 10 and Roth control were negative (Table 3.6).

Table 3.6: Breeding assay 3. AMVV1 infection results for the F1 seeds and the F1 plants (only one plant germinated per cross). “+” indicates the sample was infected, “-” indicates the sample was not infected, “/” indicates that no plants germinated for that cross.

		Breeding crosses													
		Roth control	Mart control	Nott control	Camb control	10 control	7 control	Roth x 10	Mart x 10	Nott x 10	Camb x 10	Roth x 7	Mart x 7	Nott x 7	Camb x 7
AMVV1	F1 Seeds	-	-	-	-	+	+	+	+	+	+	-	-	-	+
AMVV1	F1 plants	-	/	/	/	/	/	/	/	-	+	/	/	/	/

3.4 Discussion

3.4.1 Incidence and infection pattern of AMVV1, AMPV1 and AMPV2

AMPV1 and AMPV2 seemed to be widespread in all screened populations, which suggests that they might be found in all black-grass populations in England. This fact is characteristic of cryptic viruses (Boccardo *et al*., 1987; Hull, 2009; Roossinck, 2010; Roossinck, 2011).

AMVV1, on the other hand, had a lower and variable incidence in the screened populations. If we consider these populations as representative of the whole of England, the virus' incidence would be below 50%. The population with the highest incidence was Peldon (70.03%), and the two populations with the lowest incidence were Roth and Camb (0% and 11.11% respectively), which were geographically the closest to Peldon (see Figure 3.1). This implies that there might be a transmission barrier to AMVV1 as the spread of the virus does not seem to be straightforward. Either the transmission mechanism is through vectors, which might not be equally present in all English regions, or some black-grass populations might present a higher resistance against this virus than others, said populations being heterogenous, this is highly likely.

Infection pattern being the same in all sections and tillers from one plant suggested that infection of all three viruses was systemic.

At the tiller level, the variations in titer might have been due to the concentration of the viruses in specific organs. In the analysed tillers, the three viruses seemed to mainly concentrate in flowers and leaves. During the vegetative stage, black-grass stems tend to be much smaller than flowering stems, thus, leaves and roots would be the most effective reservoirs for virions. However, leaves tend to be more metabolically active than other plant sections, including roots, which would favour high viral replication in them. It is interesting that in the case of AMVV1 and AMPV2, the highest titers were mostly found in the flowers. As these only appear at the final life stages, it could imply that these viruses are mobilised from the leaves to the flowers during flowering. If transmission of these viruses is vertical, this mobilization would help maximise viral transmission efficiency.

At the population level, the fact that titer is not uniform, especially in the case of the partitiviruses, which have a 100% incidence, implied that this variation might be actively maintained by the hosts, which in turn suggests that it could be beneficial. This variation in titer could be explained by the heterogenicity of the studied populations, some plants maybe displaying a higher resistance or lower replication capacities for the viruses. Wild populations maintain genetic and phenotypic heterogenicity, as this

makes them more resilient to drastic changes in their environment. Resistance and/or tolerance to stresses has fitness penalties to the plants. For example, a plant highly tolerant to drought may be smaller and produce less descendants than a non-tolerant one. Under non-drought conditions, this would limit the population size. But, under drought conditions, the tolerant plants will have a higher chance to survive and produce more descendants than the non-tolerant one. In both cases, the presence of the two types of plants ensures the survival and spread of the population. In the case of the viruses under study, while there were no obvious fitness penalties observable in the form of disease symptoms, viral replication still uses host resources and energy, which has a negative impact on infected plants compared to non-infected ones (Sultan, 1987; Primack and Kang, 1989; Wolfe and Mazer, 2005; Baythavong and Stanton, 2010). If the viruses have a beneficial effect on their host, there is a possibility that it is a conditional benefit. And therefore, the observed range of viral titers would ensure that this benefit, and its attached penalties, are maximized in some plants, while both benefits and penalties are minimized in others. This would result in the survival of the host population under different conditions, increasing its fitness (Sultan, 1987; Primack and Kang, 1989; Wolfe and Mazer, 2005; Baythavong and Stanton, 2010).

However, it should be kept in mind that part of the observed titer variation was probably due to the extraction method. As mentioned before, weight was not standardized. There were differences of size in tiller sections as well as between tillers, older tillers being bigger than younger ones. Meaning that some samples had more plant material than others, which could influence the amount of viral material extracted. Additionally, some sections, in particular roots, might have had a higher presence of inhibitors, which again, could affect the efficiency of the extraction and RT-qPCR analysis.

3.4.2 Transmission mechanism of AMVV1, AMPV1 and AMPV2

No evidence of mechanical transmission of AMVV1 was found. While mechanical transmission might be possible as Huijberts, Blystad and Bos (1990) and Lot *et al.* (2002) reported the transmission of LBVaV, the type-species of the genus *Varicosavirus*, to different hosts, its efficiency seems to be very low. It must be noted that this assay did not include a positive control, that is a control were a virus know to be mechanically transmissible to the host is used to check that the transmission method worked. However, there is no information on what viruses are mechanically transmissible to black-grass, thus we were not able to include this control. Therefore,

it is possible that the lack of transmission was also due to the lack of efficiency of the method used on black-grass.

No evidence of vector transmission by *O. virulentus* of AMVV1 was found. As mentioned before, *O. virulentus* is the vector of LBVaV. However, since there is only one member in the *Varicosavirus* genus, it is possible that *O.* virulentus will not be the vector of all possible varicosaviruses.

The results from the seeds and pollen test showed that the viruses were carried in both pollen and seed in all populations, which strongly implied that the viruses are transmitted vertically. To verify this, we set-up the breeding experiments. Analysis of F1 plants in the first experiment and of F1 seed in the second and third experiment, showed transmission of AMVV1 to seeds of non-infected plants. However, transmission of AMVV1 was not 100% efficient, with the F1 plants, from the same cross, being both infected and uninfected, although we were unable to estimate a transmission efficiency due to the small numbers of viable seeds produced during the experiments. The transmission efficiency varied between different plants: in the third experiment, we used two different Peldon lines to transmit AMVV1 and found that one of the lines, line 10, was able to transmit the virus more efficiently to all other populations; all seed testing was positive but, when looking at germinated plants, one of them was negative, indicating that line 10 still did not have a 100% transmission efficiency. While the other line, line 7, only transmitted it to the Camb population (no germination was obtained from crosses with line 7). This could help explain the varying incidence of this virus in the populations.

Taken together, these results suggest that the main transmission mechanism of our viruses is pollen transmission. Vertical transmission is the only known transmission mechanism for partitiviruses. On the other hand, the *Varicosavirus* genus and the other plant-infecting *Rhadoviridae* genuses are vector transmitted. Only the genus *Sigmavirus* (arthropod host) seems to be vertically transmitted in this viral family (Wolfe and Mazer, 2005).

3.5 Conclusion

AMPV1 and AMPV2 share the common characteristics of the *Alphapartitivirus* genus: they were vertically transmitted and widespread in the studied populations. Titer variability was observed inside all populations and could help increase their fitness, by improving population plasticity. As with the rest of the partitiviruses, their study will be

constrained by the difficulties in transmitting them and the lack of non-infected individuals.

AMVV1 showed variable incidence and titer in the populations and vertical transmission. This is unusual for members of the *Rhabdoviridae* family, with only one genus, *Sigmavirus*, showing vertical transmission. It is possible that the variability in incidence and titer might be due to different levels of resistance to this virus or/and to the efficiency of the transmission. As with AMPV1 and AMPV2, this variability might result in enhanced population fitness. Additional studies on viral transmission are necessary to verify the lack of vector transmission.

Chapter 4: Effect of AMVV1, AMPV1 and AMPV2 on black-grass drought tolerance.

4.1 Introduction

Drought is one of the most important abiotic stresses affecting both crop and non-crop species worldwide. According to the Food and Agriculture Organization (FAO), between 2005 and 2015, there were 29 billion USD in losses to developing world agriculture due to drought (FAO, 2018). Furthermore, climate change is increasing its frequency and severity, increasing the interest in plant drought tolerance studies (Farooq et al., 2009).

According to Salehi-Lisar and Bakhshayeshan-Agdam (2016) drought stress occurs when “the available water for plants in soil is decreased due to low soil moisture at a certain time”. It affects growth and development, usually inducing stunted growth and lower yields and leading to plant death in extreme conditions (Farooq et al., 2009; Salehi-Lisar and Bakhshayeshan-Agdam, 2016). Plants have developed different tolerance mechanisms at the physiological and biochemical level, which generally aim to maintain cell water homeostasis, by reducing water loss, through stomatal closure and osmotic adjustments, and increasing water uptake, through changes to the size and structure of the root system (Farooq et al., 2009). Additionally, certain endophytes can contribute to plant tolerance (Xu, 2008; Davis et al., 2015a).

Cryptic viruses are a group of plant persistent viruses whose characteristics highlight a very close relationship between cryptic viruses and their hosts, suggesting that the latter actively preserves these viruses, see section 1.5.3 (Boccardo et al., 1987; Roossinck, 2011; Sabbadin et al., 2017). Three cryptic viruses were recently discovered by Sabbadin et al. (2017) in black-grass populations: AMPV1, AMPV2 and AMVV1, see section 1.6.

Cryptic viruses are attracting interest as potential beneficial endophytes of their plant hosts. But due to a lack of non-infected plants, in most cases, and the difficulties in working with this type of viruses, at the time of writing, only in two cases has a function been found. WCCV1 was found to regulate nodulation (Nakatsukasa-Akune et al., 2005) and PCV-1 helps deter aphids from their host (Safari et al., 2019). These findings open the door to the possibility of using cryptic viruses to improve crop tolerance to biotic and abiotic stresses. However, our current knowledge on these

viruses is very limited. As AMVV1, AMPV1 and AMPV2 seem to act as cryptic viruses, I hypothesize that they are beneficial endophytes of black-grass.

In this study, I investigate if the potential beneficial effect of AMPV1, AMPV2 and AMVV1 on black-grass is improved tolerance to drought stress, as this is one of the main abiotic stresses black-grass suffers in its natural environment.

4.2 Material and methods

4.2.1 Viruses

The studied viruses were identified by Sabbadin *et al.* (2017) using a non-targeted next generation sequencing approach. AMVV1 was identified as belonging to the genus *Varicosavirus* and shows variable incidence in the Peldon population. AMPV1 and AMPV2 were identified as belonging to the genus *Alphapartitivirus* and were found to be widespread in all surveyed populations.

4.2.2 Plant material

The black-grass Peldon population (Essex, UK) was used. Plants were grown from seeds and specific lines propagated by tillering. Viral titer was assessed by using RT-qPCR and 14 plants were selected and propagated to use as study lines. Plants were grown for three weeks before using them in the bioassays. Growth conditions were: 20-18 °C and 16-8 h light/dark cycle.

4.2.3 Drought stress assays

Due to the lack of plants not infected with AMPV1 and AMPV2, the comparative study was done using plants with different viral titer instead of comparing presence and absence of viruses. Three groups were created out of the selected 14 lines (Table 4.1): a low titer group, with plants that have a low viral titer for the three viruses; a medium titer group, with plants that have a medium viral titer for the three viruses; and a high titer group, as no plants with a high viral titer for the three viruses were found, we divided this group into three subgroups, each with plants having a high titer for one virus and a low titer for the other two. To account for the phenotypic variation in the population, we included two or more lines per group and subgroup.

Two identical 3 week old populations were established, each composed of 5 plants from each line. One population acted as the control and was watered daily. The other was placed under drought stress by withdrawing water for 7 days. Both populations were placed in the same glasshouse cubicle and environmental conditions were assumed to be identical (Figure 4.1).

Table 4.1: Selected clonal lines for the drought stress assays divided by titer groups. The viral titers are given in pg RNA/µg plant material and correspond to the average titer of six plants from each line. Colour scale classification (done independently for each virus): lowest value in green, 50th percentile in yellow and highest value in red.

		Line	Average pg RNA/µg plant material		
			AMVV1	AMPV1	AMPV2
Low titer		1	0.00	0.07	0.01
		5	0.00	0.03	0.04
		19	0.00	0.02	0.05
		20	0.00	0.01	0.08
Medium titer		7	11.53	86.97	3254.90
		9	7.90	41.64	509.06
		10	9.79	683.71	222.23
		16	12.85	1.33	0.90
High titer	AMVV1	2	20.44	0.03	0.01
		3	25.93	0.02	0.01
	AMPV1	13	2.69	1738.04	494.60
		14	0.01	3728.17	0.37
	AMPV2	6	4.54	19.42	7496.53
		8	0.01	0.55	8062.00

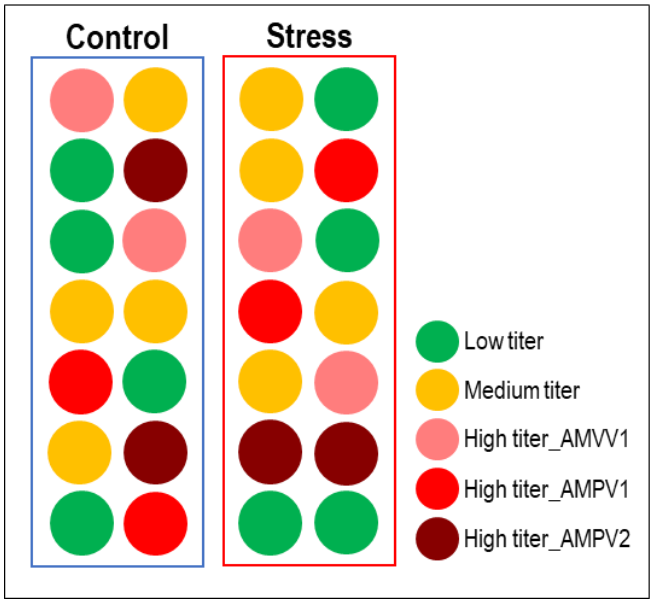

Figure 4.1: Diagram representing the experimental set-up. Each population had the same number of plants and lines; 5 plants per line. Plants were placed at random on opposing sides of the glasshouse. After the initial growth period and right before starting the bioassay, half of the plants from each side of the glasshouse were moved to the other side and all plants were reshuffled at random. This was done to ensure both populations were as similar as possible; plant position in a glasshouse can affect growth as there may be small differences in light intensity, temperature, water accumulation…, by moving the plants before the bioassay we ensure that these possible differences are present in both populations and have a low impact on our results.

4.2.4 Physiological measures

Different physiological measurements were taken to study the effect of the stress: Height, from the base of the stem to the tip of the longest leaf. Fresh weight, including roots, and dry weight, after drying in an oven at 65 °C for two days. From the weight measurements the water content percentage was estimated as follows: ((FW-DW)*100)/FW. Number of tillers, number of leaves per tiller, taking three random tillers per plant, and number of yellowing leaves.

4.2.5 Extraction method

Leaf samples were dried as above, and 0,3 g of dried material were extracted as follows: samples were homogenised in 6 mL extraction buffer (Mumford, 2002) and centrifuged at 20 000 x g for 5 min. Total RNA was extracted using the Kingfisher®mL system (Thermo labsystems) method according to Mumford (2002). Samples were eluted in 200 µL molecular grade water and stored at -20°C.

4.2.6 Detection method

The PCR reaction mix was prepared using Biorad's iTaqTM Universal Probes One-Step kit; 1 µL of sample was added to a reaction mixture containing the kit's mastermix and reverse transcriptase, 375 nM of each primer and 125 nM of the probe, to make up a final volume of 12 µL. Cycling conditions were as follows: 50°C for 10 mins for the reverse transcription step, 95°C for 2 mins for the initial denaturation step, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Primer and probe sequences can be found in table 2.3. Quantification was done as in section 3.2.3.

4.2.7 Statistical analysis

I acknowledge the collaboration of James Rainford and Roy McArthur (statistics department, Fera) in the statistical analysis. Raw data was collected, cleaned and presented to them by me. They advised on and ran the models and ANOVA analysis using R as shown below. Results were sent back to me and I interpreted them in the context of this study.

Modelling was conducted using generalised mixture models, as a way of structuring the expected variance for individuals belonging to the same line. Lines were implemented as a random effect. This statistical tool models individuals from the same line as showing greater similarity than would be expected by chance given the modelled population, but treats this similarity as having no mean effect on the effect of the measured parameters on growth. One way to visualise this effect is to consider that the lines within the study are treated as drawn from some large external population

and that the random effect characterises the average similarity between plants of the same line in this hypothetical population.

Model implementation was taken from the R packages lme4 (Bates *et al.* 2015) and lmerTest (Kuznetsova *et al.*, 2017). Implemented models assumed a Poisson distribution for measurements expressed as counts (number of tillers and number of yellowing leaves) and Gaussian ('Normal') distribution for all other measures. Following the previous protocol, leaves per tiller measure was fitted as a binomial model of the probability of leaves on each tiller exceeding 3, the most common value obtained within the dataset. Percentage water content was transformed via a logit prior to fitting to allow for use of the Gaussian methodology. All models were fitted under maximum likelihood and model comparison was conducted using ANOVA with respect to the Chi-squared distribution.

4.3 Results

4.3.1 Effect of drought stress on black-grass plants

After seven days of drought, there were obvious visual differences between the Control and the Stress populations in all replicates (Figure 4.2). Stressed plants showed stunted growth, loss of leaf turgor, as well as curling and yellowing.

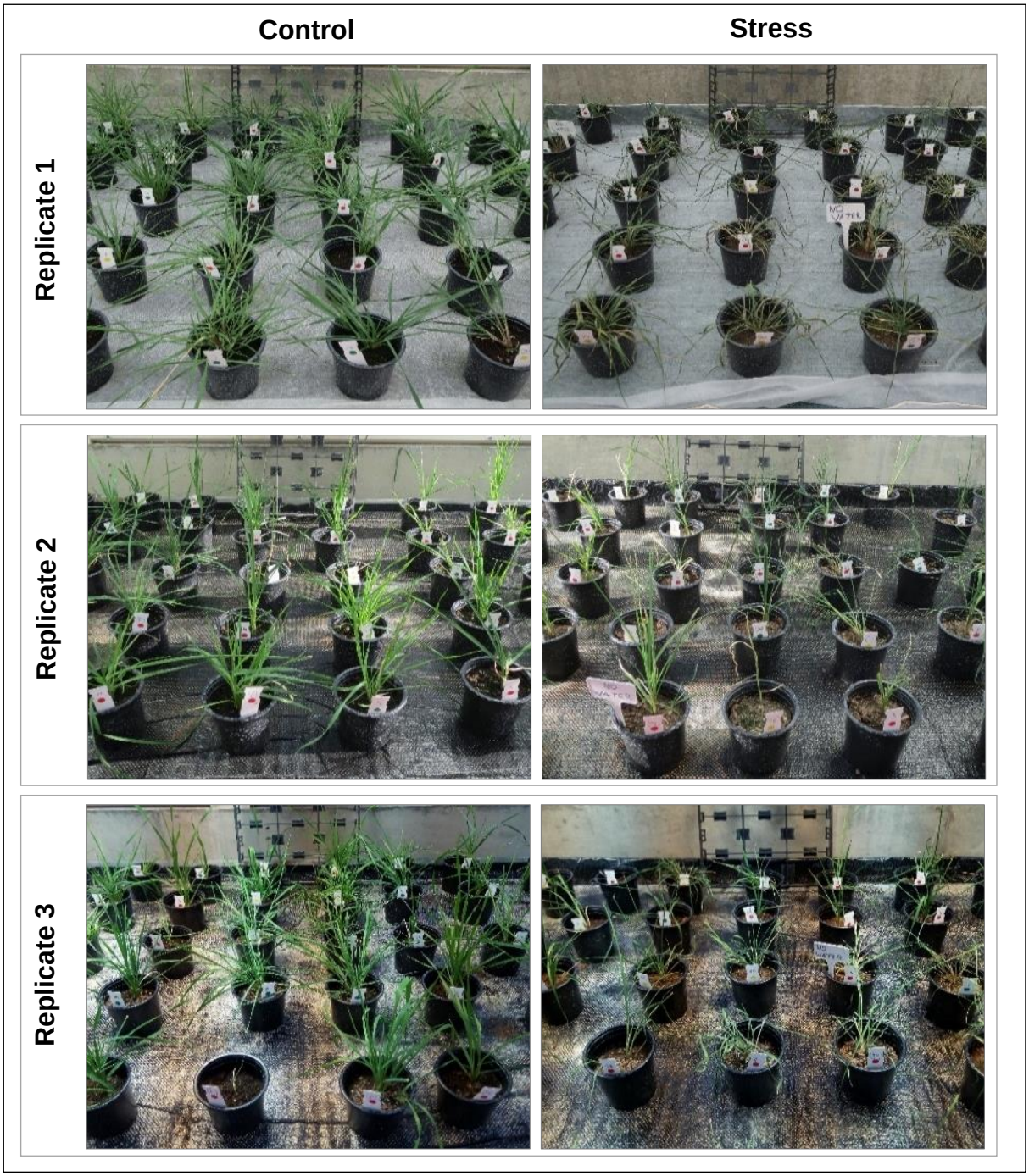

Figure 4.2: General comparison of the effect of drought stress (day 7 of the bioassay) on black-grass populations. The Control population (left column) was watered normally and the Stress population (right column) was not watered for 7 days. The three replicates are included: replicate 1 in the first row, replicate 2 in the second row and replicate 3 in the third row. The populations were made up of 5 plants from each of the lines presented in Table 4.1 and placed at random. Plant pots are 10 cm in diameter.

Both the control and stress population in all three replicates showed very similar mean height, with no significant differences between control and stress (Figure 4.3). In Replicate 1, the average height was the same for the two populations (254.73 mm in the control population and 254.03 mm in the stress population). In Replicate 2, the stress population (265.29 mm) was higher than the control (263.57 mm) by only two millimetres. While in Replicate 3, the control population (289.15 mm) was higher than the stressed population (273.65 mm).

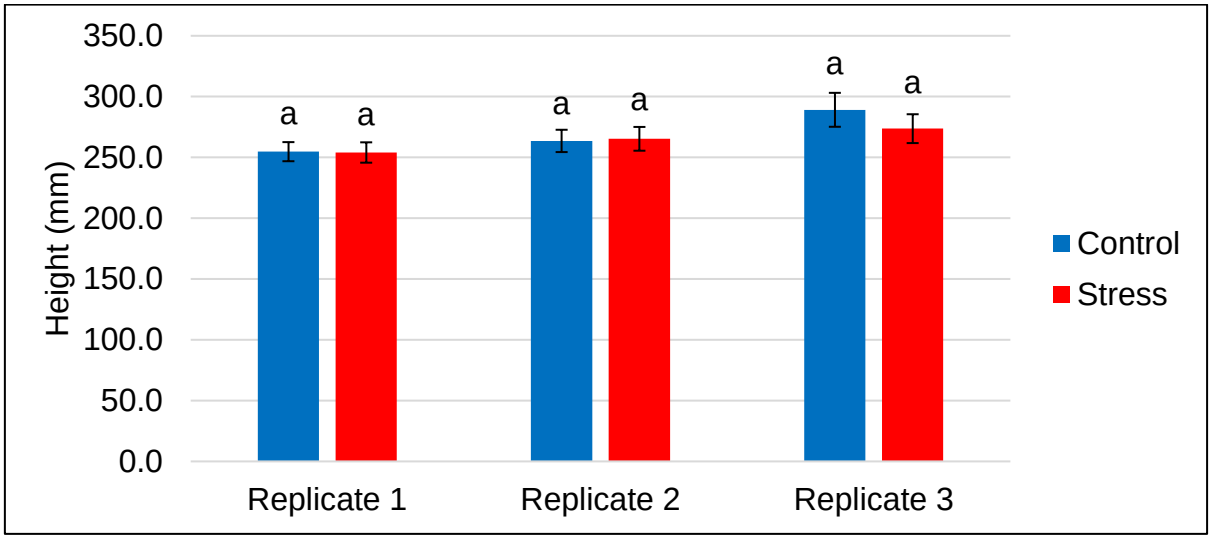

Figure 4.3: Mean height and its standard error (error bars) for the control and stress populations in the three replicates. Means with a different letter are significantly different ($p < 0.05$).

There was a reduction in the number of tillers under drought stress (Figure 4.4). The highest number of tillers for both control (17.67 mean tillers) and stress (9.92 mean tillers) population was found in Replicate 1, which also showed a significant difference between the two populations. Replicate 2 and 3 showed no significant differences between their control and stress populations.

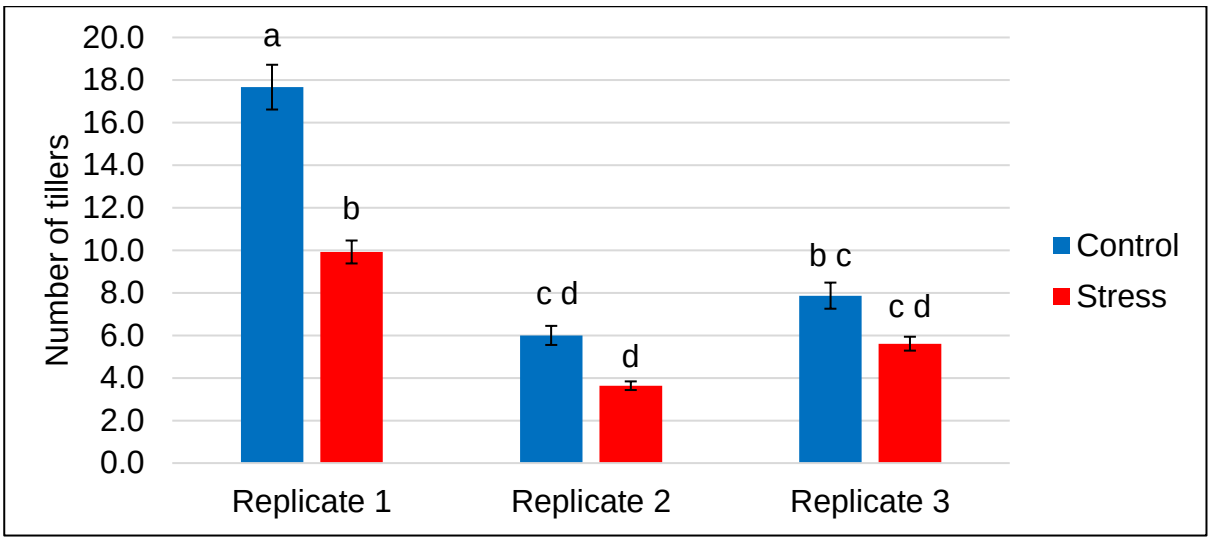

Figure 4.4: Mean number of tillers and its standard error (error bars) for the control and stress populations in the three replicates. Means with a different letter are significantly different ($p < 0.05$).

The differences in the average number of leaves per tiller were minimal (Figure 4.5). The averages range from 3.13 leaves (replicate 1 control) to 3.68 leaves (replicate 3 stress). There were no significant differences between populations in any of the replicates. In replicates 1 and 3 the control populations showed a smaller number of leaves than the stressed populations. In the case of replicate 2 both populations had the same number of leaves.

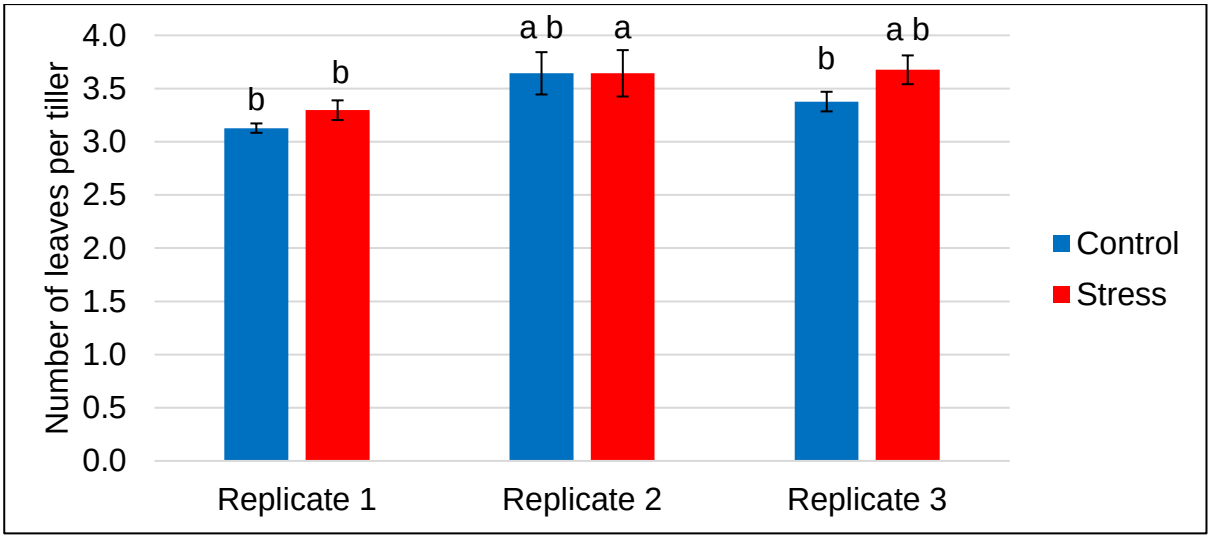

Figure 4.5: Mean number of leaves per tiller and its standard error (error bars) for the control and stress populations in the three replicates. Means with a different letter are significantly different ($p < 0.05$).

The average number of yellow leaves was greatly increased under drought stress, with all replicates showing a significant difference between their control and stress

populations (Figure 4.6). Replicate 1 showed the biggest difference between the control (0.70 mean yellow leaves) and stress (7.44 mean yellow leaves) population.

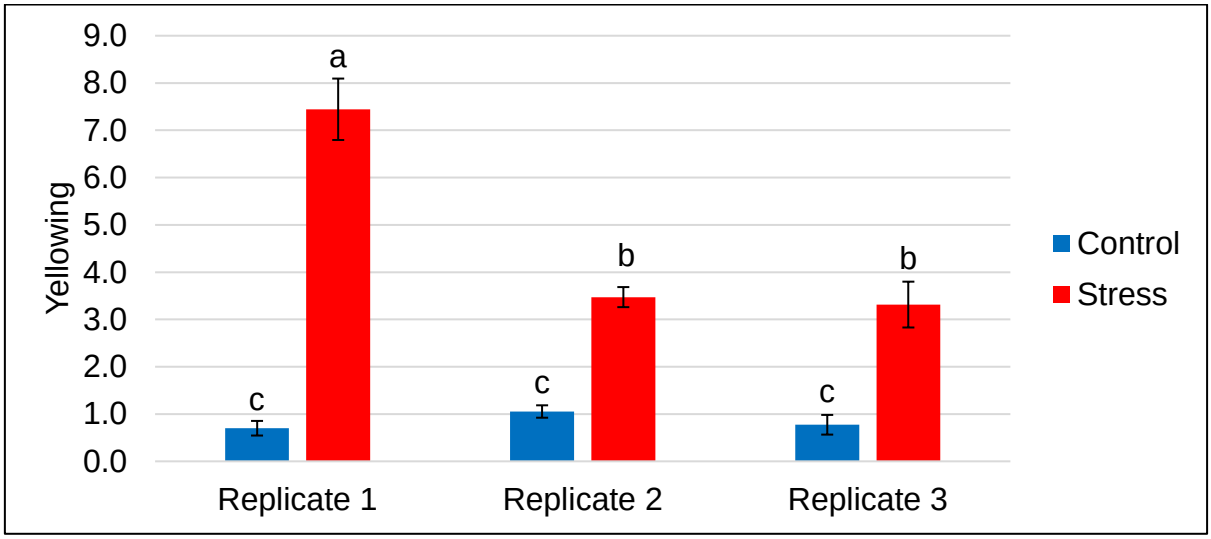

Figure 4.6: Mean number of yellow leaves and its standard error (error bars) for the control and stress populations in the three replicates. Means with a different letter are significantly different ($p < 0.05$).

Fresh weight was greatly reduced under drought stress, with all replicates showing a significant difference between their control and stress populations (Figure 4.7). Again, replicate 1 showed the highest values and the biggest difference between the control (8.65 g) and stress (1.60 g) population.

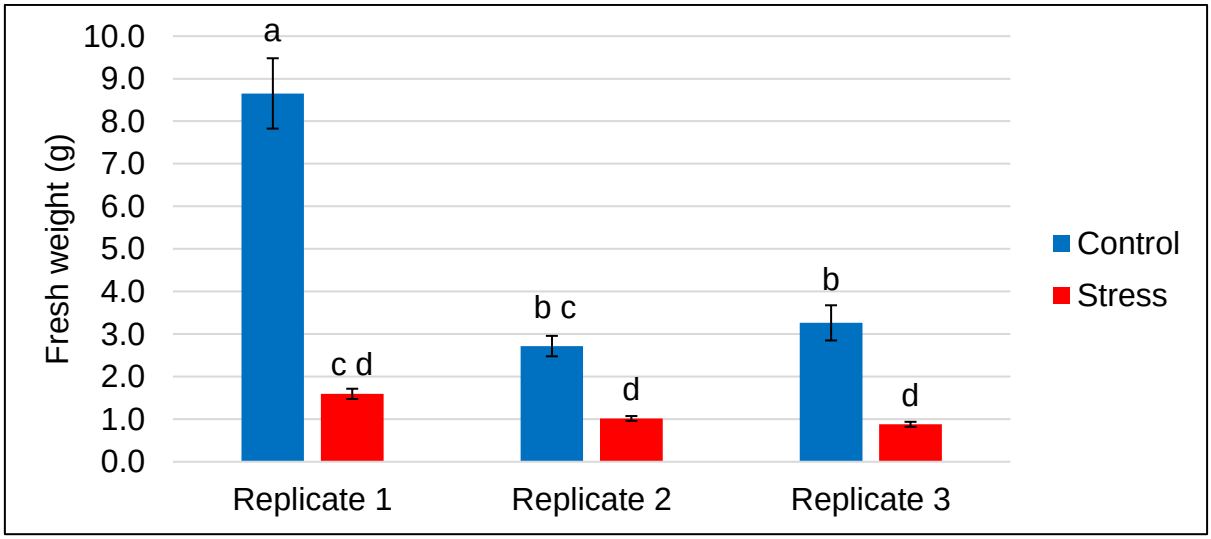

Figure 4.7: Mean fresh weight and its standard error (error bars) for the control and stress populations in the three replicates. Means with a different letter are significantly different ($p < 0.05$).

There were important differences in dry weight between the replicates (Figure 4.8): in replicate 1 we found the highest values and the control population (1.32 g) had

a higher average dry weight than the stress population (0.96 g), being significantly different. In replicate 2, the control population (0.56 g) was slightly higher than the stress population (0.53 g). And in replicate 3, the control and stress populations had the same average dry weight (0.47 g)

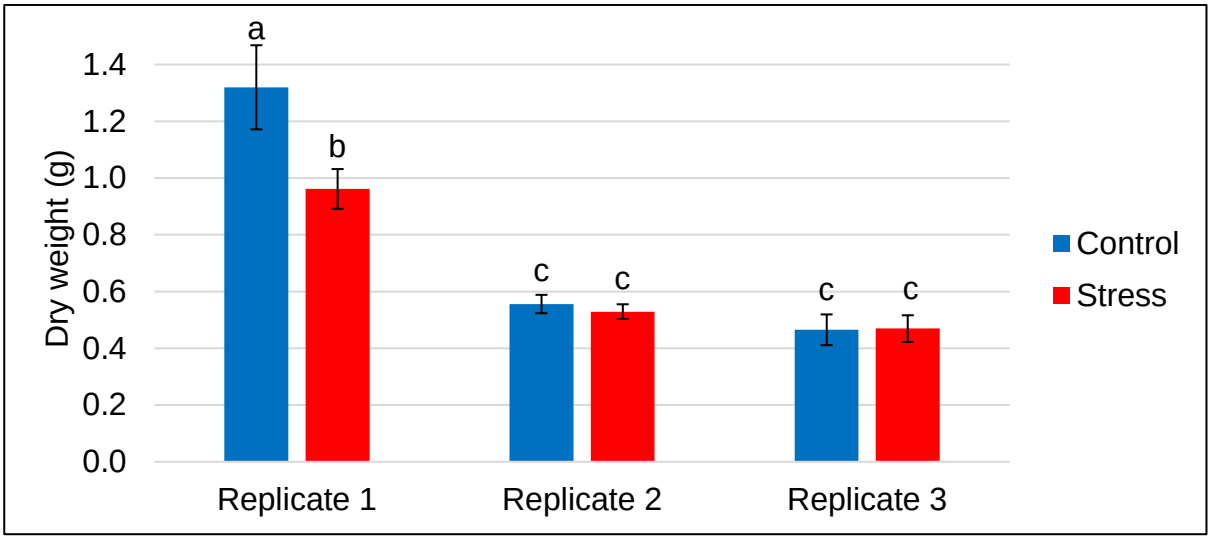

Figure 4.8: Mean dry weight and its standard error (error bars) for the control and stress populations in the three replicates. Means with a different letter are significantly different ($p < 0.05$).

Water content was reduced under drought stress, with all replicates showing a significant difference between their control and stress populations (Figure 4.9). Again, the biggest difference between control and stress was found in replicate 1 (85.60% and 39.64% respectively).

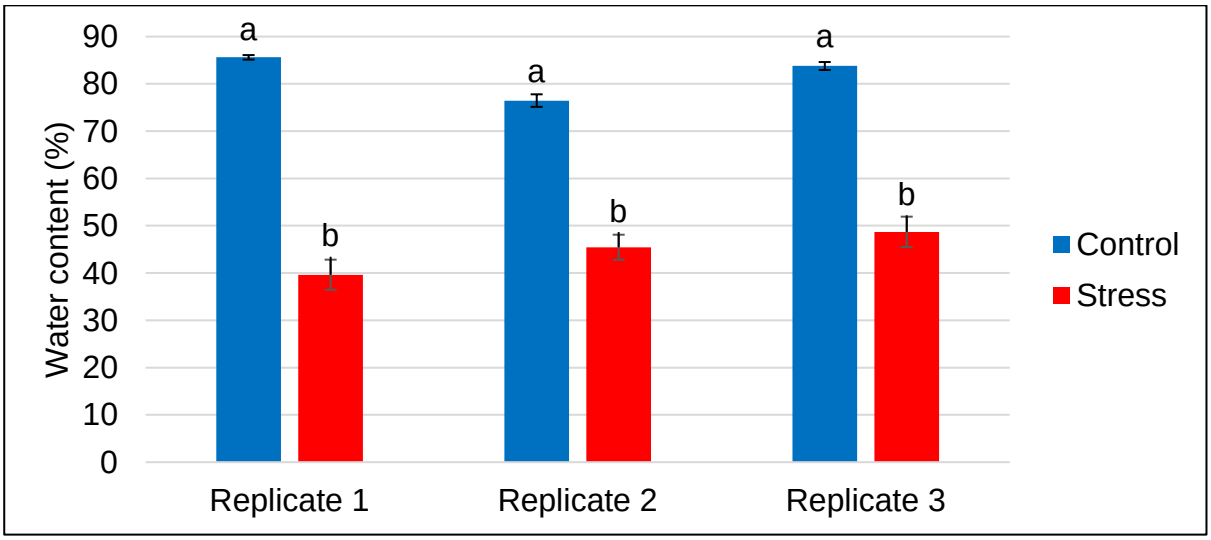

Figure 4.9: Mean water content and its standard error (error bars) for the control and stress populations in the three replicates. Means with a different letter are significantly different ($p < 0.05$).

The statistical analysis showed that the control and stress populations were significantly different (Figure 4.10).

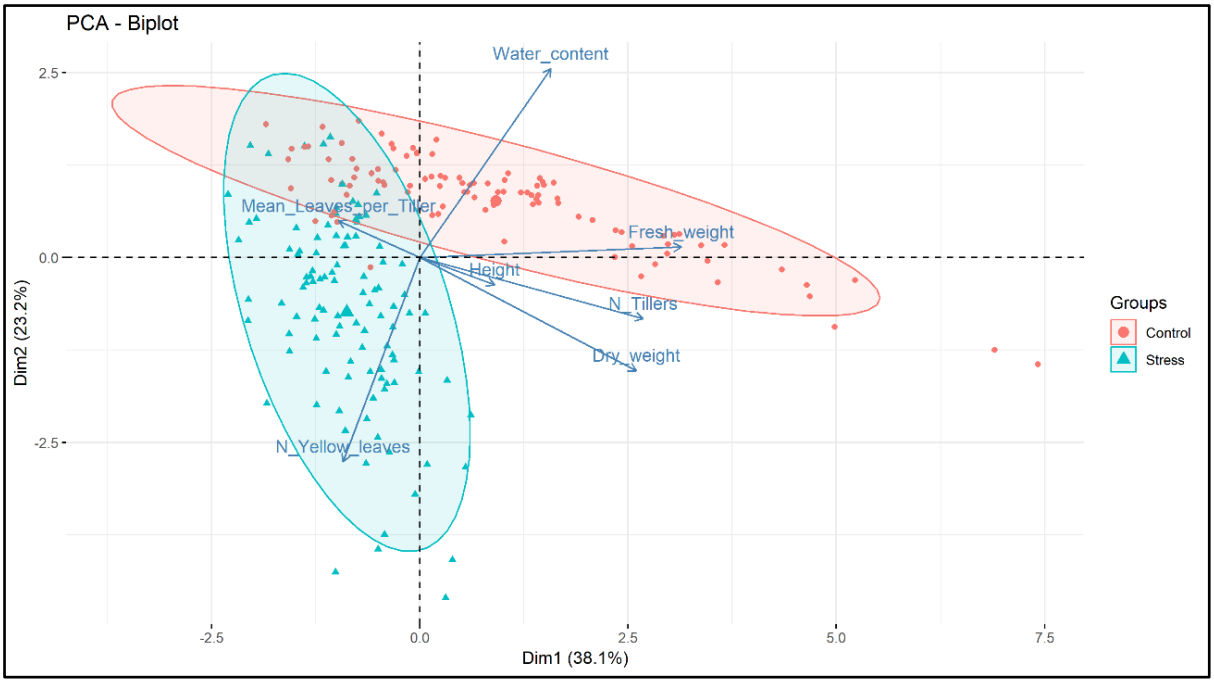

Figure 4.10: Visual representation of the control and stress populations at the end of the bioassay (day 7). Bi-plot of the first two components of PCA of growth measures. Points represent the scores of individuals, lines denote the vector direction of the various measures with respect to the principal components.

4.3.2 Effect of drought stress on the titer groups

While the control and stress populations were visually distinct, no obvious visual differences were observed between the different titer groups (Figure 4.11).

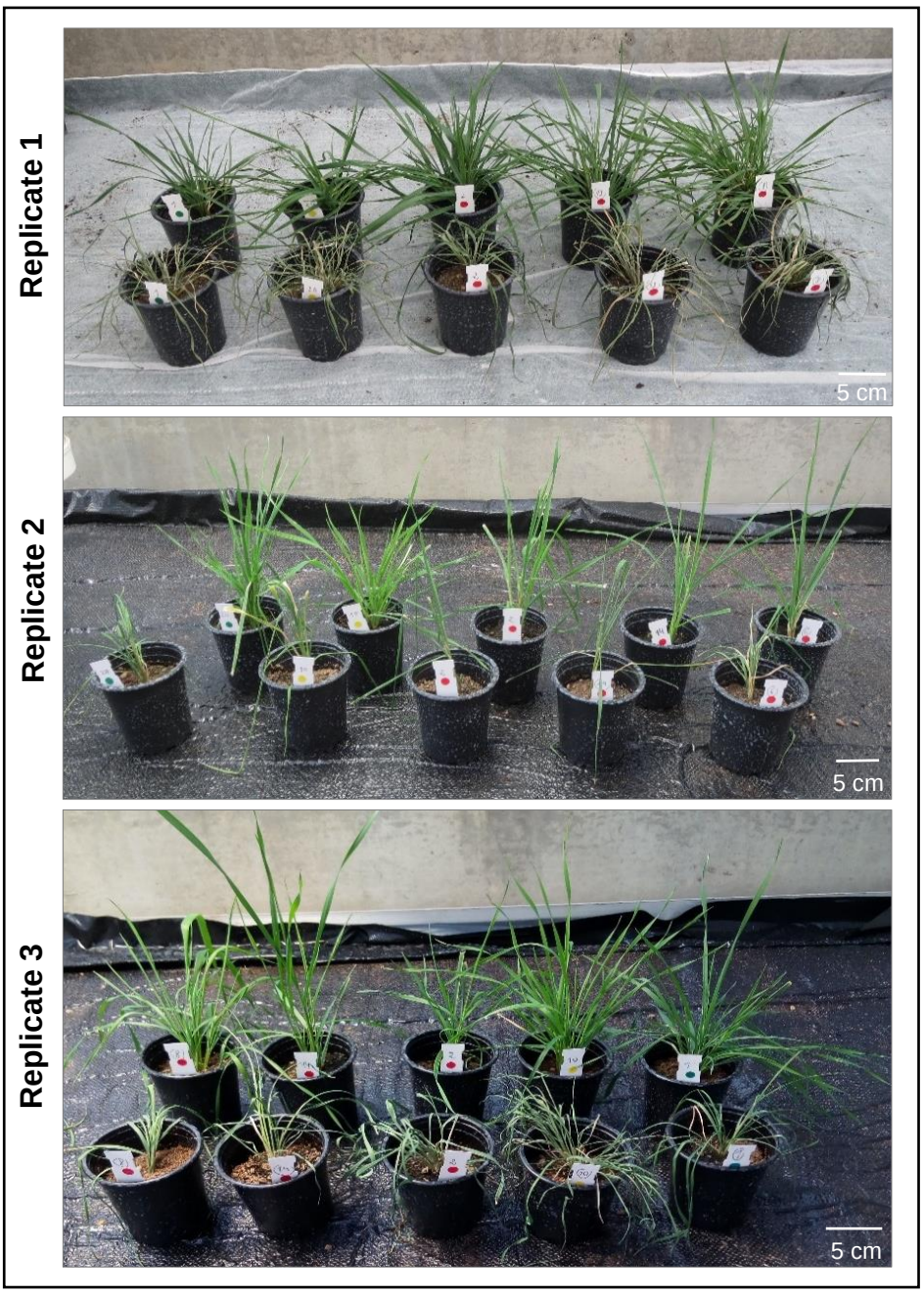

Figure 4.11: Comparison of the effect of drought stress (day 7 of the bioassay) on the different titer groups and replicates. Control population: back row, and stress population: front row. Replicate 1: from left to right we have line 1 (Low titer), line 10 (Medium titer), line 2 (High AMVV1), line 13 (High AMPV1), line 8 (High AMPV2). Replicate 2: from left to right we have line 20 (Low titer), line 10 (Medium titer), line 2 (High AMVV1), line 14 (High AMPV1), line 6 (High AMPV2). Replicate 3: from left to right we have line 8 (High AMPV2), line 14 (High AMPV1), line 2 (High AMVV1), line 10 (Medium titer), line 1 (Low titer).

Focusing on height, there were no significant differences between populations and replicates when looking at the different titer groups (Figure 4.12). We note that High AMPV1 (H-PV1) had most of the highest height groups: the control in replicate 1 was the highest of its equivalents with an average height of 301.4 mm, replicate 2's control too with 329.0 mm, replicate 3's control with 342.78 mm, replicate 1's stress was the second highest with 273.0 mm, replicate 2's stress is the highest with 321.0 mm and replicate 3's stress with 326.5 mm. On the other hand, High AMVV1 (H-VV1) had most of the smallest height groups: replicate 1's control was the second smallest of its equivalents with an average height of 231.5 mm, replicate 2's control was the smallest of its equivalents with an average height of 234.5 mm, replicate 3's control too with 235.0 mm, replicate 1's stress with 223.8 mm, replicate 2's stress was the second smallest with 248.5 mm, and replicate 3's stress was the smallest with 211.8 mm. It is interesting to note that in the case of High AMPV2 (H-PV2) replicate 1's stress and replicate 2's stress were taller than their controls, and replicate 3's stress was almost the same height as its control, with a difference of only 4 mm. replicate 1's stress in Low titer and replicate 2's stress in H-VV1 were also taller than their controls but this effect was not replicated in the repeats.

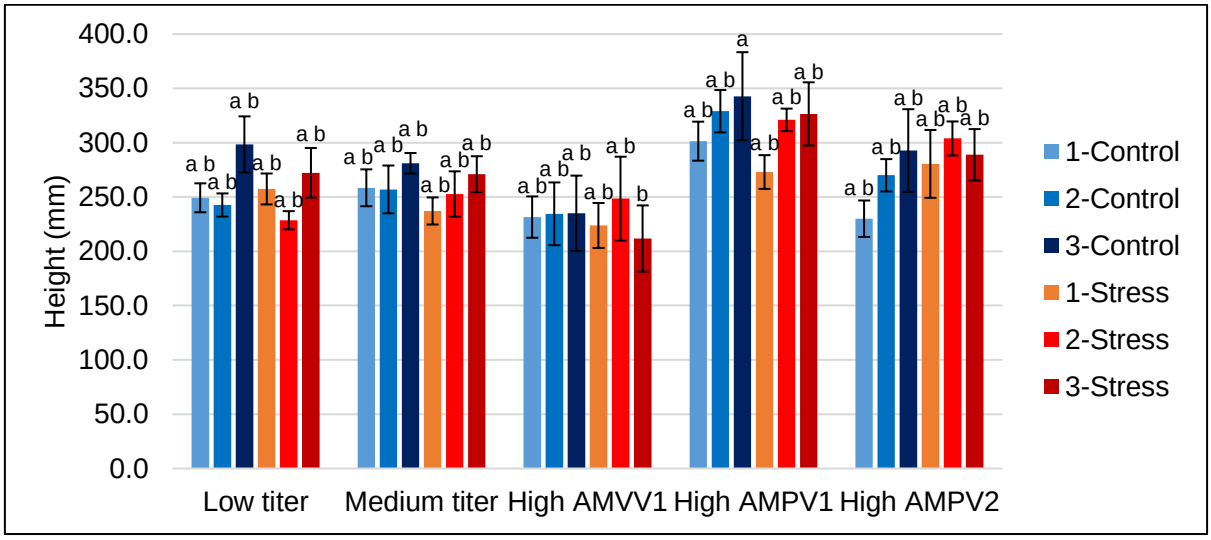

Figure 4.12: Mean height and its standard error (error bars) for the control and stress populations in the three replicates for each titer group. Means with a different letter are significantly different ($p < 0.05$). 1-control stands for the control population in replicate 1, 2-control stands for the control population in replicate 2, 3-control stands for the control population in replicate 3, 1-stress stands for the stress population in replicate 1, 2-stress stands for the stress population in replicate 2, 3-stress stands for the stress population in replicate 3.

When breaking down the results for the number of tillers into the different titer groups (Figure 4.13), there did not seem to be any obvious differences or trends between them. The range of variation between the group with more tillers and the group with least tillers in a population ranged from 3.91 mean tillers for replicate 1's control to 1.4 mean tillers for replicate 3's stress. The differences between replicates were carried over to the different groups, replicate 1's control, being significantly different in all cases, and replicate 1's stress, not significantly different, towering over their equivalents in all titer groups.

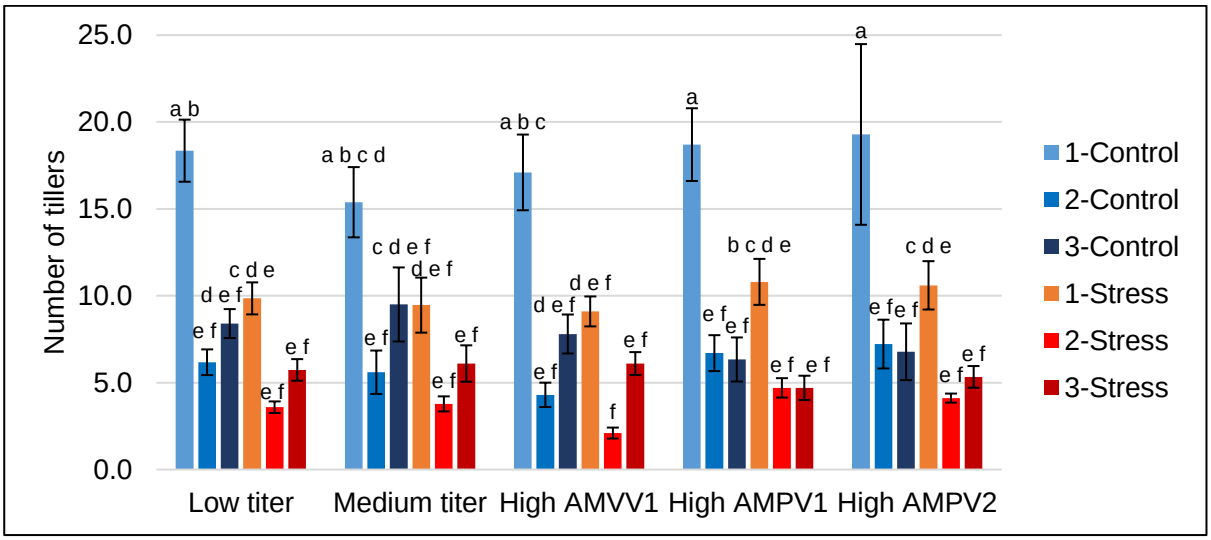

Figure 4.13: Mean number of tillers and its standard error (error bars) for the control and stress populations in the three replicates for each titer group. Means with a different letter are significantly different ($p < 0.05$). 1-control stands for the control population in replicate 1, 2-control stands for the control population in replicate 2, 3-control stands for the control population in replicate 3, 1-stress stands for the stress population in replicate 1, 2-stress stands for the stress population in replicate 2, 3-stress stands for the stress population in replicate 3.

When looking at the results for the number of leaves per tiller for the different titer groups (Figure 4.14) we could see a significantly different peak at 5.75 leaves for H-VV1 replicate 2's stress, the rest of results falling around 3.0 and 4.0 leaves.

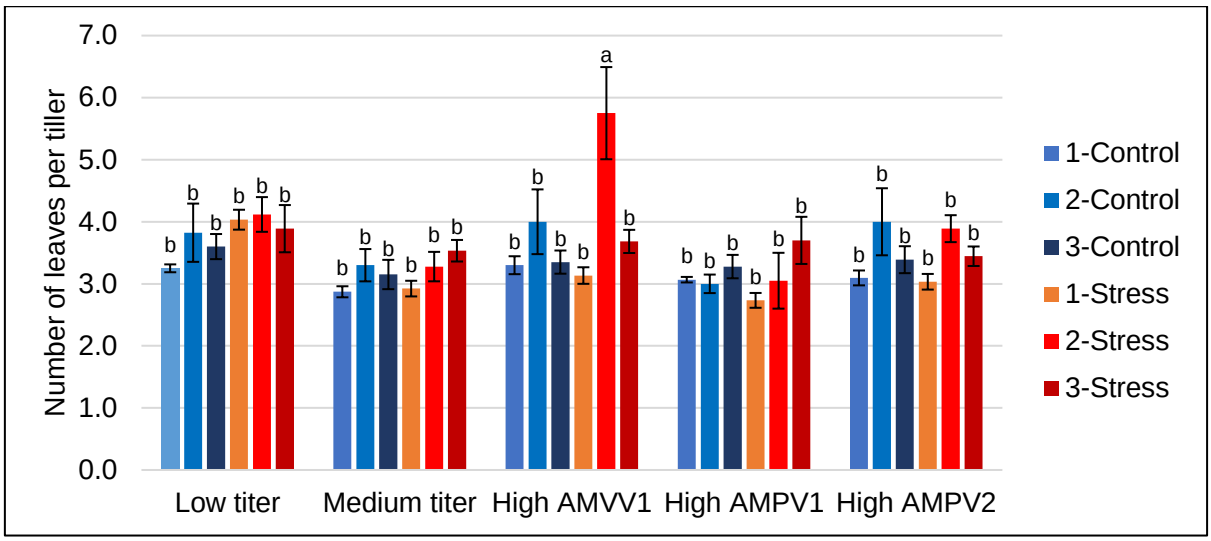

Figure 4.14: Mean number of leaves per tiller and its standard error (error bars) for the control and stress populations in the three replicates for each titer group. Means with a different letter are significantly different ($p < 0.05$). 1-control stands for the control population in replicate 1, 2-control stands for the control population in replicate 2, 3-control stands for the control population in replicate 3, 1-stress stands for the stress population in replicate 1, 2-stress stands for the stress population in replicate 2, 3-stress stands for the stress population in replicate 3.

Looking at the number of yellow leaves, the titer groups did not seem to follow any trends (Figure 4.15). The stress populations had a higher number of yellow leaves than the controls. In the case of the low titer group and the H-PV1 group, replicate 1's stress was significantly different.

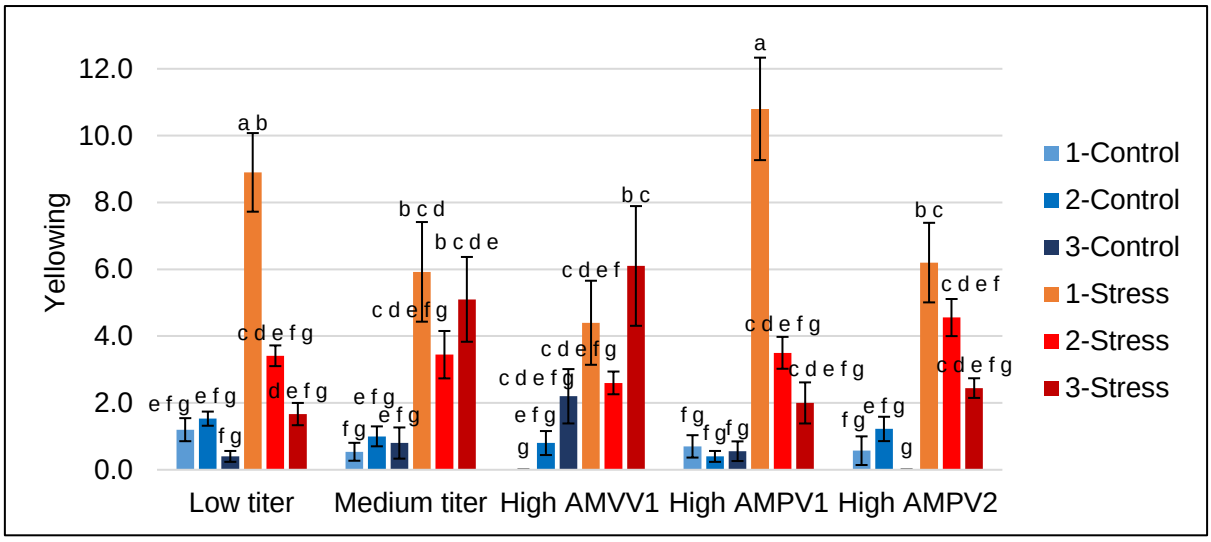

Figure 4.15: Mean number of yellow leaves and its standard error (error bars) for the control and stress populations in the three replicates for each titer group. Means with a different letter are significantly different ($p < 0.05$). 1-control stands for the control population in replicate 1, 2-control stands for the control population in replicate 2, 3-control stands for the control population in replicate 3, 1-stress stands for the stress population in replicate 1, 2-stress stands for the stress population in replicate 2, 3-stress stands for the stress population in replicate 3.

Focusing on fresh weight, replicate 1's control was significantly different in the low titer, H-VV1 and H-PV1 groups (Figure 4.16). We note that replicate 1's control had very high values in all groups except H-PV2, where its value was very similar to the other control populations. In all cases, the control populations had a higher fresh weight than the stress populations but no trend was observed.

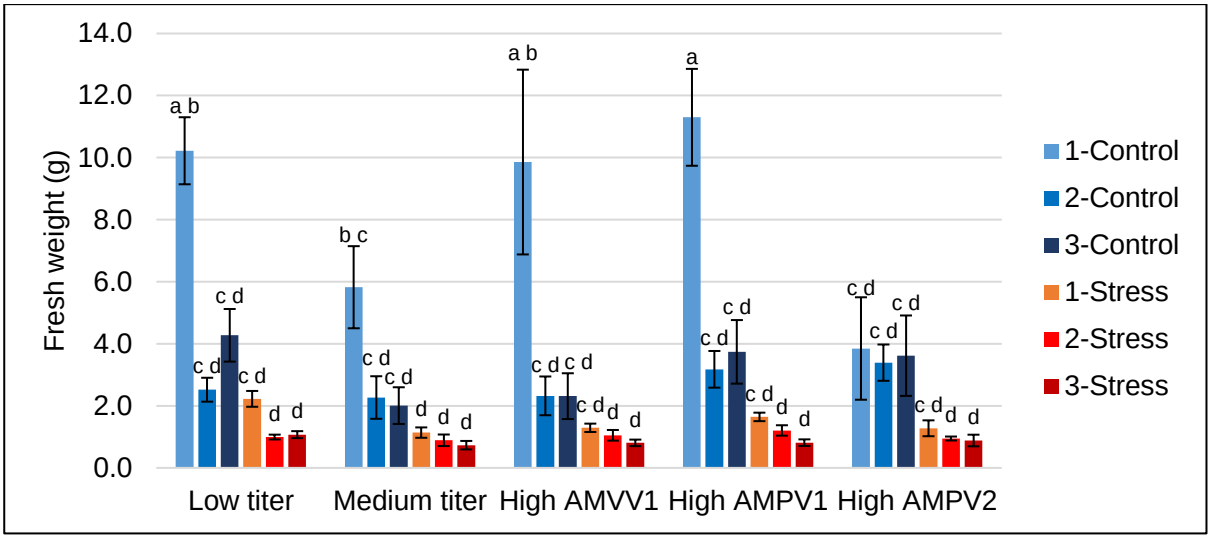

Figure 4.16: Mean fresh weight and its standard error (error bars) for the control and stress populations in the three replicates for each titer group. Means with a different letter are significantly different ($p < 0.05$). 1-control stands for the control population in replicate 1, 2-control stands for the control population in replicate 2, 3-control stands for the control population in replicate 3, 1-stress stands for the stress population in replicate 1, 2-stress stands for the stress population in replicate 2, 3-stress stands for the stress population in replicate 3.

Focusing on dry weight, when looking at the titer groups (Figure 4.17) we notice that replicate 1 showed the highest values for both the control and stress populations in all cases, with the exception of replicate 1's control in H-PV2, which was the lowest of the controls in that group.

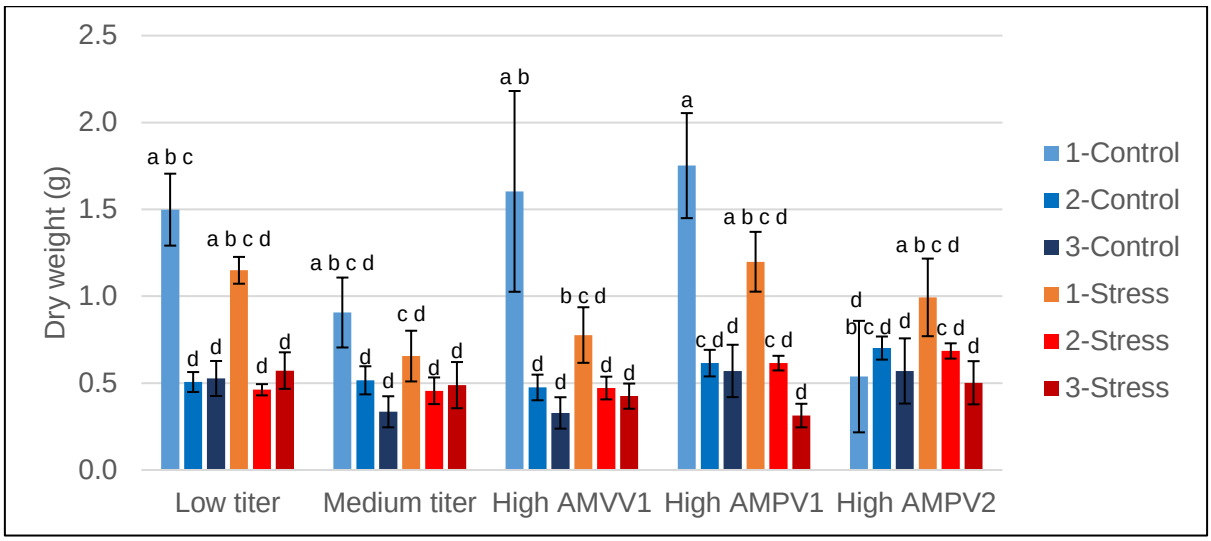

Figure 4.17: Mean dry weight and its standard error (error bars) for the control and stress populations in the three replicates for each titer group. Means with a different letter are significantly different ($p < 0.05$). 1-control stands for the control population in replicate 1, 2-control stands for the control population in replicate 2, 3-control stands for the control population in replicate 3, 1-stress stands for the stress population in replicate 1, 2-stress stands for the stress population in replicate 2, 3-stress stands for the stress population in replicate 3.

When looking at the water content for the different titer groups (Figure 4.18), we can see that while the controls remained fairly stable across them, shifting less than 10%, the stress populations had important shifts in their water content. Replicate 1's stress was fairly stable across Low (43.04%), Medium (48.32%) and H-VV1 (42.56%) and dropped in H-PV1 (28.60%) and H-PV2 (27.92%). Replicate 2's stress had similar values in Low (52.41%) and H-VV1 (54.29%), Medium (41.71%) and H-PV1 (46.03%), and then dropped in H-PV2 (26.41%). Replicate 3's stress presented its highest value in H-PV1 (63.09%), it had similar values in Low (48.66%), H-VV1 (47.57%) and H-PV2 (48.16%), and its lowest value was in Medium (35.96%). In the case of H-PV2, the controls in all replicates were significantly different.

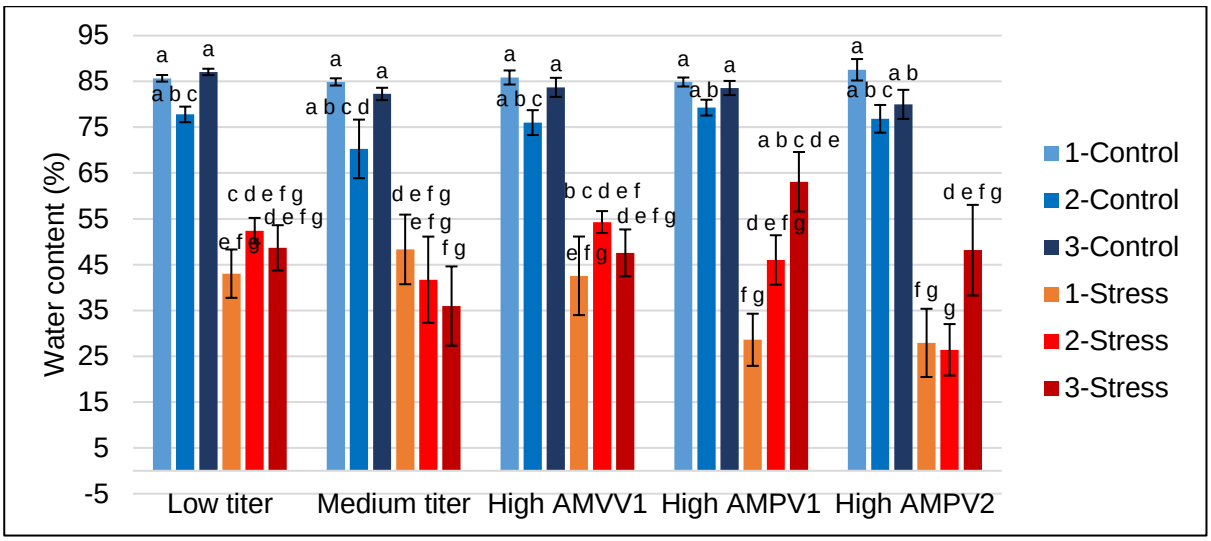

Figure 4.18: Mean water content and its standard error (error bars) for the control and stress populations in the three replicates for each titer group. Means with a different letter are significantly different ($p < 0.05$). 1-control stands for the control population in replicate 1, 2-control stands for the control population in replicate 2, 3-control stands for the control population in replicate 3, 1-stress stands for the stress population in replicate 1, 2-stress stands for the stress population in replicate 2, 3-stress stands for the stress population in replicate 3.

The statistical analysis indicates that no significant differences were found between the titer groups (Figure 4.19).

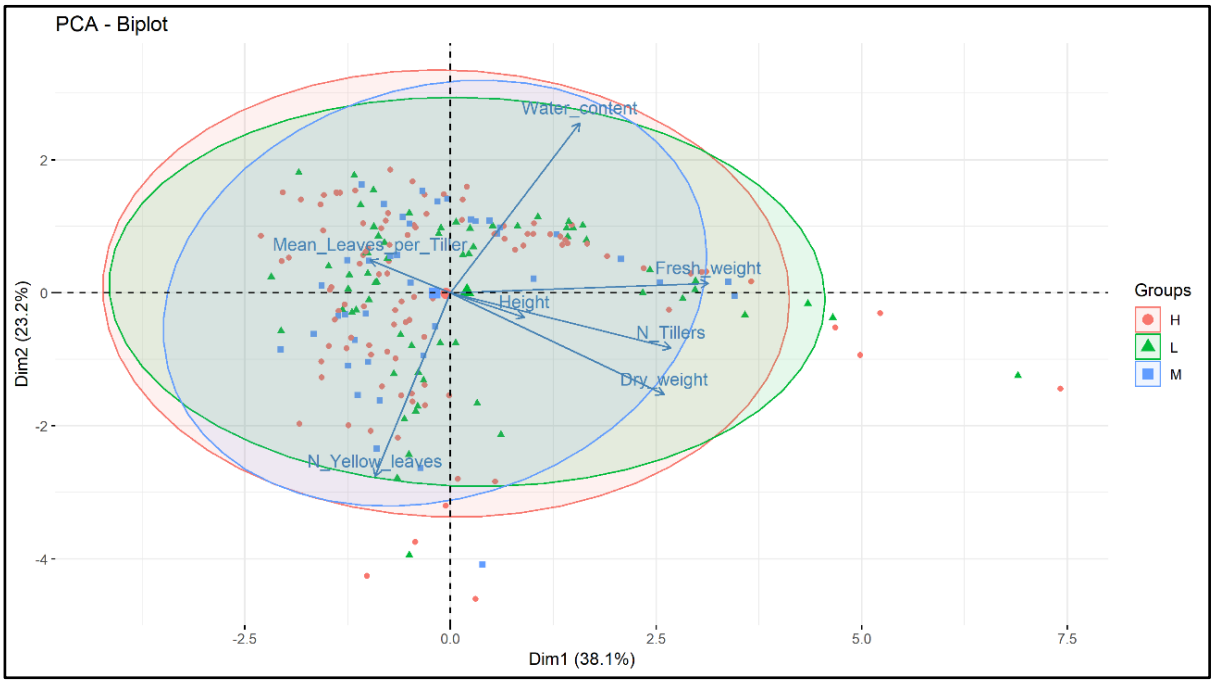

Figure 4.19: Visual representation of the three main titer groups (Green: low titer, Blue: medium titer, Red: high titer). The titer values for the initial lines are used, these initial lines are the ones used to create the studies populations by propagation.. Bi-plot of the first two components of PCA of growth measures. Points represent the scores of individuals, lines denote the vector direction of the various measures with respect to the principal components.

4.3.3 Statistical analysis of viral titer

No significant interaction was found between the stress treatment and changes in viral titer. However, AMVV1 showed an important and consistent increase under stress in all replicates (Figure 4.20). AMPV1 and AMPV2 did not show such an important variation between the control and stress populations, and the changes were not consistent between the replicates.

Viral titer suffered large changes between replicates: AMPV1 decreased in replicates 2 and 3, while AMVV1 and AMPV2 increased.

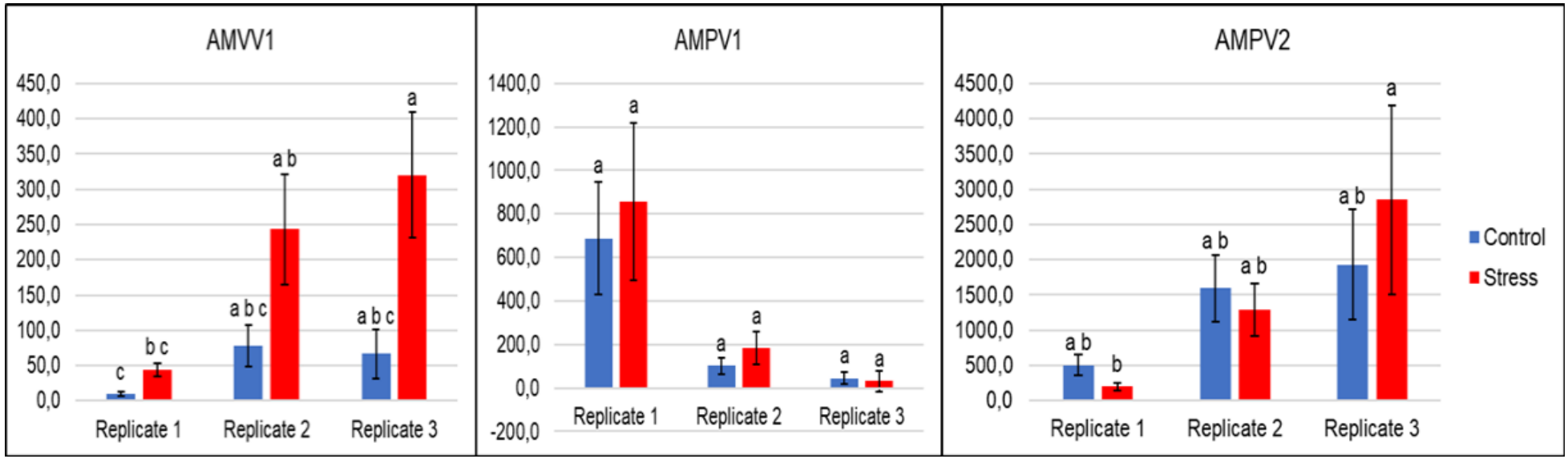

Figure 4.20: Mean titer and its standard error (error bars) for the control and stress populations in the three replicates for each virus. Means with a different letter are significantly different ($p < 0.05$).

4.3.4 Statistical analysis of the replicates

Significant differences were found between the three replicates. Replicate 1 was more variable than the others (Figure 4.21) and bigger differences were observed between its control and stress populations (Figure 4.22).

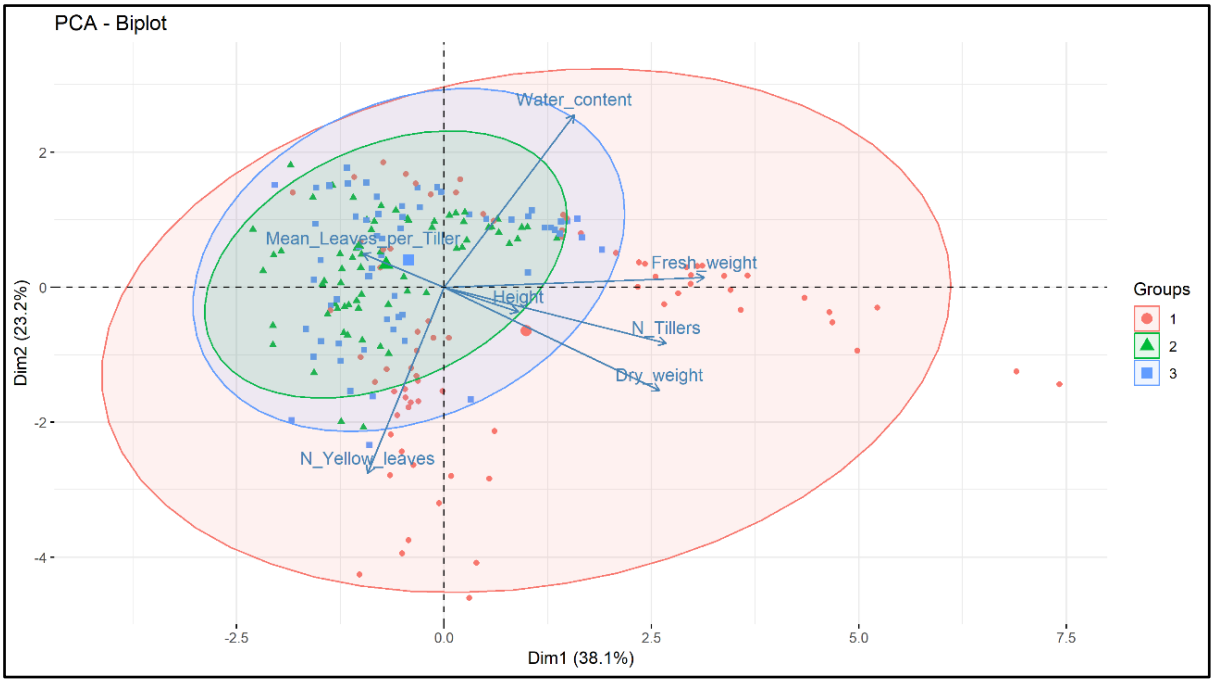

Figure 4.21: Visual representation of the three replicates (Red: replicate 1, Green: replicate 2, Blue: replicate 3). Bi-plot of the first two components of PCA of growth measures. Points represent the scores of individuals, lines denote the vector direction of the various measures with respect to the principal components.

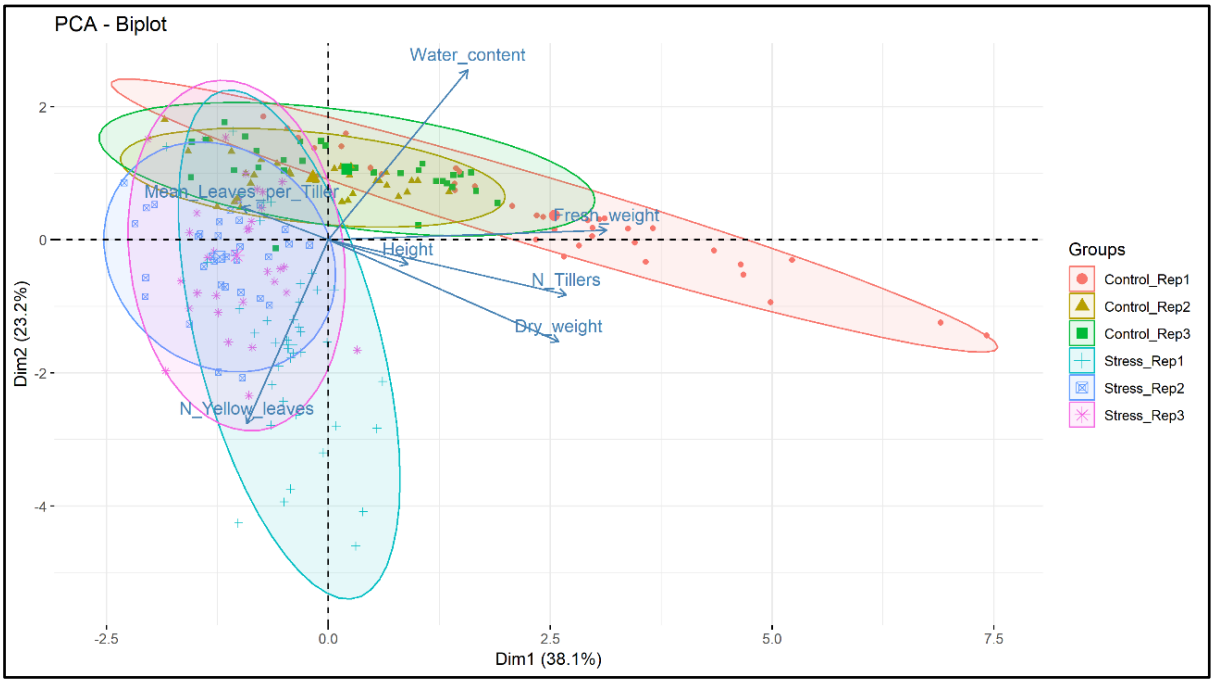

Figure 4.22: Visual representation of the three replicates divided into Control and Stress populations. Bi-plot of the first two components of PCA of growth measures. Points represent the scores of individuals, lines denote the vector direction of the various measures with respect to the principal components.

4.3.5 Effect of the viruses on the growth of black-grass plants

In this section we look at the results of the modelling. We studied the changes to the growth parameters associated to increasing viral titers and if there were significative differences between how these parameters changed under control and drought stress conditions.

Starting with AMVV1, we see that there was a significant positive effect on height, number of tillers, dry weight and yellowing under stress (Figure 4.23). Under normal conditions, high AMVV1 titer seemed to negatively affect the growth of its host, with generally a decrease in the measured parameters, except for water content. But under stress conditions, these negative effects were alleviated. However, some of the parameters (number of tillers, number of leaves per tiller, number of yellow leaves and water content) also showed an interaction with the replicates.

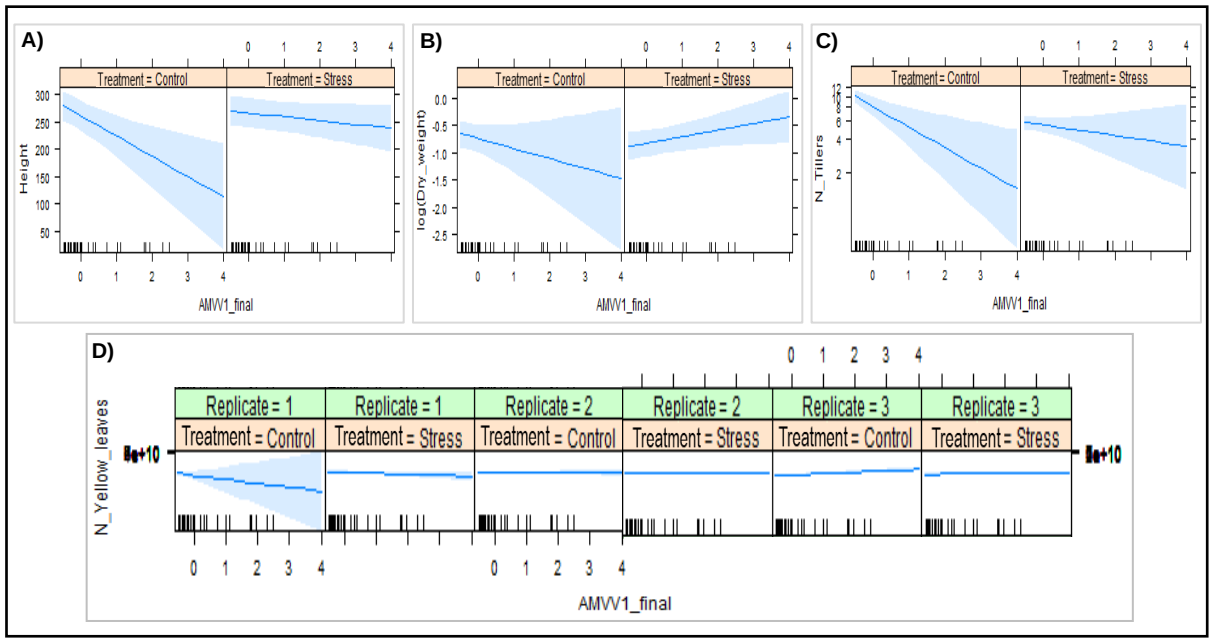

Figure 4.23: Significant interactions between treatment and AMVV1. A: Height, B: Dry weight, C: Number of tillers, D: Yellowing. Yellowing also has a significant interaction with the replicates. Effect direction plots, X axis for each plot represents the scaled values for the viral titre, Y axis represents the physiological parameter. Solid line shows the mean effect and shaded area the confidence interval around that mean.

In the case of AMPV1, no significant interaction was found between it and drought stress response. Higher levels of AMPV1 correlated with an increase in number of tillers (Figure 4.24) and a decrease in the height and number of leaves per tiller (Figure 4.25). Dry weight and Water content remained stable (Figure 4.26), which we interpreted as the overall plant biomass not being affected by the changes to the other growth parameters. Yellowing showed an interaction with the replicates and did not follow a clear trend.

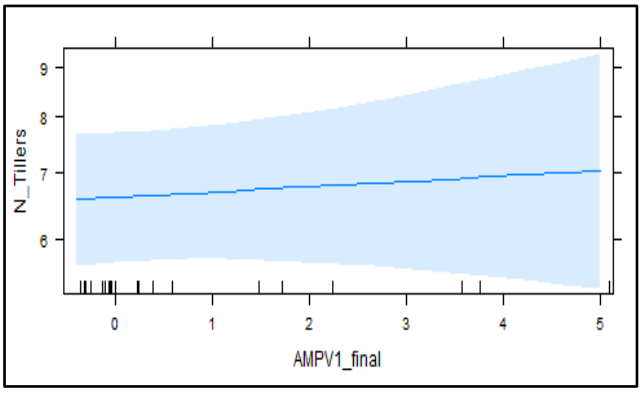

Figure 4.24: Positive effect of AMPV1 on black-grass physiological parameters: number of tillers. Effect direction plots, X axis represents the scaled values for the viral titre, Y axis represents the Number of tillers. Solid line shows the mean effect and shaded area the confidence interval around that mean.

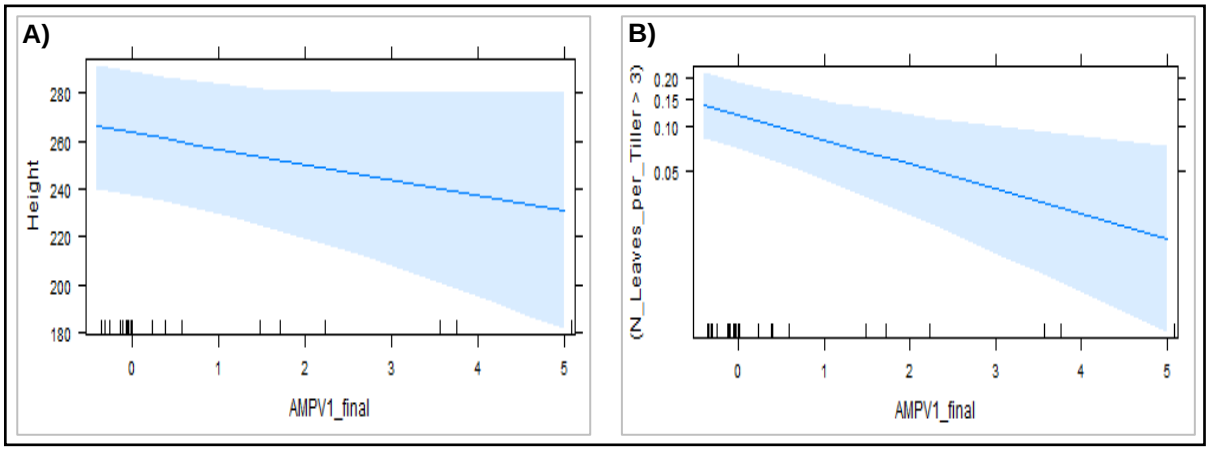

Figure 4.25: Negative effect of AMPV1 on black-grass physiological parameters: A: height, B: number of leaves per tiller. Effect direction plots, X axis for each plot represents the scaled values for the viral titre, Y axis represents the physiological parameter. Solid line shows the mean effect and shaded area the confidence interval around that mean.

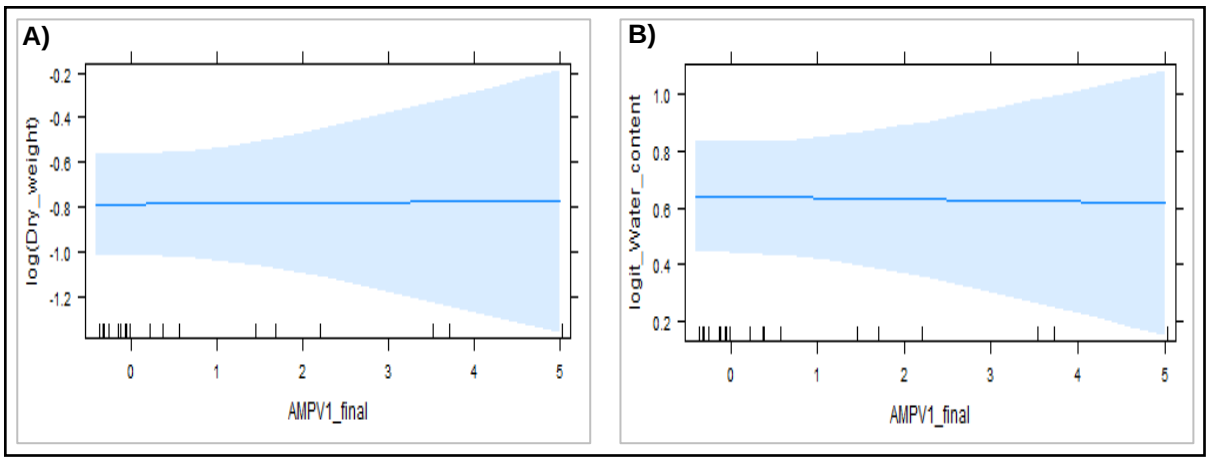

Figure 4.26: No apparent effect of AMPV1 on black-grass physiological parameters: A: Dry weight, B: Water content. Effect direction plots, X axis for each plot represents the scaled values for the viral titre, Y axis represents the physiological parameter. Solid line shows the mean effect and shaded area the confidence interval around that mean.

Finally, there was no interaction between AMPV2 and stress nor between replicates for height and number of tillers, both these parameters decreased at high AMPV2 titer. There was an interaction between AMPV2 and stress and replicates for number of leaves per tiller and water content, which seemed to decrease or remain stable at high titer, and number of yellow leaves, which did not seem to follow any trend. There was an interaction between replicates for dry weight, which generally seemed to be reduced at high titer.

4.4 Discussion

4.4.1 Effect of drought stress on black-grass plants

Drought stress seemed to mainly affect the number of tillers, fresh weight and water content, notably reducing them. It also increased yellowing of leaves. In contrast, height and number of leaves per tiller suffered minimal reduction under drought stress, and in some cases, especially for the number of leaves, they seemed to be positively affected by it. Dry weight was reduced in most cases but there were some notable exceptions as noted in the results section, and the differences between the control and stress populations were not as steep as for fresh weight and water content.

The overall reaction of black-grass to drought stress appeared to be a reduction in the number of tillers while maintaining height and slightly increasing the number of leaves. The survival strategy seemed to focus on the maintenance of the tillers that had already grown instead of producing new ones, while increasing their photosynthetic capacity to compensate for their reduced number. This seemed to be supported by the relation between the two weights and water content. Reduction of fresh weight seemed to be primarily due to the loss of cellular water, as dry weight showed smaller differences between the control and stress populations. In some cases, the stress populations had a higher average dry weight than the control, possibly due to the increase in number of leaves and most probably to the increase in the root system´s size, this being a common drought tolerance mechanism in plants (Weaver, 1930; Salehi-Lisar and Bakhshayeshan-Agdam, 2016).

4.4.2 Effect of drought stress on the titer groups

Overall, no obvious trends were detected between titer groups and growth parameters. Only in the case of height, some small trends could be noted: AMPV1 consistently had most of the highest plants across all replicates while AMVV1 had most of the smallest. However, the differences between the titer groups were small and not statistically significant. But this could be the first indication of the viruses having an

effect on their host. Additionally, as mentioned before, no interactions were found between the stress treatment and the titer groups.

All of this seemed to indicate that the viral titer's possible effect on the growth of its host would be a secondary one. As our population was highly heterogenous and no obvious visual trends between titer and phenotype were observed when selecting the lines, this was not surprising. While the viruses might have an effect on their host, it is most probably subordinate to the genotype and phenotype of the host, and to the effects of changes in environmental conditions.

4.4.3 Variation in viral titer

As mentioned before, no significant interaction was found between the treatment and changes in titer. However, the p-value was low ($p = 0.088$) which could indicate the existence of a marginal effect of drought stress, the high variability present in the study probably hindering the finding of a stronger evidence in favour or against this interaction. Nevertheless, only AMVV1 showed a clear and consistent variation in its titer, increasing under drought stress (Figure 4.21).

After the preliminary results obtained in the previous chapter, such stark variations in titer between replicates were not expected. However, the conditions of the plants were different in the drought bioassay (higher number of propagations, plants maintained for longer periods of time…) and this could explain the unforeseen variation. Below we present some plausible explanations for the variation between replicates.

4.4.4 Differences between replicates

Plants were propagated by tillering and grown under the same conditions, as a result replica experiments were expected to be very similar. An explanation for the variation could be plant age. While each propagated tiller grew up to a fully formed plant, its physiological age was predetermined by the developmental stage of the mother plant, delaying its developmental cycle until the plant had grown but continuing with the molecular signals of the mother plant. This was very evident when trying to propagate an already flowering plant, where, even small tillers with no signs of imminent flowering immediately progressed to flowering once planted. Therefore, we believe variations due to plant age and their effect on the growth of new plants were present in the different replicates, as these were not grown and tested at the same time due to space constrains.

Furthermore, replicates 2 and 3, which were also significantly different between them, were grown in quick succession, with only a 5 week gap between propagations. Thus, it is possible that environmental changes had a greater impact than expected on

growth. The experiments were performed in a glasshouse cubicle and as a result, whilst basal light and temperature conditions were established, natural light changes and large changes in ambient temperature affected the conditions in the glasshouse.

Additionally, it is possible that the propagation method had an impact on the cellular and molecular level, accounting for some of the observed variation.

4.4.5 AMVV1 as a conditional mutualist

With a number of significant positive interactions between treatment and parameters, AMVV1 seemed to influence plant response to drought stress. However, the fact that some of the parameters also showed an interaction with the replicates could mean that some of the observations might have a bigger phenotypic component than expected.

The positive effect of AMVV1 under stress seemed to fall in line with the observations made on other virus-host systems, where the viruses shift from antagonistic to conditional mutualist under stress. Xu *et al*. (2008) found that CMV, TMV, TRV and BMV improve tolerance of host plants to drought stress. In all cases, the appearance of drought symptoms was delayed by 2–5 days. Two ascomycete endophytic fungal strains and the yellow tail flower mild mottle virus have been found to confer water stress tolerance to *Nicotiana benthamiana* seedlings by increasing the accumulation of sugar, protein and proline as osmolytes, increasing antioxidative enzyme activity, reducing membrane damage, and enhancing expression of drought-related genes (Dastogeer *et al*., 2018). BYDV infection benefits barley performance when plants are challenged with acute water stress. Infection with BYDV is associated with higher leaf water potential in infected hosts when water inputs are low and enhanced growth, seed set and germination for infected hosts when water is withheld (Davis *et al*., 2015a).

The main explanation for these protective effects is that the activation of the defence system due to viral infection improves tolerance to later stresses, as the defence/tolerance mechanisms to different stresses tend to overlap. Viral infection affects many plant cell mechanisms that are part of the tolerance strategy against drought, such as respiration, transpiration and photosynthesis. The tolerance related changes usually limit the loss of water, and their activation prior to the stress could reduce its initial impact. Infection also has an effect on the concentration and regulation of different metabolites: phytohormones, sugars, etc., which can have a positive impact on drought tolerance, especially as osmotic adjustment (Farooq *et al*., 2009; Davis *et*

al., 2015b). Additionally, the stunted growth of infected plants reduces its water requirements, improving its tolerance to drought stress.

It has also been found that response to simultaneous stresses is specific and different from the response to each individual stress, with important variations in plant transcription (Atkinson and Urwin, 2012; Prasch and Sonnewald, 2013; Suzuki *et al*., 2014; Ramegowda and Senthil-Kumar, 2015). This could mean that black-grass, and other species, under dual stress have a more efficient protective response than under a single stress.

4.4.6 AMPV1, plant structure and increased fitness

No obvious interaction was found between the presence of AMPV1 and plant response to drought stress. However, AMPV1 did seem to have an overall effect on its host, as several growth trends appeared to respond to this virus' titer. According to these results, AMPV1 seems to have an influence on the structure of its plant host. Promoting the formation of tillers to the detriment of their size, but with an equivalent trade-off between the two, as the biomass was not affected. In other words, AMPV1 seems to promote the growth of wider plants instead of taller plants.

Tillering is regulated by phytohormones which are known to be affected by viral infection (Jameson, 2000; Kariali and Mohapatra, 2007; Giron *et al*., 2013). In particular, cytokinins have been associated to an increase in tillering, as well as to delayed senescence of tillers and increased yield (Kariali and Mohapatra, 2007; Yeh *et al*., 2015). It is possible that AMPV1 affects phytohorme regulation, leading to a change in plant structure.

As a grass, black-grass is mainly affected by environmental stresses (drought, heat…), limited resources and grazing. Thus, an increase in tillering could be beneficial in some situations. An increase in spread could prevent the growth of competing species, ensuring a higher availability of resources as well as limiting shading from taller plants. Additionally, it could correlate with a bigger root system, which again, would enhance resource availability and could indirectly increase tolerance to drought stress, as it would improve water uptake (Weaver, 1930; Salehi-Lisar and Bakhshayeshan-Agdam, 2016). Different studies have found that grazing stress modifies the size and structure of grassland root systems (Lorenz and Rogler, 1967). A decrease in root system size is observed under moderately and highly grazed systems, but the root system under moderate grazing reaches deeper into the soil (Schuster, 1964; Lorenz and Rogler, 1967; van der Maarel and Titlyanova, 1989). An improved root system could help prepare against grazing stress.

The main grazing tolerance mechanism is known as compensatory growth. This mechanism is based on the plant's ability to regrow after damage by mobilising stored resources and quickly regaining its photosynthetic ability (Benot *et al*., 2018). A smaller tiller size would reduce the amount of resources necessary for regrowth, as well as allowing the plant to regain the photosynthetic tissues faster. Weaver (1930) linked a reduced height with higher survival under grazing conditions and it has been observed that under grazing, the composition of grassland tends to shift to smaller species. Additionally, the higher number of tillers increases the overall chances of regrowth. Thus, the changes promoted by AMPV1 seem to favour the compensatory growth ability of black-grass. Finally, the higher number of tillers could correlate with an improved flowering rate, and therefore, an improved propagation of the plant species and of AMPV1, as plant partitiviruses are transmitted through pollen (ICTV, *Partitiviridae* chapter, 2017).

Hence, it is possible that the shift to increased tillering acts as a tolerance mechanism against grazing stress and could increase the overall fitness of the host under some conditions. This is in line with the effects found for other cryptic viruses in plants, which seem to focus on increasing its hosts´ fitness, implementing changes that are beneficial under specific circumstances, and therefore, granting their hosts a survival advantage over non-infected plants. WCCV1 was found to regulate nodulation, reducing it when nitrogen levels in the soil are adequate and therefore, preventing the use of resources for the development of an unnecessary organ (Nakatsukasa-Akune *et al*., 2005). PCV-1 influences the composition of secondary metabolites that detract aphids from feeding on the host plant, helping reduce the incidence of aphid damage and the infection by pathogenic viruses transmitted by these (Safari *et al.*, 2019).

4.4.7 AMPV2 as an antagonist

It is unclear if AMPV2 had an effect on plant drought stress response due to the numerous interactions between replicates. Overall, AMPV2 seemed to negatively affect its host, with a decrease in height, number of tillers, number of leaves per tiller and dry weight at high titer. However, these changes varied greatly between replicates, which could imply that there were other substantially more important factors affecting these parameters. Regardless, these negative effects seemed to be exacerbated under drought stress.

In many cases, pathogen symptoms are exacerbated when additional stresses are applied (Atkinson and Urwin, 2012; Suzuki *et al*., 2014; Ramegowda and Senthil-Kumar, 2015). Clover *et al*. (1999) showed that drought stress and *beet yellows virus*

(BYV) had an additive effect on sugar beet, the dual stress yielding an additional reduction in growth compared to the individual stresses. Similarly, Prasch and Sonnewald (2013) found that a combination of drought stress and *turnip mosaic virus* (TuMV) infection in *Arabidopsis thaliana* also reduced growth more strongly than each individual stress. And Bergès *et al*. (2018) found that, in general, the combination in *Arabidopsis thaliana* of water deficit and *cauliflower mosaic virus* (CaMV) infection was more deleterious than each of the stresses on their own.

While not causing any evidently characteristic symptoms, AMPV2 seems to negatively affect the growth of its host. This is probably due to the use of plant resources by the virus, but not sufficiently to cause acute symptoms or death of the plant under normal growing conditions. Although no obviously virulent partitiviruses have been found in plants, this genus is known to negatively affect some fungi species (ICTV, *Partitiviridae* chapter, 2017), which makes it possible that some partitiviruses could prove detrimental to their plant hosts.

4.5 Conclusion

None of the studied viruses seem to have a direct effect on the tolerance of black-grass to drought stress. But each of them appears to cause different symptoms. AMVV1 seems to act as a conditional mutualist, hindering growth under normal conditions but alleviating these effects under stress. AMPV1 on the other hand, seems to affect the growth pattern of its host regardless of the environmental conditions, shifting from tall plants with few tillers to dwarfed plants with more tillers. This could increase the plant's fitness as it might potentially increase both its competitiveness in grasslands, enabling it to spread and outcompete neighbouring plants as well as enhancing its reproductive capabilities, producing more tillers to generate seed. In contrast, AMPV2 seems to act as an antagonist, negatively affecting the growth of its host, an effect that is exacerbated under stress.

However, these results were highly constrained by the level of unexpected variability found and the consequential problems it caused in the statistical analysis, and at this stage cannot be considered conclusive. But they do allow us to glimpse into the relationship between these viruses and their host, and to provide us with hypothesis around which future experiments can be done.

An in-depth study of how plant age affects viral titer is necessary. The observed effect of AMVV1 could be confirmed by repeating the drought assay. Additionally, other stress experiment would help narrow down the reach of this effect, if it acts as a general

response to stress or as a tailored response to certain stresses. In the case of AMPV1, more physiological and titer data would help elucidate if the observed effect is consistent across the studied population and other populations. Also, an experiment recreating grassland conditions and grazing stress would help study if the effect is in fact beneficial under those conditions. Follow-up screenings would help confirm the negative impact of AMPV2. Regardless, the creation of a phenotypical homogenous population would highly facilitate the study of these viruses.

There are still many unknows surrounding cryptic viruses, from how many are infecting crop and wild species to how they affect their hosts, and the difficulties in working with them heavily hinder their study. Nonetheless, research into these viruses has started to bear some results, shedding new light on virus-plant relationships.

Chapter 5: *In vitro* culture of black-grass and *in vitro* elimination of AMVV1, AMPV1 and AMPV2.

5.1 Introduction

In vitro culture is the culture of cells, organs or organisms on artificial media under asepsis and controlled environmental conditions. *In vitro* culture is used for bacteria, fungus, animal cells, organs and plants (Seguí Simarro, 2010; Cann, 2012; Modrow *et al*., 2013; Panattoni *et al.*, 2013; Singh, 2015).

Plant tissue culture is based on the plant cell's totipotent ability, in which a cell is able to revert the differentiation process, regaining a meristematic status, non-differentiated but with the capacity to differentiate into any cellular type. There are three main plant tissue culture methods. Firstly, the use of explants with axillary buds, which are meristematic regions and will naturally produce more shoots, in some cases, induction of rooting is necessary. Secondly, organogenesis, the induction of new shoots from non-meristematic plant cells. Induction is direct when there is no previous dedifferentiation step, using leaves or similar material as the explant, and indirect when there is a previous dedifferentiation step, which produces a callus from which new explants are induced. Thirdly, somatic embryogenesis, which is the formation of a functional embryo from plant cells, in this case, induction is normally indirect (Seguí Simarro, 2010; Panattoni *et al.*, 2013; Singh, 2015).

There are a number of applications for plant *in vitro* culture (Seguí Simarro, 2010; Panattoni *et al.*, 2013; Singh, 2015):

- Large scale micropropagation of commercially valuable plants, which is faster and takes less space than *in vivo* propagation.
- Germplasm conservation.
- Creation of model systems for basic studies (plant metabolism, physiology, pathology, etc.).
- Development of new varieties or species, via somaclonal variation (mutations, translocations, etc.), protoplast fusion, genetic transformation, etc.
- Metabolic engineering, which is the production of interesting metabolites by large scale cell culture.
- Production of virus-free plants.

The main virus elimination methods are meristem culture, thermotherapy, chemotherapy and cryotherapy. A combination of these techniques is also used, usually resulting in increased viral elimination but a reduced survival rate (Fletcher *et al.,* 1998; Senula *et al.*, 2000; Wang *et al*., 2008; Wang and Valkonen, 2009; Ramgareeb *et al*., 2010; Panattoni *et al.*, 2013). In all cases, careful consideration and experimentation are necessary to reach a balance between plant survival and virus elimination (Panattoni *et al.*, 2013).

In the case of meristem culture, due to the high cell division activity in the meristem and the lack of connection to the vascular system, meristematic cells are, in most cases, virus-free. And therefore, plants derived from these cells are also virus-free. In some cases, the use of bigger shoots (2 cm or less) is also able to reduce and eliminate viruses, this is believed to be due to changes in the plants, due to the *in vitro* conditions, that can affect the replication of the viruses. Meristem culture is the virus elimination method most commonly used (Fitch *et al*., 2001; Parmessur *et al.*, 2002; Ramgareeb *et al.*, 2010; Cheong *et al.*, 2012; Panattoni *et al.*, 2013; Taşkın *et al*., 2013).

In thermotherapy, plants are subjected to high temperatures, usually between 35 °C and 54 °C, for an appropriate period of time. Both the temperature and length of the treatment need to be adapted to the plant species and virus type; they need to be inside the physiological tolerance limit of the plant while disrupting viral replication. In this case, viral elimination is believed to happen because the rate of viral degradation is higher than the rate of viral replication. Viral degradation occurs due to the rupture of hydrogen and disulphide bonds of capsid proteins and of phosphodiester covalent bonds of nucleic acid. Viral infectivity can also be affected due to changes in cell pH and ionic strength causing inhibition of the viral replicase. Usually, this treatment needs to be repeated a number of times to be effective (Ramírez Malagón *et al*., 2006; Panattoni *et al.*, 2013).

In chemotherapy, plants are grown on media containing antiviral agents. Antiviral use for virus elimination in plants is less developed than for animals and the underlying mechanisms are not yet well understood; it is believed that the antiviral agents are able to inhibit different molecules which affect viral replication. Ribavirin, a synthetic analogue of guanosine, is the main antiviral agent used in plant virus elimination. Its main mechanism of action against DNA viruses is believed to be the inhibition of the inosine monophosphate dehydrogenase (Fletcher *et al.*, 1998; Ramírez Malagón *et al*., 2006; Panattoni *et al.*, 2013). In the case of RNA viruses, ribavirin triphosphate is

utilized by the viral RdRP and causes lethal mutagenesis of the viral genome (Crotty *et al.,* 2000; Cameron *et al.,* 2001).

In cryotherapy, the newest of these treatments, meristems are treated with liquid nitrogen. Some viruses are able to infect meristems, but in many cases, these infections only affect the outer layers of the meristem, with inner-meristematic cells still being virus-free. Treatment with liquid nitrogen is able to freeze and destroy these outer layers, increasing the chances of virus elimination in meristem culture. Different techniques have been developed for cryotherapy: two-step cooling, ultra-rapid cooling, vitrification, DMSO droplet method, droplet vitrification, encapsulation/dehydration and encapsulation/vitrification. However, this treatment is very aggressive, with small survival rates, and all techniques require a large number of steps, making it more labour intensive and complicated than thermo- and chemotherapy (Sakai and Engelmann, 2007; Kaczmarczyk *et al.*, 2011; Panattoni *et al.*, 2013).

We were interested in the possibility of *in vitro* viral elimination for AMVV1, AMPV1 and AMPV2, as it could open the door to the development of new virus-free populations that could be used in comparative studies. An *in vitro* culture protocol was developed for black-grass and thermotherapy, chemotherapy and a combination of both were trialled.

5.2 Material and methods

5.2.1 Culture media

A standard Murashige & Skoog (MS) media was used as the culture media (Murashige and Skoog, 1962). Media was prepared using Sigma´s liquid MS Macronutrient (10X) and MS Micronutrient (10X) solutions at a 1X concentration and topped up with distilled water. We added 30 g/L of sucrose (Sigma-Aldrich, USA) and 2 g/L of Gelzan (Sigma-Aldrich, USA), as the gelling agent, and brought the pH to 5.8 before autoclaving the solution. After autoclaving, the solution was left to cool down in a 60 °C water bath. Under sterile conditions, in a vertical flow cabinet, Sigma´s MS vitamin solution (1000X) at a 1X concentration was added and the solution was distributed in the culture pots, around 75 mL per pot, and left to solidify overnight.

5.2.2 Plant material

Initially, I attempted the culture of explants from plants with known viral titers. I used a standard sterilization process on the explants: 20% bleach solution followed by three autoclaved water baths. I trialled different concentrations and soaking times, but in all cases contamination was widespread.

Therefore, I decided to use seeds to set-up our *in vitro* population. 24 seeds from the Peldon population, 12 seeds from Roth and 12 seeds from Nott were used. A solution of distilled water, bleach (20%) and a few drops of Tween-20, that acts as a surfactant, was used to sterilize the seeds. These were placed in a tea infuser and incubated in the above solution for 5 minutes. They were then washed in three autoclaved water baths for 3 min, 3 min and 5 min, respectively. Following the surface sterilization, they were placed on top of the culture medium, 4 seeds per pot, and left to germinate and grow in a growth cabinet at 20/18 °C and 60% humidity, under a light cycle of 16/8 h (Figure 5.1).

After 13 weeks, plants were micropropagated into fresh medium; 1-2 cm long explants were taken from the base of different tillers and placed in new pots, two explants (from the same mother plant) per pot, and left to grow under the same conditions as described above. During the micropropagation, samples were taken from the unwanted plant material and the plants' AMVV1 viral titer was tested using RT-qPCR (Figure 5.2A). Plants were selected according to this titer: two lines from Peldon with differing AMVV1 titers, one line from Roth and one line from Nott with negative AMVV1 titers were selected and used in the virus elimination assays. Lines were maintained and elimination assays set up by micropropagating as above.

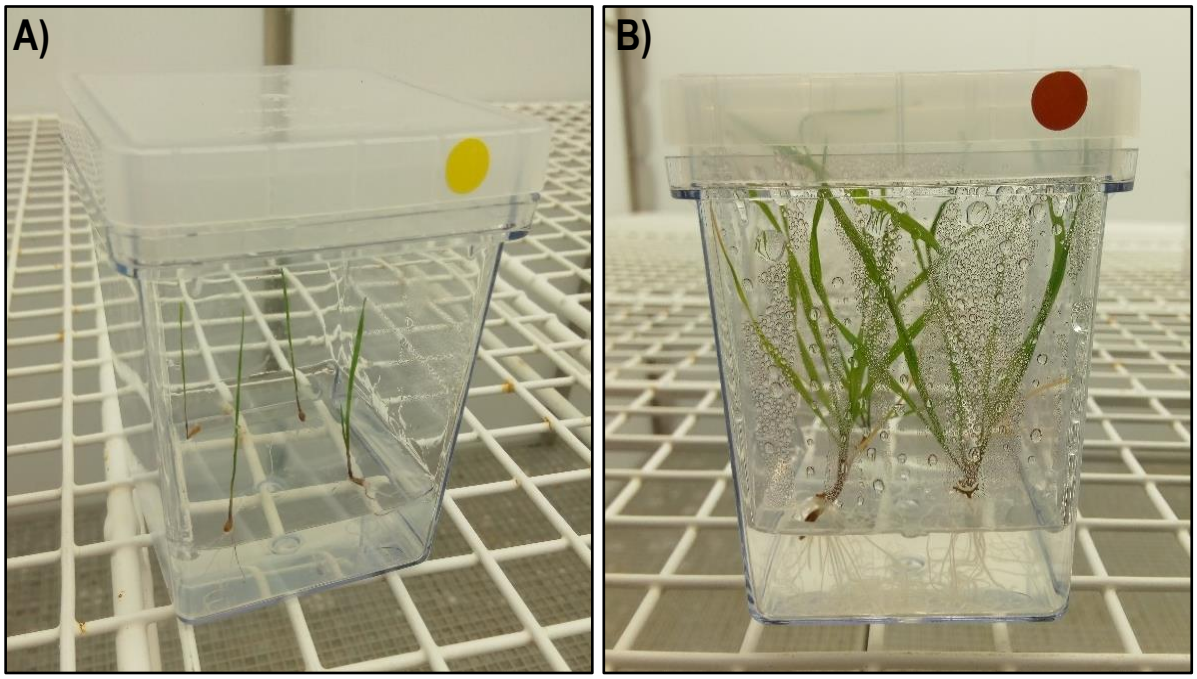

Figure 5.1: Development of black-grass seeds under *in vitro* culture. A) seed growth after 2 weeks. B) seed growth after 7 weeks.

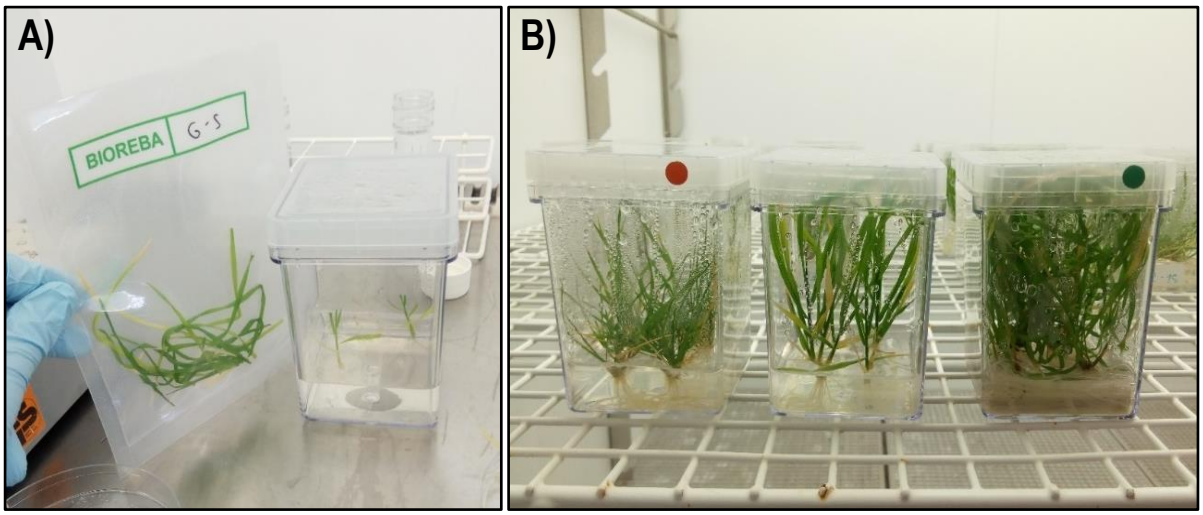

Figure 5.2: First micropropagation after seed set-up. A) micropropagated explants in fresh medium and corresponding sample used for the RT-qPCR analysis. B) explant growth after 8 weeks.

5.2.3 Virus elimination assays

For these assays, we used the G-2 Roth line, which had been identified as not infected by AMVV1 (Table 5.2), and two Peldon lines: R-10 which had been identified as having a high AMVV1 titer (Table 5.2) and B-4, which had been identified as having a medium AMVV1 titer (Table 5.2). These lines were micropropagated into different mediums and subjected to different virus elimination assays: thermotherapy, chemotherapy and a combination of both.

For the thermotherapy assay, two identical growth cabinets were used. The Control cabinet was under the same growth conditions as before while the Stress cabinet´s temperature was set-up at 30 °C, adapting the method used in Ramírez Malagón *et al.* (2006), Senula *et al.* (2000) and Verma *et al.* (2005). Explants were set-up as before and left to grow for 4 weeks before micropropagating them to fresh medium under normal growth conditions. Samples were taken during the micropropagation process and analysed by RT-qPCR as in previous chapters.

For the chemotherapy assay, I used ribavirin (Sigma, Germany) as the anti-viral agent. It was added to the medium after the autoclave step, adapting the method used in Fletcher *et al.* (1998), Ramírez Malagón *et al.* (2006), Senula *et al.* (2000) and Verma *et al.* (2005). Three culture media were used in the chemotherapy assay: the Control medium, standard MS, the A medium, standard MS with added ribavirin at a concentration of 30 mg/L, and the B medium, standard MS with added ribavirin at a concentration of 40 mg/L. Two explants per pot were set-up as before under the same growth conditions. Explants were left to grow for 6 weeks before micropropagating them to standard fresh medium under normal growth conditions. Samples were taken

during the micropropagation process and analysed by RT-qPCR as in previous chapters.

Adapting the methods used in Fletcher *et al.* (1998), Senula *et al.* (2000) and Verma *et al.* (2005), the two treatments were combined so as to have explants growing in a 30 mg/L ribavirin medium under 30 °C and in a 40 mg/L ribavirin medium under 30 °C. Explants were set-up as before and left to grow for 6 weeks before transferring them to standard fresh medium under normal growth conditions. Samples were taken during the micropropagation process and analysed by RT-qPCR as in previous chapters.

5.3 Results

5.3.1 In vitro population set-up

The germination rate of the seeds was 100%. However, one pot was contaminated with bacteria and was discarded. This contamination did not seem to have originated from the seeds as it was not close to them. It was probably due to a human error during the procedure: incorrect sterilisation of the work material, contamination carried on gloves, etc.

The micropropagation survival rate of the explants was: 77.42% for Peldon, 73.91% for Roth and 85.71% for Nott (Figure 5.2). The overall survival rate, considering all lines as a set, was 78.76%. No contamination appeared, with the exception of two pots; but as before, this contamination seemed to be foreign to the explants as it did not appear close to them. Additionally, no unexpected growth formation or other deformations associated with *in vitro* culture were observed, and explants formed healthy looking aerial and root systems (Figure 5.2B). Therefore, this method allowed us to establish a healthy and sterile *in vitro* black-grass population.

5.3.2 Virus elimination assays

Controls had a survival rate of 100% for G-2 (Figure 5.3), 100% for R-10 (Figure 5.4) and 0% for B-4 (Figure 5.5). The overall survival rate was 66.67%. Following testing for all viruses, Ct values for the controls were higher than for the mother plants (Table 5.2). The number of surviving plants per assay is shown in Table 5.1.

Table 5.1: *In vitro* assay set-up. Experimental conditions are showed as temperature in °C plus the concentration of Ribavirin in the medium in mg/L. Number of surviving explants against the total explants used are shown for every line and as a total.

Treatment	Conditions	Surviving explants/total explants			
		Total	G-2	R-10	B-4
Control	*20 °C + 0 mg/L*	4 / 6	2 / 2	2 / 2	0 / 2
Thermotherapy	*30 °C + 0 mg/L*	7 / 12	3 / 4	3 / 4	1 / 4
Chemotherapy	*20 °C + 30 mg/L*	2 / 6	0 / 2	0 / 2	2 / 2
	20 °C + 40 mg/L	2 / 6	0 / 2	0 / 2	2 / 2
Combination	*30 °C + 30 mg/L*	2 / 6	0 / 2	1 / 2	1 / 2
	30 °C + 40 mg/L	2 / 6	0 / 2	1 / 2	1 / 2

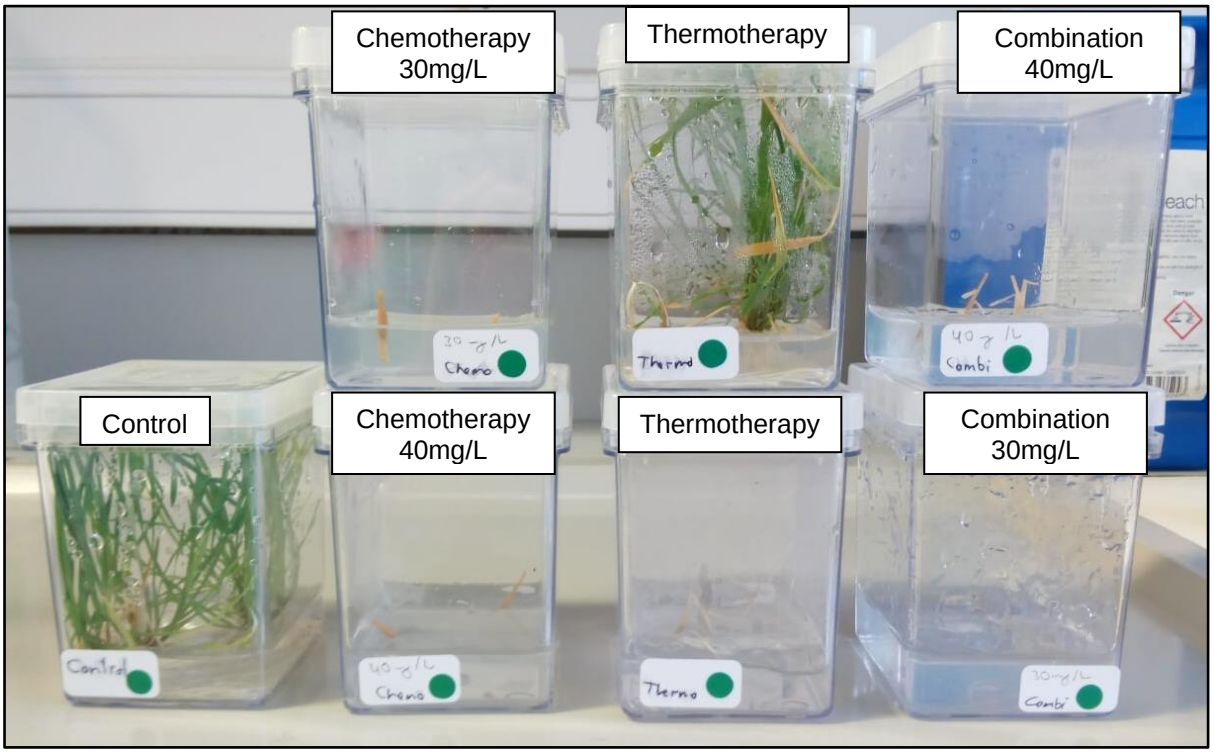

Figure 5.3: State of the explants at the end of the virus elimination assays for line G-2. From left to right and top to bottom: control, 30 mg/L and 40 mg/L chemotherapy, thermotherapy, and 40 mg/L and 30 mg/L combination therapy.

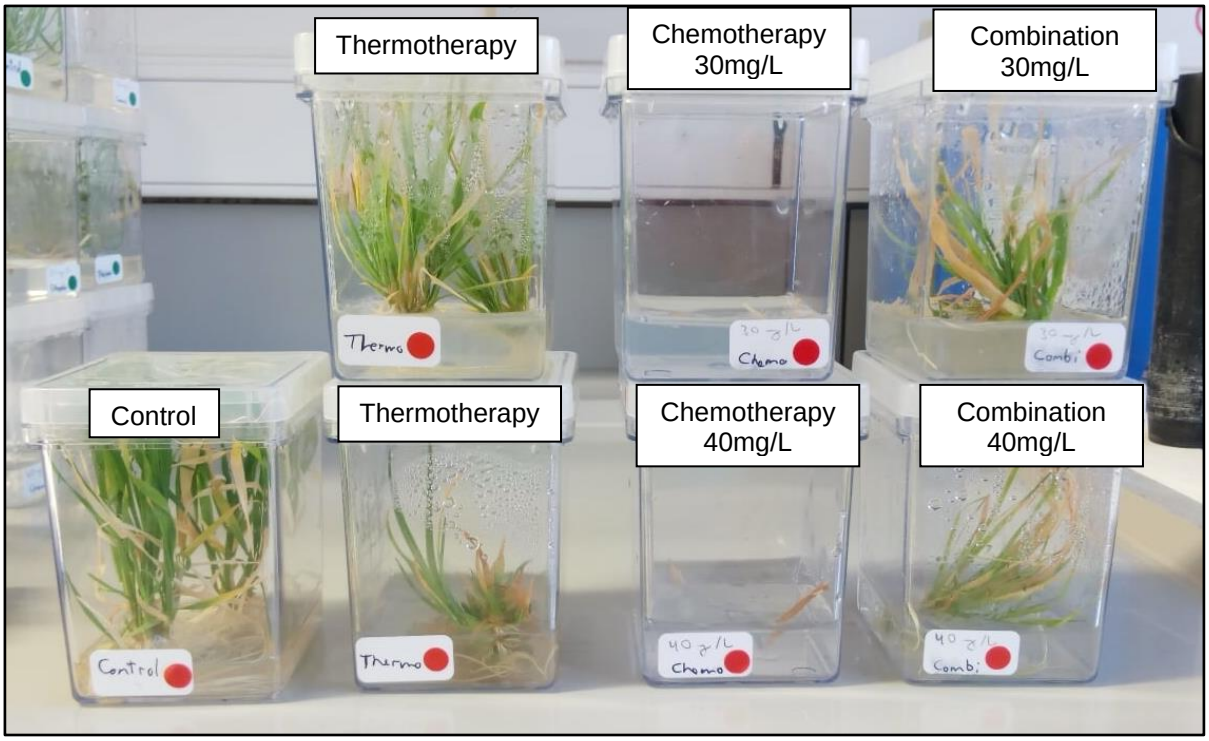

Figure 5.4: State of the explants at the end of the virus elimination assays for line R-10. From left to right and top to bottom: control, thermotherapy, 30 mg/L and 40 mg/L chemotherapy, and 30 mg/L and 40 mg/L combination therapy.

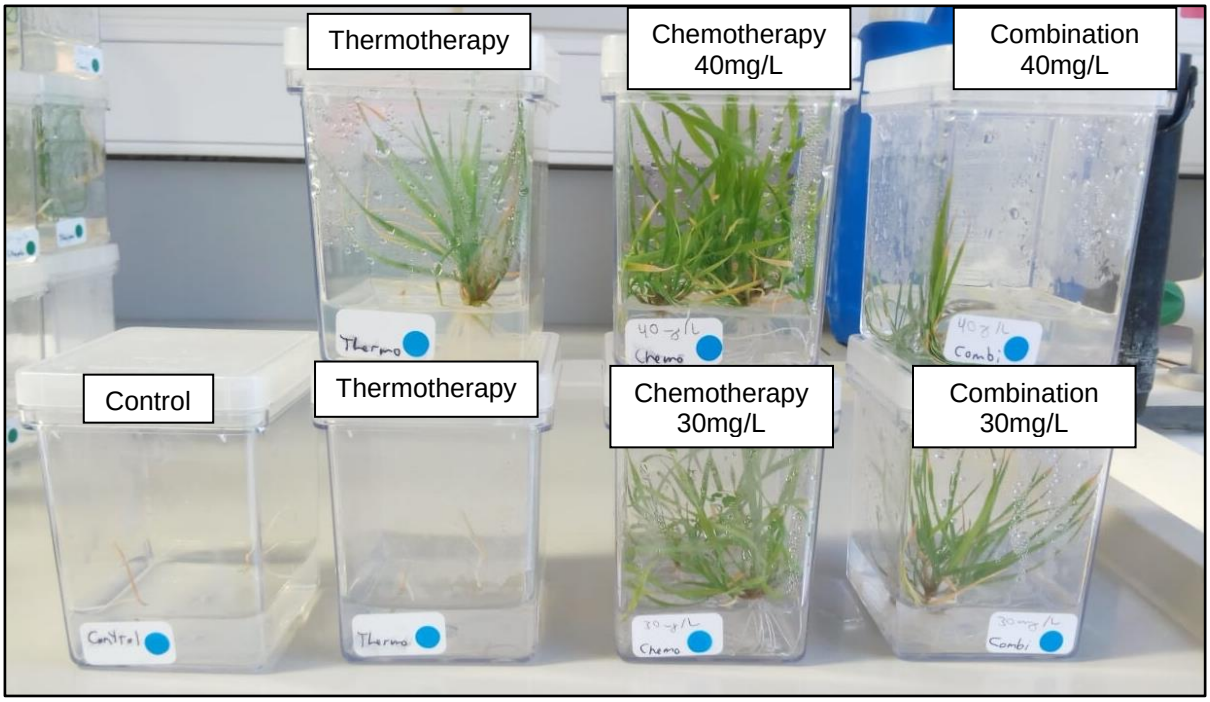

Figure 5.5: State of the explants at the end of the virus elimination assays for line B-4. From left to right and top to bottom: control, thermotherapy, 40 mg/L and 30 mg/L chemotherapy, and 40 mg/L and 30 mg/L combination therapy.

Survival rates following thermotherapy were: 75% for G-2, 75% for R-10 and 25% for B-4. The overall survival rate was 58.33%. No consistent effect of the treatment was visible on the surviving plants when comparing them to the controls. In the case of R-10, thermotherapy had the biggest reduction in virus titer for all viruses (Table 5.2).

Survival rates following chemotherapy were: 0% for G-2, 0% for R-10 and 100% for B-4. The overall survival rate was 33.33%. For both the A and B growth media. No consistent effect of the treatment was visible on the surviving plants when comparing them to the controls.

Survival rates following combination therapy were: 0% for G-2, 50% for R-10 and 50% for B-4. The overall survival rate was 33.33%. For both the A and B mediums. There was no obvious difference between the effect of the different concentrations of Ribavirin, Ct values being very similar or the reduction rate not being consistent between plant lines. Additionally, no consistent effect of the treatment was visible on the surviving plants when comparing them to the controls. In the case of R-4, combination therapy had the biggest reduction in virus titer for all viruses (Table 5.2).

Table 5.2: RT-qPCR results for the *in vitro* assays. Values given are the average of the results of all tested plants for each line and treatment. Values are given in Cts. “40.00” indicates the virus was not detected. “/” stands for assays were no plants survived.

		G-2			R-10			B-4		
		AMVV1	AMPV1	AMPV2	AMVV1	AMPV1	AMPV2	AMVV1	AMPV1	AMPV2
Mother plants		40.00	32.84	31.37	20.81	20.96	16.37	38.91	18.93	31.32
Control		40.00	40.00	40.00	22.26	28.23	17.85	/	/	/
Thermotherapy		40.00	40.00	40.00	26.97	33.47	21.47	40.00	21.26	35.43
Chemotherapy	30 mg/L	/	/	/	/	/	/	40.00	21.94	34.26
	40 mg/L	/	/	/	/	/	/	40.00	23.21	35.75
Combination	30 mg/L	/	/	/	26.28	29.26	21.00	40.00	23.13	39.07
	40 mg/L	/	/	/	24.98	30.49	22.74	40.00	24.04	33.51

5.4 Discussion

We believe the difficulties in setting up an *in vitro* population from pre-existent plants was due to the growth pattern of black-grass; the leaves' blades grow from the base of the stem and tightly cover it, each new leaf growing on top of the older ones, creating layers of leaves in-between which, soil and microorganisms can reside and be protected during the sterilization process. Seeds are generally easier to sterilise, due to their small size, usually smooth surface and sturdiness to aggressive conditions, ensuring a higher survival and growth rate. In our case, this was true; therefore, seeds seem to be the best option to set up an *in vitro* black-grass culture.

A reduction in titer was observed for all viruses after two micropropagations. Reduction in viral titer due to *in vitro* culture and micropropagation has been reported in some cases: Taşkın *et al.* (2013) reported that shoot tip culture of garlic species was able to eliminate *onion yellow dwarf virus* (OYDV) and *leek yellow stripe virus* (LYSV) in 20% and 27% of plants for *Allium tuncelianum* and in 33% and 13% of plants for *Allium sativum*.

All virus elimination methods reduced viral titer, however, thermotherapy was the most effective. It resulted in the highest survival rate (58.33%) and the largest reduction in titer either as a standalone or in combination with chemotherapy. The fact that there did not seem to be a clear improvement to titer reduction when combining thermotherapy and chemotherapy, makes thermotherapy a better option than the combination therapy, as it seems to yield similar results while being less expensive, less complex and yielding a higher survival rate. The toxicity of Ribavirin might have been responsible for the lower survival rate under chemotherapy and combination therapy conditions.

While results were obtained showing no presence of the viruses (Ct values of 40.00) for a few explants, B-4 with AMVV1 and G-2 with AMPV1 and AMPV2, these results are more probably explained by a titer reduction below the detection limit of the RT-qPCR assay. *In vitro* elimination experiments often need sequential treatments to yield results and viral presence can go undetected until the explants are acclimatised and transferred to glasshouse/field conditions (Ramírez Malagón *et al*., 2006). Therefore, a follow-up with acclimatisation of the explants is necessary to confirm the explants infection status.

However, these results showed that it might be possible to eliminate the viruses via *in vitro* culture in combination with thermotherapy. In this case, meristem culture was not used as I lacked the training necessary and given the small size of the assays,

I chose to limit the death rate by using bigger explants. It is therefore necessary to repeat these experiments using meristems, as this could further decrease titer. Additionally, a reduction in viral titer under *in vitro* conditions could affect the presence of the viruses in the pollen. Thus, it could be interesting to see if it is possible to obtain virus-free seeds by breeding *in vitro* plants.

5.5 Conclusion

A reduction in titer was observed for all three viruses under all elimination methods. A reduction was also observed due to micropropagation. However, thermotherapy was shown to be the best option, yielding some of the most important titer reductions while maintaining the highest survival rate. Additionally, it is the least expensive and complex of the tested elimination methods. No confirmed virus-free plants were obtained, but these results imply that it might be possible to obtain virus-free plants via *in vitro* elimination methods or a mixture of these and other techniques such as breeding. Additional experiments with a larger number of plants and their acclimatisation to in-field conditions are necessary.

Chapter 6: General discussion

6.1 Introduction

Cryptic viruses are a group of plant persistent viruses. Due to their very close relationship with their hosts and lack of disease symptoms associated to them, cryptic viruses are hypothesised to act as mutualistic symbionts to their hosts (Boccardo, 1987*;* Hull, 2009; Roossinck, 2010; Roossinck, 2011). These benefits could potentially be exploited to create new technological solutions to crop problems, from new forms of stress tolerance/resistance to yield increases. In this work, I studied three newly discovered viruses, AMVV1, AMPV1 and AMPV2, infecting black-grass (*Alopecurus myosuroides*) populations. These populations were associated to broad-ranging herbicide resistance due to enhanced metabolism (Sabbadin *et al*., 2017). I was interested in seeing if these viruses could play a role in this enhanced metabolism. Biological characterization is essential for virus classification, study and management (Cann, 2012; Gaur *et al*., 2016). A genome analysis of the viruses had previously been done (Sabbadin *et al*., 2017), so I focused on their incidence, infection pattern and transmission mechanism, as well as the effect of *in vitro* viral elimination methods. A drought stress assay was carried out to study the effect of the viruses on plant tolerance to abiotic stress.

6.2 AMVV1, biological characteristics and relationship with its host

AMVV1 was tentatively classified as a varicosavirus (Sabbadin *et al*., 2017). Currently, there is only one varicosavirus ratified by the ICTV, namely LBVaV, with some related but still unclassified viruses including AMVV1, TStV and RCaVV (ICTV's 10th report, *Rhabdoviridae* chapter, 2017). Thus, the information we have for this genus is limited.

I found AMVV1 infection to be systemic, being present in its host's flowers, leaves, stems and roots. However, viral titer was highly variable, both at the plant and population level. Incidence was also quite variable between populations, with Peldon having a high incidence, 79.03%, while the incidence in the other studied populations varied between 0% (population Roth) and 27.78% (population Mart). Transmission experiments showed that AMVV1 was transmitted vertically, with the virus being present in pollen and seeds, and breeding of infected and non-infected plants yielding infected offspring. Moreover, its highest titer in plant parts was found in the flowers,

which could indicate that there is a migration of viral particles to the flowers or an increased replication rate in these during the flowering stage, increasing viral transmission efficiency. However, we found that vertical transmission was not 100% efficient and, in some cases, there was no transmission between populations. Additionally, Peldon, population with the highest incidence, was geographically closer to Roth, population with the lowest incidence, than to other populations. Black-grass pollen being wind borne (CABI, 2018), it was expected for geographically close populations to have similar levels of incidence. All this implies that a barrier to transmission or some type of resistance against AMVV1 might exist, this genotype/phenotype being more common in some populations, like Roth. Vertical transmission of AMVV1 is interesting as the *Varicosavirus* type-species, LBVaV, is only vector transmitted (Maccarone, 2013), as is TStV (Sasaya *et al.*, 2005). And in the *Rhabdoviridae* family, only the genus *Sigmavirus* is vertically transmitted. Interestingly, sigmaviruses are also not associated with disease symptoms (ICTV's 10th report, *Rhabdoviridae* chapter, 2017). While no evidence of vector transmission was found in this work, we recommend studying further this possibility, as only one vector species was tested. As AMVV1 was not widespread and there may be mechanisms that control its transmission and replication, we believe that this virus should not be considered cryptic.

AMVV1 appeared to act as a conditional mutualist under drought stress. Under normal conditions, high AMVV1 titer reduced the growth of its host, though not aggressively enough to be obvious. When placed under drought stress, this negative effect was alleviated; high-AMVV1-titer plants being more similar to low-AMVV1-titer plants under stress conditions than under normal conditions. This conditional protective effect has been described for other antagonistic plant viruses such as TMV, TRV, CMV and BMV, and is believed to be due to the overlapping of tolerance mechanisms to abiotic stress and viral stress (Xu *et al*., 2008; Westwood *et al*., 2013). Overall, the presence of AMVV1 seemed to increase black grass' population fitness. According to the results found in this work, we can consider AMVV1 infection to be positive to its host. Under normal growing conditions, its symptoms don´t seem to represent a disadvantage for its host when compared to non-infected plants, while under drought conditions, it increases the chances of survival of its host, and therefore, of the population/species. The, seemingly, active maintenance of a variable incidence and titer would ensure population plasticity. This positive effect is interesting in the context of varicosaviruses, as it is unclear what their effect is. LBVaV was thought to

be the causal agent of LBVD until it was demonstrated that MLBVV was responsible for the disease. Since, LBVaV has been considered symptomless (Maccarone *et al*., 2010; Maccarone, 2013; ICTV's 10th report, *Rhabdoviridae* chapter, 2017), but Verbeek *et al.* (2013) recently associated LBVaV to necrotic symptoms in lettuce. On the other hand, TStV is clearly associated with disease symptoms (Sasaya *et al.*, 2005). The newly described RCaVV was found in diseased plants co-infected with 8 different viruses, and its effect on the host and transmission mechanism is yet to be elucidated (Koloniuk *et al.*, 2018). The findings for AMVV1 open the door to the possibility of LBVaV having an indirect effect on LBVD. It is therefore possible that LBVaV could either enhance MLBVV infection and thus LBVD progression or lettuce tolerance to LBVD. Viruses have been shown to have complex relationships with the members of their symbiotic system, and their effects are not limited just to their host as shown by CHV-1/host/plant interaction. CHV-1 directly and negatively affects its fungal host, *Cryphonectria parasitica*, which in turn affects indirectly and positively the fungus' plant host, *Castanea spp* (Bryner *et al.*, 2012; Bryner *et al.*, 2014). Further studies into AMVV1, LBVaV and RCaVV would help broaden our understanding of the pathology of varicosaviruses.

It is necessary to repeat the experiments presented here to ensure that the results found are replicable. Additionally, the experimental set-up could be changed to avoid some of the pitfalls encountered during our study. A study focusing solely on AMVV1 could greatly increase the strength of the statistical analysis. The creation of a population with infected and non-infected plants is feasible and would help simplify the assays and their analysis. Nevertheless, plant heterogeneity remains the principal limitation to studying AMVV1. A homogenous population could be obtained through breeding and back-crossing, though it is unclear how many back-crosses would be necessary, potentially making this option very lengthy. It is debatable, however, if using a homogenous population is an ideal choice. Given the high variability in titer values, black-grass being a naturally heterogenous species and AMVV1 probably being specific to black-grass, given its vertical transmission, the use of an artificial homogenous population might only offer limited insight into the viral effect, and could potentially be misleading. Still, it could be useful in support experiments which aim to look at more detailed characteristics of varicosavirus infection. As mentioned before, a follow-up on vector transmission is also recommended and additional studies on drought stress would help confirm and establish AMVV1´s conditional mutualist lifestyle. Additional trials with other stresses (heat, salt, fungal infection, etc.) would

determine if the protective effect is limited to drought stress or extends to other abiotic and biotic stresses.

6.3 AMPV1 and AMPV2, biological characteristics and relationship with their host

Both AMPV1 and AMPV2 were tentatively classified as alphapartitiviruses (Sabbadin *et al*., 2017). The *Partitiviridae* family includes 5 genera and its members are reported to infect both plants and fungi. Plant partitiviruses share many of the same general characteristics of cryptic viruses (ICTV's 10th report, *Partitiviridae* chapter, 2017).

AMPV1 and AMPV2 were systemic in infected plants and widespread in all tested populations, with a 100% incidence. However, titer was variable both at the plant and population level. The highest titer for AMPV1 was found in leaves and for AMPV2, in flowers. While there does not seem to be a barrier to transmission, given the incidence rate, the variable titer might imply the existence of a mechanism that controls the rate of viral replication. As before, this would help maintain population plasticity. Transmission was vertical, with AMPV1 and AMPV2 present in both pollen and seeds. These characteristics firmly establish AMPV1 and AMPV2 as cryptic viruses.

While AMPV1 and AMPV2 seem very similar in terms of their biological characterization, their effect on the host seem radically different. AMPV1 seems to affect the growth structure of its host: high-AMPV1-titer plants tended to grow more tillers, but these were shorter and had fewer leaves than the low-AMPV1-titer plants. What was interesting, is that the overall plant biomass did not seem to be affected by these changes, with both weight and water content remaining stable. This implies that there is a neutral trade-off between the number of tillers and their size. Therefore, the presence of AMPV1 would not be negative to the plant, as there is no loss of biomass due to its presence. Moreover, black-grass grows naturally in grassland, and the changes to the growth structure could prove beneficial under those conditions. The increased tillering of high-AMPV1-titer plants means that they have a spreading habit, potentially making them more efficient at competing with other species for growing space, which in turn limits the germination of other species and prevents shadowing from taller plants. This spreading habit would also increase their resource availability, especially if the spreading is also associated to the root system (Weaver, 1930; Salehi-Lisar and Bakhshayeshan-Agdam, 2016). Additionally, grazing stress has been reported to more severely affect tall species (Weaver, 1930; Lorenz and Rogler, 1967),

thus, the smaller size of high-AMPV1-titer plants would have a protective effect against this stress. The increased number of tillers could correlate with an increased number of flowers and seeds, maximising the spread of the host species and of the virus. Overall, the presence of AMPV1 may confer increased host fitness, particularly under grassland conditions. This effect can be considered similar to those reported for other cryptic viruses: WCCV1 was found to regulate nodulation (Nakatsukasa-Akune *et al.,* 2005) and PCV-1 helps deter aphids from its host (Safari *et al.,* 2019); virus-led changes to existing mechanisms that are not obviously detrimental and improve host fitness under certain conditions.

In contrast, AMPV2 seems to be completely antagonistic to its host. Reducing the growth of its host under normal conditions and exacerbating this negative effect under drought stress. An increased negative effect has been reported for certain combinations of viral and abiotic stress: BYV and drought in sugar beet (Clover *et al*., 1999), TuMV and drought in *A. thaliana* (Prasch and Sonnewald, 2013), etc. However, AMPV2's detrimental effect did not seem to cause the host an obvious disadvantage under normal conditions. But, under long periods of drought stress, AMPV2 infection could prove highly disadvantageous. Given the negative effect of AMPV2 it would be interesting to study if its titer variability is due to a host resistance mechanism, a virus evading mechanism or a combination of both. While no detrimental plant partitivirus has been reported before, this genus is known to negatively affect some fungi species. AMPV2 could be the first reported antagonistic plant partitivirus.

As before, it is necessary to repeat the experiments to ensure that the results found are repeatable. For both partitiviruses, given their incidence rate, studies may still have to rely on viral titer level instead of absence/presence of virus. A reduction in viral titer was observed under *in vitro* conditions and after thermotherapy, chemotherapy and a combination of both. While no partitivirus has been eliminated through *in vitro* viral elimination methods, there might be a possibility that non-infected plants could be obtained through a combination of these *in vitro* methods and breeding, reducing titer to a point where pollen transmission would no longer be 100% efficient. But the success rate would presumably be very low and, as with AMVV1, reducing the population heterogeneity might not be ideal.

In the case of AMPV1, a follow-up study on plants physiological parameters, including roots, and viral titer would help determine how strong the effect of the virus is on growth structure, and if this effect is found in all black-grass populations. Assays under grassland conditions and grazing stress would help establish how useful the

theoretical positive effects of the change in growth structure are in-field. A survey of grasslands, taking black-grass' physiological data and samples for PCR testing, would allow for a relatively fast in-field study of the connection between viral titer and growth structure. As well as supporting the study of AMPV1´s incidence in new black-grass populations. A more controlled and detailed study could be developed by growing black-grass together with other common grassland species (*Lolium perenne, Agrostis stolonifera, Holcus lanatus, Festuca pratensis,* etc.) (Walker *et al.,* 2004) under controlled environmental conditions. This would support the study of the effect of AMPV1 on black-grass under its natural conditions. Variations on this experimental set-up, with the creation and comparison of different experimental lots: no competition (only black-grass is grown), titer groups (low titer, high titer, etc.), different levels of soil resources, etc., would allow a higher resolution analysis of AMPV1´s effect, its limits and the possibility of titer changes to adapt to the environment. Additionally, introducing grazing stress, through periodic cutting of the plants to simulate the effect of grazing damage, would help study the tolerance and survival rates to this stress of black-grass plants with different titer levels, and the difference in tolerance with other grassland species.

AMPV2 analysis suffered the most from the encountered limitations, therefore, a follow-up trial of its effects under normal and stress conditions is necessary. Steps should be taken to limit as much as possible the unwanted variability due to plant age and environmental conditions.

6.4 Limitations of this study and possible solutions for future work

Cryptic virus studies have been heavily constrained due to the difficulties in detecting and working with them. Their low titer has historically made them very difficult to detect, and only the development of new techniques like NGS and qPCR has allowed us to confidently detect and quantify them (Boccardo, 1987*;* Hull, 2009; Roossinck, 2011). Cryptic viruses are usually widespread in both population and species, meaning that usually there are no non-infected individuals to be found. Additionally, their transmission is limited to host sexual reproduction and there is no effective *in vitro* elimination method. All of the above hinder comparative studies on their effects (Boccardo, 1987; Hull, 2009; Roossinck, 2010; Roossinck, 2011). The only reported effects of cryptic viruses have been obtained by genetic transformation, in the case of WCCV1 (Nakatsukasa-Akune *et al.,* 2005), and by the chance finding of two

non-infected individuals, in the case of PCV-1 (Valverde and Gutierrez, 2008; Safari *et al.,* 2019).

Similarly, we encountered several limitations when studying AMVV1, AMPV1 and AMPV2. The use of highly heterogenous populations and the lack of non-infected AMPV1 and AMPV2 plants meant that our experimental set-up was not straightforward and had to rely on titer variability. The lack of mechanical transmission and the difficulties in breeding meant that the creation of bespoke populations was difficult and not feasible in the available time. But the biggest limitation was the unexpected variability found during the stress assays: physiological plant age, effect of tillering on plants, effect of plant age on virus titer and high replicate variability due to non-controlled environmental conditions, such as shifts in natural light and drastic changes in ambient temperature, glasshouse settings are able to minimize their effect but can't completely eliminate their impact. For future experiments and similar systems, it becomes essential to have a better understanding of how the plant host reacts to different environmental conditions and propagation methods, so that these can be accounted for in the experimental set-up or during the analysis of the results. Additionally, variations in viral titer across the host´s life cycle should be recorded prior to any experiments, so that these can be designed around these potential variations.

Overall, looking at our system, it is necessary to repeat the experiments to ascertain that the observed effects are statistically significant and are reproducible in these and other black-grass populations. Given the analysis limitations due to unexpected variability, an in-depth investigation at the relationship between plant age and viral titer is necessary, and future experiments should try to control this factor and, ideally, limit tillering to a minimum. Similarly, environmental conditions seem to be a limiting factor and, if possible, the use of simultaneous repeats or larger experimental populations would contribute to resolving these problems. As mentioned before, studies focused on the individual viruses, specially AMVV1, would potentially allow for more robust results, confirming and possibly expanding on the effect of these viruses.

6.5 Conclusion

The discovery of increasing numbers of seemingly non-pathogenic viruses opens the door to the use of these for our benefit. In the case of plants, persistent viruses stand as a possible source of underexplored genetic variation, which could be exploited for the protection of crop species from adverse abiotic stress conditions. In this work, we have found that persistent viruses not associated with disease symptoms

can still act as very mild pathogens, as is the case of AMPV2 and AMVV1. However, we have found evidence of beneficial effects: AMVV1 under stress and AMPV1. And in the case of AMVV1, the negative effects seem negligible, being able to consider it fully beneficial. Together with recent findings on the beneficial effects of WCCV1 and PCV-1 (Nakatsukasa-Akune *et al.,* 2005; Safari *et al.,* 2019), it seems that some plant persistent viruses could effectively be used to benefit crop species. With the caveat that feasible solutions for the transfer of either the viruses or the beneficial mechanisms to species of interest must be found first. Which shows the need to further study this type of viruses, to not only reap technological benefits, but to have a better understanding of viruses themselves and their relationship with their hosts.

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