

**Investigations of mycoviruses from  
*Leptosphaeria* species and their effects on  
pathogenicity**

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## Abstract

The aims of this research were to assess the incidence of dsRNA viruses in five different plant pathogenic fungi, including *Hymenoscyphus fraxineus*, *Dothistroma septosporum*, *Leptosphaeria* species (*Leptosphaeria maculans* and *Leptosphaeria biglobosa*) and *Pyrenopeziza brassicae*, as their incidence has not been reported previously in these species, and then to investigate the effects of mycoviruses on the growth and pathogenicity of the fungi. Hence, 57 *L. maculans* and 16 *L. biglobosa* isolates were screened and 11 *L. biglobosa* isolates possessed dsRNA elements while only one of the 45 *D. septosporum* isolates was found to contain a mycovirus subsequently identified as a chrysovirus. In contrast, none of the 162 *H. fraxineus* and 10 *Pyrenopeziza brassicae* isolates appeared to contain dsRNA elements.

Further research was carried out on *L. biglobosa* because of the excellent recovery of dsRNA elements and the fact that it is also responsible for causing phoma stem canker which is an economically important disease of oilseed rape worldwide. However, not only *L. biglobosa* but also the more damaging *L. maculans* causes phoma stem canker. They are closely related and co-existing plant pathogens. Mycoviruses are a specific group of viruses that infect and replicate in fungi. They can be associated with hypovirulence or hypervirulence but are normally cryptic. Three different mycoviral dsRNAs were identified from *L. biglobosa* isolate C-Rox 12.8.1, ranging in size from ca. 4.0-4.9 kbp. Sequence analysis of LbMV-1 dsRNAs 1 and 2 revealed that they are most closely related to members of the family *Totiviridae*. However, attempts to characterise LbMV-1 dsRNA 3 failed. On the other hand, four mycoviral dsRNAs were identified from *D. septosporum* isolate D 752.1, ranging in size from ca. 2.8-3.5 kbp. Molecular characterisation of DsCV-1 showed that it is very similar to the chrysoviruses.

To obtain information on the effects of dsRNAs on their host, cycloheximide treatment was used to eradicate dsRNA elements from *L. biglobosa* isolate W10. Subsequently, comparative growth experiments to assess radial growth and mycelial weight for virus-infected and virus-free W10 isolates were performed. These experiments showed that the LbMV-1 infection increased the growth of the fungus. To investigate the influence

of mycoviruses on the pathogenicity of *L. biglobosa*, pathogenicity tests were performed using isogenic lines of the W10 isolate inoculated onto oilseed rape cotyledons and disease symptoms were analysed at different time periods; mycoviruses were found to increase fungal pathogenicity. In addition to this, effects of pre-treatment of *B. napus* leaves with conidia of virus-infected or virus-free *L. biglobosa* on infection by conidia of *L. maculans* and development of disease (phoma leaf spot) were studied in a controlled-environment conditions. Pre-treatment of first true leaves with virus-infected *L. biglobosa* decreased the phoma leaf spot lesion area on second true leaves (systemic effect) as compared to pre-treatment with virus-free *L. biglobosa*.

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# **Chapter 1**

## **General Introduction**

## 1.1 Mycoviruses

Whereas animal and plant viruses have been studied extensively, mycoviruses have been studied to a much lesser extent. Moreover, full molecular characterisation of large numbers of mycoviruses is yet to be achieved (Pearson *et al.*, 2009). The importance of viruses is mainly because of their ability to cause disease in plants, animals and humans; however, their role in mutualistic symbiosis has also been demonstrated in recent decades (Roossinck, 2011).

Mycoviruses are viruses that infect fungi and are widespread in filamentous fungi, yeasts, mushrooms and oomycetes (Wu *et al.*, 2012). The majority of the mycoviruses possess single-stranded (ss) or double-stranded (ds) RNA as their genetic material. Mycoviruses have diverse genome structures: viruses of the families *Chrysoviridae*, *Partitiviridae*, *Endornaviridae*, *Megabirnaviridae*, *Quadriviridae*, *Reoviridae* and *Totiviridae* have a dsRNA genome, while those of *Alphaflexiviridae*, *Gammaflexiviridae*, *Hypoviridae*, *Narnaviridae*, and *Barnaviridae* have a ssRNA genome (Ghabrial *et al.*, 2015). Furthermore, *Metaviridae* and *Pseudoviridae* do not encode a RNA-dependent RNA polymerase (RdRP) but use a reverse transcriptase (Wu *et al.*, 2012).

Frequently, several unclassified dsRNA viruses have been isolated from fungi. Mycoviruses RnQV-1 and RnQV-2, isolated from *Rosellinia necatrix* and Amasya cherry disease-associated mycovirus, containing four-segment dsRNA genomes, were classified into a new family, *Quadriviridae*. *Alternaria alternata* dsRNA mycovirus and *Aspergillus* mycovirus 341 contain four-segment dsRNA genomes, although they are significantly different from viruses in *Chrysoviridae* and *Quadriviridae* families.

Some novel non-segmented dsRNA viruses including *SsNsV-L*, *Diplodia scrobiculata* RNA virus 1, Grapevine associated totivirus-2, and *Phlebiopsis gigantea* mycovirus dsRNA 2, *Fusarium graminearum* dsRNA mycovirus-3, are phylogenetically related to each other but are distant from totiviruses (Xie & Jiang, 2014).

Recently, mycoviruses of an endophytic fungus have been reported to replicate in two different plant hosts (Nerva *et al.*, 2017). There is also some evidence which confirms the ability of viruses to adapt their replication to host species from different kingdoms.



For example, plant viruses, brome mosaic virus (BMV) (Janda & Ahlquist, 1993), tomato bushy stunt virus (TBSV) (Panavas & Nagy, 2003) and tobacco mosaic virus (TMV) (Mascia & Gallitelli, 2016) can replicate in different ascomycetous species. Circular DNA virus of the genus Genomovirus was also shown to replicate in both insect and fungal cells (Liu *et al.*, 2016). A relationship between plant viruses and mycoviruses was first revealed for Hypoviruses, which are phylogenetically related to plant-infecting Potyviruses (Koonin *et al.*, 1991).

The origin of mycoviruses is a mystery but two main hypotheses have been proposed to describe the origin of mycoviruses (Pearson *et al.*, 2009). The “ancient coevolution hypothesis” suggests that the association between mycoviruses and fungi is ancient and indicates long-term coevolution. In contrast, the “plant virus hypothesis,” states that plant viruses are the origin of mycoviruses; i.e. the original mycovirus was a plant virus which transferred from plant to fungus within the same host plant. Similar explanations might also be given for the origin of plant viruses; i.e. plant viruses originated from mycoviruses that may have moved from fungus to plant (Nibert *et al.*, 2014).

### **1.1.1 Totiviridae**

Members of this family contain mono-segmented dsRNA genomes, 4.6-7.0 kbp in size, and usually encompass two overlapping open reading frames (ORFs) on one strand. These two ORFs encode the capsid protein (CP) and the RNA-dependent RNA polymerase (RdRP). Members belonging to this family are divided into two genera: *Toti-* and *Victoriviruses* (King *et al.*, 2011; Ghabrial, 2008). Viruses infecting smut fungi and yeasts have been placed in the genus *Totivirus* and viruses infecting filamentous fungi in the genus *Victorivirus* (Ghabrial & Nibert, 2009).

There are reports of three different strategies used for RdRP expression among members of this family: (1) fusion protein (CP/RdRP) consequent to ribosomal frame-shifting, for example in parasitic protozoa viruses and *Saccharomyces cerevisiae* virus L-A (ScV-L-A) (Dinman *et al.*, 1991); (2) fusion protein following fusion with the CP gene without ribosomal frame-shifting, as in *Ustilago maydis* virus H1 (Kang *et al.*, 2001); and (3) non-fused protein by a termination-reinitiation mechanism, as shown

for victoriviruses and *Helminthosporium victoriae* virus 190S (HvV190S) (Huang & Ghabrial, 1996; Li *et al.*, 2011; Soldevila & Ghabrial, 2000).

### **1.1.2 Partitiviridae**

Members of this family have bisegmented genomes 1.4-2.4 kbp in length. Both segments encompass ORFs. The larger ORF encodes RdRP and smaller ORF encodes CP. Two separate virus particles are produced at the end of the packaging process of two genome segments. There are three different genera in this family: *Alpha-*, *Beta-*, and *Gammapartivirus* (Nibert *et al.*, 2014). Gammapartitiviruses only infect filamentous fungi, whereas Alpha- and Betapartitiviruses infect not only filamentous fungi but also plants.

### **1.1.3 Megabirnaviridae**

*Megabirnavirus* is the only current genus in this family and *Rosellinia necatrix* Megabirnavirus 1 (RnMBV1) is the prototype. It has two genome segments and both are packaged into separate virus particles, which are encapsidated in isometric particles ~50 nm in diameter. DsRNA 1 encompasses two overlapping ORFs encoding CP and RdRP, which are expressed as a fusion product with CP *via* ribosomal frame-shifting (Salaipeth *et al.*, 2014; Chiba *et al.*, 2009). DsRNA 2 encompasses two non-overlapping ORFs.

### **1.1.4 Chrysoviridae**

*Chrysovirus* is the only genus of this family and *Penicillium chrysogenum* virus (PcV) is the prototype of the genus (Ghabrial & Caston, 2011). It contains four dsRNA segments, 2.4-3.6 kbp in size, and these segments are separately encapsidated in virions. DsRNA 1 and dsRNA 2 encode RdRP and CP, respectively. DsRNA 3 and dsRNA 4 encode P3 and P4 proteins of unknown function. The 5'- and 3'- terminal sequences of these four dsRNAs have been found to be highly conserved (Jiang & Ghabrial, 2004).

### **1.1.5 *Quadriviridae***

The genus of this family *Quadrivirus* includes one species to date, *Rosellinia necatrix quadrivirus 1*. Two well characterized strains of this species were isolated from different locations in Japan (Lin *et al.*, 2012; 2013). Members of this family have four monocistronic genome segments, ranging from 3.7-4.9 kbp in length, which are encapsidated in isometric virus particles ~45 nm in diameter. DsRNA 1 encodes a product of unknown function, while dsRNA 2 and dsRNA 4 encode CPs, and dsRNA 3 encodes the RdRP. Each quadrivirus genome segment contains “CAA” repeats at 5' UTR (Ghabrial *et al.*, 2015).

### **1.1.6 *Reoviridae***

In 1994, “reo-like” virus particles were first observed in a fungus (Enebak *et al.*, 1994) and these viruses were placed in a genus *Mycoreovirus*. *Mycoreovirus 1-3* (MyRV1-3) were accommodated in this genus. MyRV1 and -2 were found in *Cryphonectria parasitica*, and MyRV3 was found in *Rosellinia necatrix* (Hillman & Suzuki, 2004; Wei *et al.*, 2004). These mycoreoviruses were reported to confer hypovirulence to their natural hosts. Mycoreoviruses contain monocistronic genome segments with 5'-caps on their positive-stranded RNAs. MyRV1 and -2 are composed of 11 genome segments while MyRV3 has 12 genome segments and each virus ranges in size from 0.7- 4.1 kbp (Kanematsu *et al.*, 2004).

### **1.1.7 *Endornaviridae***

In the 9th ICTV report, some mycoviruses were assigned to family *Endornaviridae* with dsRNA viruses though they are phylogenetically closely related to alpha-like ssRNA viruses (Ghabrial & Suzuki, 2009; Hacker *et al.*, 2005). Endornaviruses contain a linear dsRNA genome *ca.* 14-17 kbp in size and encode long polypeptides such as RNA helicases, RdRPs and UDP glucosyltransferases. Endornaviruses do not produce true virions and they are normally found in cytoplasmic vesicles of infected fungi, plants and oomycetes.

## 1.2 Satellite or defective dsRNA viruses

Subviral RNA forms such as defective RNA (D-RNA) and defective interfering RNA (DI-RNA) have been identified amongst a wide variety of mycoviruses that occur naturally and/or during conservation in the laboratory (Marriott & Dimmock, 2010; Simon *et al.*, 2004). These RNAs are known as 'defective' because they do not have capacity to encode the essential viral proteins and to replicate in the absence of the parent (helper) virus. DI-RNAs are called 'interfering' as they can attenuate the symptoms caused by the helper virus. However, some defective RNAs do not interfere with multiplication of their helper viruses; they are known as D-RNAs. Satellite RNAs do not show intensive sequence similarities with their helper viruses and the origin of their nucleic acid sequences remain uncertain, whereas DI-RNA contains closely related, shorter forms of parental viral genomes (Simon *et al.*, 2004). DI-RNAs are normally produced by recombination and/or deletion that is frequent at specific regions rather than occurring randomly throughout the parental genome (Cheng *et al.*, 2003; Nagy & Simon, 1997).

DI-RNA generally has a negative effect on replication and symptom expression of the helper viruses (Marriott & Dimmock, 2010; Simon *et al.*, 2004). Recently, a hypovirulent strain of *Sclerotinia sclerotiorum* was found to contain *Sclerotinia sclerotiorum* botybirnavirus 1 (SsBRV1) and a satellite-like RNA that had no obvious effect on the colony morphology but reduced the growth rate and virulence of the host fungus (Liu *et al.*, 2015). There are a few hypotheses that support this phenomenon, such as the suggestions that the DI-RNA may interfere with viral replication or that DI-RNA stimulates RNA silencing that degrades helper viral mRNAs as an antiviral defence mechanism and reduces symptom induction (Havelda *et al.*, 1998; Szittyá *et al.*, 2002).

Two groups of D-RNAs are recognized; one group that is associated with *S. cerevisiae* is known as M satellites and other group is associated with *Trichomonas vaginalis* T1 virus (TVT1) of the eponymous protozoan. D-RNAs have also been reported in association with mycoviruses belonging to the families *Hypoviridae*, *Narnaviridae*, *Partitiviridae* and *Totiviridae* (Palukaitis *et al.*, 2008). M satellite dsRNA encodes a toxin which kills other yeasts not containing this dsRNA, offering immunity to the host carrying it (Palukaitis *et al.*, 2008).

### 1.3 Discovery and distribution of fungal viruses

Mycoviruses have been described in over 60 species from approximately 50 genera of fungi (Lemke & Nash, 1974). A disease of cultivated mushroom (*Agaricus bisporus*) reported in 1950 (Sinden & Hauser, 1950) caused premature degradation of mushroom tissue and altered morphology during the development of the mushroom and decreased mushroom production. Sinden was the first to propose that a virus might be associated with the disease (Sinden & Hauser, 1957). The virus itself was first observed by the use of transmission electron microscopy (Gandy & Hollings, 1962). Subsequently, three morphologically distinct viruses were identified in diseased mushrooms (Hollings, 1962; 1965; Hollings *et al.*, 1963; 1965). The infective and pathogenic characteristics of these viruses were studied after their partial purification by density-gradient centrifugation.

Another group of fungi, the *Penicillium* moulds, were also found to contain viruses. They were discovered during studies of *P. stoloniferum* and *P. funiculosum*. The search for effective compounds active against poliovirus developed interest in research for antiviral substances produced by these two *Penicillium* species (Powell, 1952; Shope, 1953). Polyhedral virus particles were isolated from the antiviral fraction of *P. stoloniferum* and visualised through electron microscopy (Ellis & Kleinschmidt, 1967). Simultaneously, it was discovered that the virus particles associated with *P. stoloniferum* contained dsRNAs, which were responsible for the antiviral activity of the two moulds (Banks *et al.*, 1968).

In early 1936, the presence of virus in yeast was presumed, and later Lindegren and co-workers suggested that a lytic phenomenon in yeast was presumably associated with a viral infection (Hirano *et al.*, 1962; Lindegren *et al.*, 1962).

Many other fungi have been implicated in transmission of viral diseases to plants. *Olpidium brassicae*, *Polymyxa graminis* (Estes & Brakke, 1966) and *Synchytrium endobioticum* are some examples of fungi which function as vectors for the transmission of viruses to higher plants (Campbell, 1962; Estes & Brakke, 1966; Fry, 1958; Grogan, 1958; Nienhaus & Stille, 1965).

## 1.4 Structural features of mycoviruses

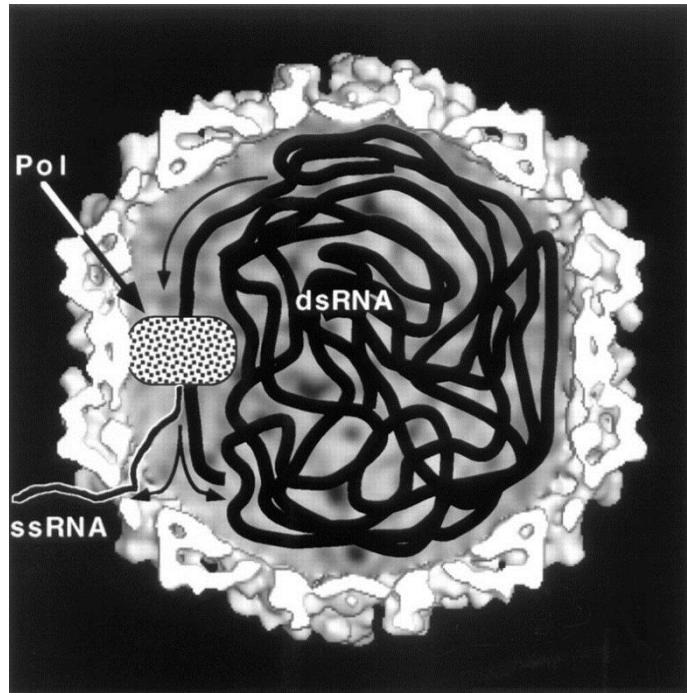
DsRNA viruses of the families *Totiviridae*, *Partitiviridae*, *Chrysoviridae* and *Reoviridae* contain icosahedral capsids (Ghabrial & Suzuki, 2009). Mycoreoviruses are assumed to have concentric, double-shelled structures whereas partitiviruses, chrysoviruses and totiviruses have simple single-shelled icosahedral capsids (Ghabrial & Suzuki, 2009).

To date, 3D structural analyses have focused on the mycoviruses with dsRNA genomes and single-layered icosahedral capsids, despite the wide diversity of fungal viruses. Virus particles are purified in large amounts for their characterisation by 3D electron microscopy and/or X-ray crystallography. Structural analyses of dsRNA viruses have presented ubiquitous characteristics in a broad spectrum of dsRNA mycoviruses, together with those that infect prokaryotes and complex eukaryotes.

Totiviruses L-A and P4, infecting the yeast *S. cerevisiae* and the smut fungus *U. maydis*, respectively, were the first illustrated viruses with an unusual T=1 capsid formed by 12 decamers (Cheng *et al.*, 1994). “T=2” capsid of dsRNA viruses is identified as the inner or core capsid in the multi-layered capsids of *Reoviridae* members (Grimes *et al.*, 1998; Reinisch *et al.*, 2000). These capsids remain structurally intact during the viral life cycle and mediate multiple activities (Fig. 1.1); they assist in sequestering the dsRNA genome and avoiding induction of dsRNA-signalled host defence mechanisms which operate in some hosts. In addition to this, unusual capsid stoichiometry and architecture are presumed to be related with RNA synthesis activities, such as assembly of the packaged dsRNA molecules and capsid-bound RdRP complexes for replication and transcription processes; release of the positive-strand transcripts for protein synthesis or packaging into new virions and addition of 5' caps to those transcripts.

## 1.5 Transmission of fungal viruses

It was anticipated that mycoviruses are not infectious as free particles and lack an extracellular phase in their life cycle. It is generally considered that viral transmission is limited to the intracellular routes and it depends on their fungal hosts (Buck, 1998). More specifically, mycoviruses are transmitted horizontally by heterokaryosis or vertically by sporulation. However, recently DNA mycovirus particles purified from an



**Figure 1.1 Hypothetical model of a transcriptionally active L-A virion.** Polymerase (Pol) is covalently adhered to a Gag subunit, which is assumed to be integrated into the icosahedral surface lattice. The capsid wall perforated by many holes functions as a molecular sieve which can retain dsRNA inside but allow passage of an ssRNA molecule (Castón *et al.*, 1997).

infected host, *Sclerotinia sclerotiorum* hypovirulence-associated DNA virus 1 (SsHADV-1), were demonstrated to be infectious when applied extracellularly to hyphae of virus-free host *Sclerotinia sclerotiorum* grown on PDA or sprayed on leaves of *Arabidopsis thaliana* and *Brassica napus*. SsHADV-1 can suppress the development of lesions on leaves, thus reduce disease severity and enhance rapeseed yield significantly under field conditions. Nevertheless, SsHADV-1 has a narrow host range; it could only infect sister species of *S. sclerotiorum* and cause debilitation of the host fungi, but could not infect or transfect other fungi tested. Hence, SsHADV-1 was demonstrated to have potential as a natural fungicide (Yu *et al.*, 2013).

Initial experiments were done by Lhoas, who confirmed the transmission of viruses in fungi through heterokaryosis (Lhoas, 1970; 1971). He did an experiment with genetically marked strains of *P. stoloniferum* and *Aspergillus niger* and demonstrated the transmission of virus from the virus-infected strain to the non-infected strain. During anastomosis, hyphal fusion occurs between different fungal strains; this permits the exchange of cytoplasm and genetic material, including mycoviruses. Successful anastomosis depends on vegetative compatibility of the two fungi. This process is controlled by vegetative incompatibility and heterokaryon incompatibility genes in ascomycetes (Glass & Dementhon, 2006; Saupe, 2000). Therefore, the spread of viruses through heterokaryosis is restricted by the incompatibility between fungi, which affects the host range of a mycovirus.

Vegetative incompatibility is considered as non-self-recognition to counteract microbial antagonism and causes programmed cell death (PCD) of contacted fungal cells. Mycoviruses can only be transmitted efficiently if they can suppress the host vegetative-incompatibility reaction (Xie & Jiang, 2014). It was determined that CHV1 could suppress the host vegetative-incompatibility reaction and transfer in the host (Biella *et al.*, 2002). Moreover, CHV1- host (Ep773) induced the down-regulation of genes that are involved in PCD (Shang *et al.*, 2008). Recently, successful horizontal transmission of the *Cryphonectria hypovirus 1* (CHV1) was detected in different fungal species of *Cryphonectria* (Liu *et al.*, 2003). Similar phenomena have been described in different taxa of ascomycetes and basidiomycetes (Ikeda *et al.*, 2005). This occurrence indicated that hypoviruses might suppress the vegetative-incompatibility



reactions. However, the degree of suppression is different, based on individual mycoviruses and their hosts.

Horizontal transmission *via* protoplasmic fusion is a successful route of transmission of mycoviruses between different fungal strains (Suzaki *et al.*, 2005; Xie *et al.*, 2006). Interestingly, transmission of mycoviruses between different species has been reported, despite the lack of mechanical or vector transmission (Pearson *et al.*, 2009). In addition, the ability of mycoviruses for interspecies transmission between *Fusarium poae* and black *Aspergillus* spp. through protoplast fusion was demonstrated and infection was found to be stable even after several rounds of sub-culturing (Van Diepeningen *et al.*, 2000).

Vertical transmission by spores is another primary mode of mycovirus dispersal; nevertheless, the rate of transmission depends on the fungus/virus combination and the spore type (sexual or asexual). Mycoviruses are more easily transmitted by the asexual spores produced from modified hyphae (Buck, 1998), while the rate of transmission through sexual spores has been reported to be less, at least for fungi with an extended sexual stage in their life cycle (Varga *et al.*, 2003).

There has been no evidence of infection of intact filamentous fungi by purified virus particles; however, protoplasts of many fungi have been infected with purified mycoviruses under experimental conditions (Pearson *et al.*, 2009). For example, protoplasts of *S. cerevisiae* were successfully transfected with virus-like particles (VLPs) (Schmitt & Breinig, 2002).

## **1.6 Replication of dsRNA viruses**

Mycoviruses have several modes of replication, which vary considerably in different fungi. There are very few studies on the replication of mycoviruses due to the cellular complexity of their hosts and, interestingly, they replicate in parallel without killing their host (Lemke & Nash, 1974). Mycoviruses may replicate using conservative (Nemeroff & Bruenn, 1986; Wickner, 1996) or semi-conservative mechanisms (Buck, 1978). As in ssRNA viruses, dsRNA does not function as mRNA and dsRNA is transcribed into mRNA following amplification by RdRP. These mRNA transcripts are translated into structural and regulatory viral proteins. These (+) strands can be encapsidated and

form immature virions. The mRNA transcripts also serve as template for the synthesis of complementary (-) strands (Castón *et al.*, 1997); thus, the complete mature virions are produced with encapsidated dsRNA.

## **1.7 Effects of mycoviruses on host phenotypes**

Mycoviruses can induce advantageous or destructive effects on host fungi, but most mycoviral infections are latent and asymptomatic (Buck, 1998). Some effects caused by mycoviruses have been elucidated. However, the function of mycoviruses in regulating host ecology is not known (Hyder *et al.*, 2013).

### **1.7.1 Symptomless infection (cryptic)**

The term 'cryptic' indicates that the viruses might induce symptoms only under specific environmental conditions. Although they are widespread and there is often absence of obvious impact, many investigators consider that mycoviruses do not affect fungal biology (Pearson *et al.*, 2009). Although producing no obvious phenotypic changes under one set of conditions, small effects might be elicited. This was confirmed by Van Diepeningen *et al.* (2006), who observed small but significant effects on spore production and growth rate in isogenic lines of *Aspergillus* spp.

### **1.7.2 Hypovirulence (reduced fungal pathogenicity)**

Some mycoviruses affect the growth rate of their host, which may or may not be accompanied by obvious phenotypic changes. Mycoviruses have been reported to reduce pigmentation, sexual and asexual sporulation, together with mycelial growth. In other words, they reduce the virulence of fungi, a phenomenon known as hypovirulence. CHV1 is the best example of a mycovirus responsible for the hypovirulent phenotype of its fungal host, *Cryphonectria parasitica* (Choi & Nuss, 1992; Nuss, 2005) In general, viruses belonging to the genus *Hypovirus* cause hypovirulence (Fauquet *et al.*, 2005).

In addition, mitoviruses are another group of viruses which infect the mitochondria of fungi and confer hypovirulent traits on them; for example, *Botrytis cinerea* was found to contain a mitovirus that causes hypovirulence, together with reduced laccase

activity and sporulation (Castro *et al.*, 2003). The Dutch elm disease fungus *Ophiostoma novo-ulmi* also exhibited virus-induced fungal debilitation. It contains different-sized dsRNA elements, which are responsible for reducing the level of mitochondrial cytochrome c oxidase resulting in respiratory deficiency of the fungus and this debilitated fungus was unable to infect elm trees.

White stem rot causing fungus *S. sclerotiorum* has a wide range of hosts, including important arable and vegetable crops such as beans, lettuce and rapeseed. This fungus can quickly destroy plants and produce dormant fungal bodies (sclerotia) on affected parts residing in the soil. These sclerotia can again infect the bases of nearby plants. Due to the absence of resistant crop cultivars, control is not usually effective. Recently, mycoviruses were found to confer hypovirulence to *S. sclerotiorum* strains, which could offer potential to exploit mycoviruses to reduce virulence and control fungal diseases of field crops (Xie & Jiang, 2014).

There are also some reports of economically important diseases resulting from the virus infection. Virus infection in oyster mushroom *Pleurotus florida* decreased the growth rate and increased the growth abnormalities, thus resulting in 30% reduction in fruit body yield (Go *et al.*, 1992). Similarly in *P. pulmonarius*, 50% yield losses were due to mycovirus infection (Rinker *et al.*, 1993).

### **1.7.3 Hypervirulence (enhanced fungal pathogenicity)**

In contrast to hypovirulence, there are also some reports of beneficial effects resulting from mycovirus infection. In the root pathogen *Nectria radicularis*, a 6 kbp dsRNA was found to interfere with signal transduction pathways and increase pathogen virulence (Ahn & Lee, 2001).

It was also reported that dsRNA mycovirus infection conferred thermal tolerance to the host plant and the pathogen, so that they could both survive at high temperatures (Márquez *et al.*, 2007).

The killer phenomenon is the most studied phenotype linked with virus infection of fungi and it was first discovered by Bevan and Makower in 1963 in a *Saccharomyces cerevisiae* strain. In this phenomenon, dsRNA genomes encode a proteinaceous

toxin to which the host fungus is immune but which is lethal to other strains of the same fungus that do not produce the toxin. Killer toxins help host strains to eliminate competitor strains occupying the same niche (McCabe *et al.*, 1999).

## **1.8 Interactions between host fungi and mycoviruses**

Study of interactions between the hypovirulence-associated virus and its host provide good opportunities to recognise virus-associated elements which are responsible for altering fungal host phenotypes and to understand the molecular basis of fungal biology. Additionally, genomic sequences of pathogenic fungi facilitate investigation of pathogen-virus interactions at the molecular level. Previously, two host-mycovirus interaction systems have been well-characterised: *C. parasitica*–hypovirus and *H. victoriae*–HvV190S.

Researchers have extensively studied biological control, virus replication, RNAi response to virus infection, virus transmission and ecology and virus distribution and diversity using the *C. parasitica*-hypovirus system (Dawe and Nuss, 2001; 2013; Ghabrial and Suzuki, 2009; Hillman and Suzuki, 2004; McCabe *et al.*, 1999; Milgroom & Cortesi, 2004; Nuss, 1992; 1996; 2005; 2011; Nuss & Koltin, 1990; Pearson *et al.*, 2009). Using the *H. victoriae*-HvV190S system, the virion structure of HvV190S, the molecular mechanism of HvV190S translation and the interaction between HvV190S and its host have been characterised (Castón *et al.*, 2006; de Sa *et al.*, 2010; Dunn *et al.*, 2013; Ghabrial *et al.*, 2002; Ghabrial *et al.*, 2013; Ghabrial & Havens, 1992; Ghabrial & Suzuki, 2009; Li *et al.*, 2011). There are also recent advances in study of other host-virus interaction systems.

*S. sclerotiorum*-mycovirus interaction system revealed the down-regulation of 150 genes in the strain Ep-1PN infected with mycovirus (Li *et al.*, 2008). These down-regulated genes represented a broad spectrum of biological functions. Suppression of *S. sclerotiorum* integrin-like gene (SSITL) resulted in a reduction in virulence and growth rate of the host fungus (Zhu *et al.*, 2013).

In addition to this, 1,775 *F. graminearum* genes that were affected by a hypovirulence-associated virus (FgV1) were detected. *F. graminearum*-FgV1 interaction induced up-

regulation of genes that are associated with virus replication, transcription and signal transduction reactions (Xie & Jiang, 2014).

A large number of dsRNA viruses have been characterised in *R. necatrix*. To elucidate the *R. necatrix*–mycovirus interaction system, four viruses (RnPV1, RnPV2, MyRV3, and RnMBV1) were independently introduced into a virus-free *R. necatrix* strain that constitutively induced RNA silencing of the exogenous green fluorescent protein (GFP) gene. MyRV3 infection suppressed RNA silencing of GFP, while other mycoviruses did not. MyRV3 interferes with the dicing of dsRNA into siRNA and exhibits a counter-defence strategy against host RNA silencing (Yaegashi *et al.*, 2013).

### **1.9 Mycoviruses as biocontrol agents**

Hypoviruses of the chestnut blight fungus are the best example of viruses that were successfully used as biological control agents. *C. parasitica*, also known as *Endothia parasitica*, is the causal agent of chestnut blight in chestnut trees (*Castanea spp.*). This fungus mainly affects the trunk and branches of the tree and causes swollen or sunken cankers (Smith, 2012). Chestnut blight was first observed in North America in 1904 and in Europe in 1938 (Robin & Ursula, 2001). This disease might have been introduced earlier into the eastern United States from chestnut seedlings imported from Japan (Milgroom & Lipari, 1995). After the introduction of the pathogen, most of the American (*Castanea dentate*) and European (*Castanea sativa*) chestnut trees were destroyed (Dutech *et al.*, 2010).

Initially, Antonio Biraghi, an Italian plant pathologist, noticed a spontaneous recovery of European chestnut trees from cankers. Later, a French mycologist, Jean Grente and his co-workers found that the unusual isolates of *C. parasitica* that were responsible for healing of cankers had reduced pigmentation in comparison to the bright orange pigmentation of the wild-type strains. It was also reported that these isolates rarely cause fatal infection in European chestnut (Macdonald & Fulbright, 1991). Subsequently, these isolates were recognised as hypovirulent and *in vivo* and *in vitro* studies revealed that transmissible cytoplasmic genetic factors were responsible for the hypovirulent phenotype. These factors were identified as dsRNA molecules and were successfully used as biocontrol agents (Macdonald & Fulbright,

1991). Notably, the size of dsRNA molecules from hypovirulent strains was found to range from 8 to 12 kbp (Dodds, 1980).

### **1.10 Advantages and disadvantages of using mycoviruses to control crop diseases**

There have been few reports of mycoviruses that could be used to control crop diseases. There are some advantages of using hypovirulence-associated mycoviruses to control fungal diseases. Firstly, viruses can quickly inhibit lesion extension once they are transmitted to fungal strains as they proceed to reach the growth area of colonies for replication (Boine *et al.*, 2012). This quick reaction to suppress the occurrence of crop diseases is crucial for successful biological control because fungal diseases often damage plants very early during the growing season. Secondly, whether the hypovirulent strains produce spores or other propagation bodies on crop plants is not likely to be a problem as crops are harvested at the end of the growing season. Thirdly, if hypovirulent pathogen strains share a similar niche with virulent pathogen strains, it is possible that hypovirulent strains can grow well on hosts. For example, chestnut trees infected with a hypovirulent strain of the chestnut blight pathogen might have only superficial lesions on their stems (Anagnostakis, 1982). Fourthly, hypovirulent strains produce pathogen-associated molecular effectors during their growth on the host and a defence response produced by the hosts specifically targets the infection by the virulent strain. Furthermore, the prevalence of mycoviruses in crops is easily established if a mycovirus-infected strain of a fungal pathogen is applied to crops at the correct time.

Farmers always prefer as short time as possible to control crop diseases and a short time requirement for establishing the prevalence of mycoviruses in crops is vital for successful control. Moreover, in crops the proficiency of viral transmission between vegetatively-incompatible hosts is not expected to meet these criteria. When hyphal fragments of the hypovirulent *Sclerotinia sclerotiorum* strain Ep-1PN infected with *Sclerotinia sclerotiorum* debilitation-associated RNA virus (SsDRV) were sprayed onto leaves of rapeseed plants and a vegetatively incompatible virulent strain was inoculated on these plants, all plants were damaged by the virulent strain. On the other hand, when a vegetatively compatible virulent strain was used for the challenge

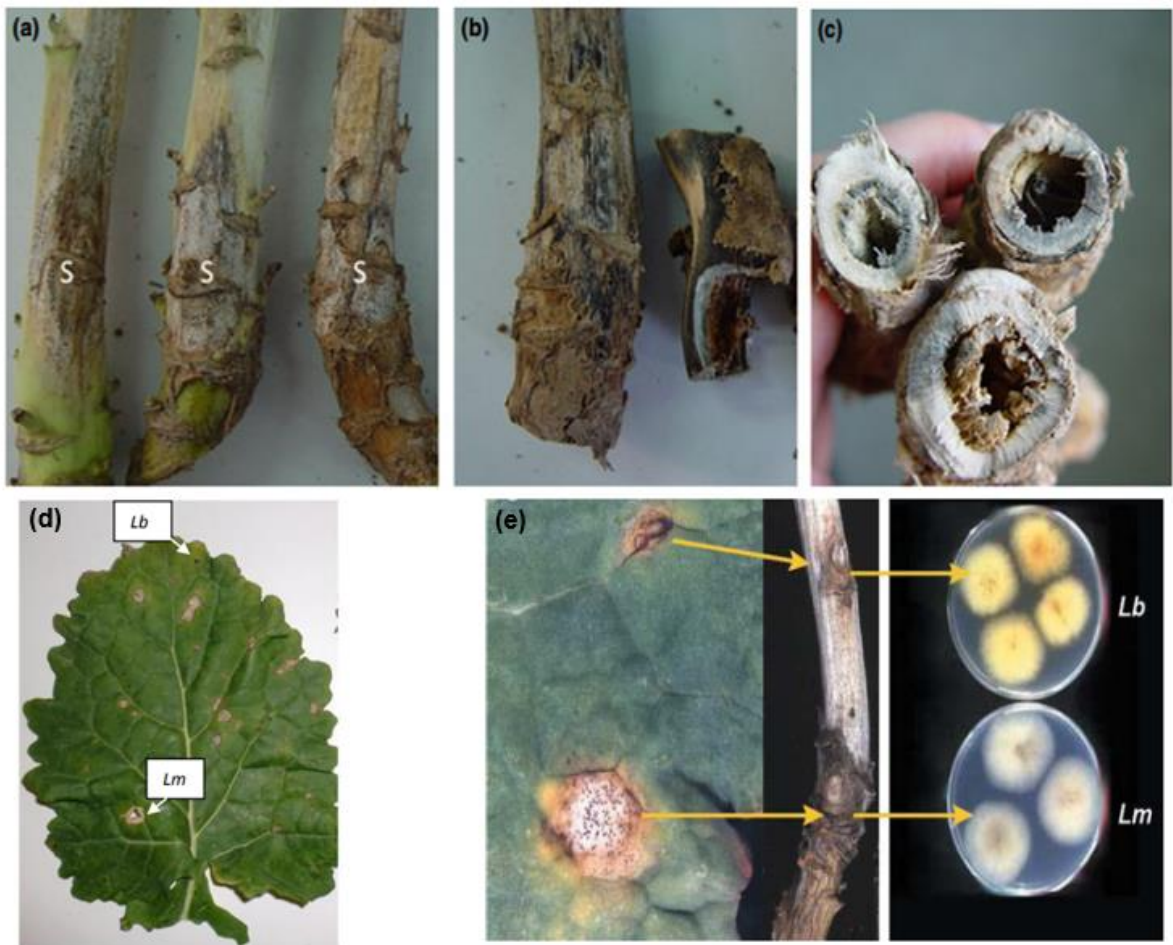
inoculation, the plants survived. Thus, there are also some strategies to resolve the mycovirus transmission problem in crops (Xie & Jiang, 2014).

### **1.11 *Leptosphaeria maculans*, *Leptosphaeria biglobosa* and phoma stem canker**

*Leptosphaeria maculans* (anamorph *Plenodomus lingam*) and *L. biglobosa* (anamorph *P. biglobosus*) cause phoma stem canker (blackleg) on crucifers, including oilseed rape (*Brassica napus*, *B. rapa*, *B. juncea*, canola, rapeseed). Phoma stem canker is a very serious disease of oilseed rape which causes worldwide losses of more than £1000 million per cropping season (Liu *et al.*, 2014). Severe epidemics have been observed in Australia, Canada and Europe; milder epidemics occur in China. The severity and epidemiology of phoma stem canker disease is affected by different cultivars, weather, pathogen populations and regions. *L. maculans* (group A) and *L. biglobosa* (group B) are two coexisting and related *Leptosphaeria* species (West *et al.*, 2001). These groups have a similar life cycle, produce similar spores and affect the same host but demonstrate differences in genetics (Taylor *et al.*, 1991), culture (Cunningham, 1927), metabolite production (Balesdent *et al.*, 1992), disease symptoms on leaves (Ansan-Melayah *et al.*, 1997; Brun *et al.*, 1997; Thürwächter *et al.*, 1999) and disease symptoms on stems (Johnson & Lewis, 1994). Sexual reproduction has not been reported between A and B groups and their pseudothecia are also different in morphology (Farahani & Zinkernagel, 1997; Gabrielson, 1983; Petrie & Lewis, 1985; Somda *et al.*, 1997).

*L. biglobosa* includes several sub-species; European B-group isolates are described as *L. biglobosa* 'brassicae', whereas in the Canadian B-group is a different sub-species, *L. biglobosa* 'canadensis' (Mendes-Pereira *et al.*, 2003). *L. maculans* is generally more virulent than *L. biglobosa*. Moreover, *L. maculans* is generally responsible for damaging stem base canker while *L. biglobosa* is generally responsible for less damaging upper stem lesions (Fig. 1.2).

Epidemics of phoma stem canker usually start with the adherence of ascospores to leaves (Bokor, 1975; Hall, 1992; Mahuku *et al.*, 1997; McGee, 1974; West *et al.*, 2001), although in some cases they may be initiated by seed infection



**Figure 1.2 Symptoms caused by *L. maculans* and *L. biglobosa*.** External canker symptoms on upper stems (a) and stem bases (b) of winter oilseed rape with cross-sections showing internal necrosis (c). Leaf showing the large pale lesions produced by *L. maculans* while smaller and darker lesions on leaves were produced by *L. biglobosa* (d). Cultural identification on PDA plates, showing yellow pigmentation produced by *L. biglobosa* but *L. maculans* does not exhibit any pigmentation (e) (Fitt *et al.*, 2006; Zhang *et al.*, 2014).



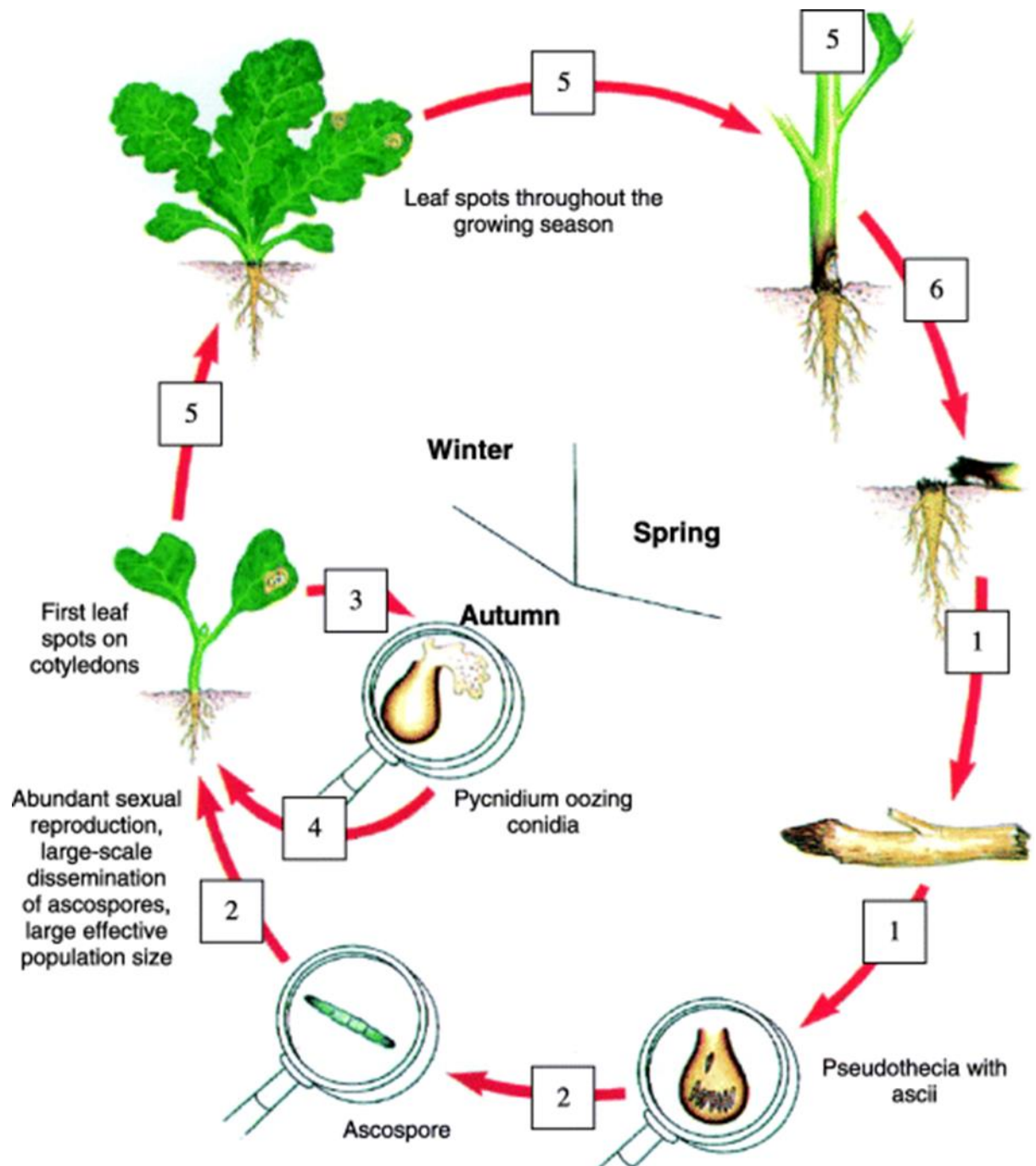
(Jacobsen & Williams, 1971; Kharbanda & Stevens, 1993; Wood & Barbetti, 1977a), stubble infection or conidial infection (Hall, 1992; Thürwächter *et al.*, 1999). Fungal penetration normally occurs *via* leaf stomata; nevertheless, wounds are also a possible route of infection (Chen & Howlett, 1996; Hammond *et al.*, 1985). Biddulph *et al.* (1999) found that *L. maculans* ascospores require a minimum of 4 h of leaf wetness to initiate penetration. Leaf spot development appears different according to the type of *Leptosphaeria* species, host resistance and the development phase of the lesion (West *et al.*, 2001). *L. maculans* causes pale green spots, which expand up to 2 cm in diameter and develop into pale brown lesions. These lesions contain pycnidia. Gradually, the middle part of the lesion drops out entirely. *L. biglobosa* causes comparatively smaller and darker lesions with less or no pycnidia (Ansan-Melayah *et al.*, 1997; Brun *et al.*, 1997). Under optimal temperature and humidity conditions, one or two ascospores are sufficient to produce a lesion (Wood & Barbetti, 1977b). However, during experiments in a controlled environment, *L. maculans* conidia were not capable of initiating infection on unwounded parts, but only likely to produce symptoms on wounded stems, petioles or leaves (Hammond *et al.*, 1985). Hall (1992) reported that the secondary infection by conidia was rarely associated with yield loss. Hypocotyl lesions constrict the stem and cause seedling blight. Stem disease exhibits black or brown lesions at the hypocotyl base of seedling. This disease is also known as canker, crown canker, blackleg or basal canker (West *et al.*, 2001). When the stem is girdled by the lesion, water transportation is interrupted and premature ripening of pods occurs (Davies, 1986). In extreme conditions, weakened stems cause plant death (Petrie & Vanterpool, 1974). Pod infection triggers premature ripening and seed infection, resulting in yield loss (Kharbanda & Stevens, 1993; Wood & Barbetti, 1977a).

In the first stage of the life cycle, *L. maculans* survives as a saprophyte on stem debris for many years (Rouxel & Balesdent, 2005). Sexual mating occurs during this period with the formation of pseudothecia, which release a large number of air-borne ascospores. These spores adhere to leaves and initiate the phoma leaf spot symptoms. Infection of leaves produces leaf spots. After colonising the leaf, *L. maculans* initiates asexual reproduction and produces pycnidia. Pycnidia release an ooze containing conidia and disperse them up to very short distances. The phoma leaf spot phase is followed by an endophytic phase which lasts for a longer period of time.

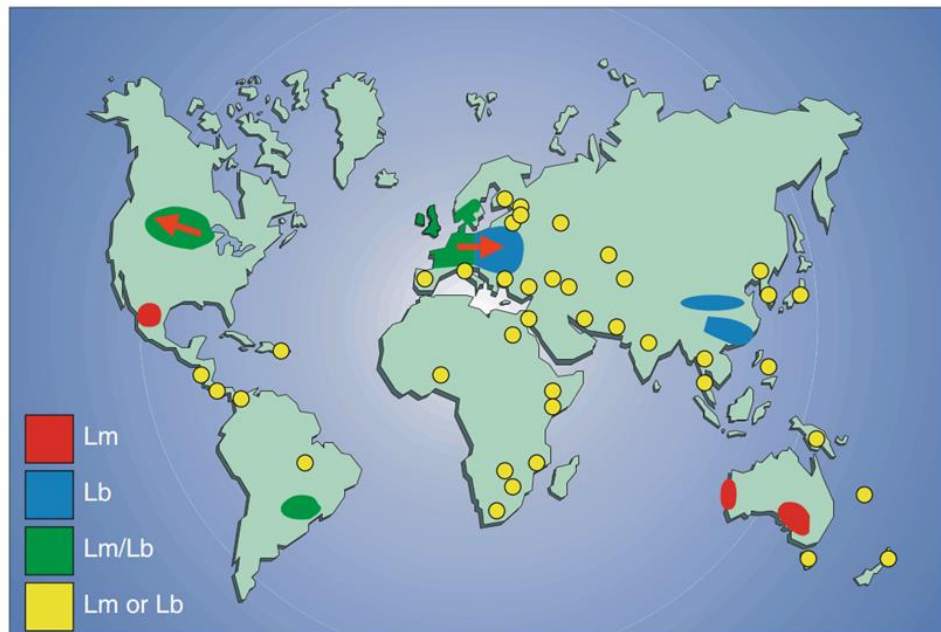
In this phase, the fungus starts colonising leaf stalk and stem tissues. Symptoms cannot be observed in this stage as the fungus spreads intercellularly towards the base of stem and root. The fungus later changes to a necrotrophic phase and destroys the stem base (Fig. 1.3).

### **1.12 World-wide spread of *L. maculans* and *L. biglobosa***

*L. maculans* and *L. biglobosa* are widely distributed around the world as a result of their transmission in seed of *B. oleracea*, *B. rapa*, *B. napus* and other brassica crops (West *et al.*, 2001). One or other of them appears in Europe (25 countries), Asia (16 countries), Africa (eight countries), North America (Canada, USA), central America (five countries), South America (Argentina and Brazil) and Oceania (five countries) (Anon, 2004) (Fig. 1.4). Reports mostly do not provide information on the brassica crop on which the pathogen was recognised or differentiate between *L. maculans* and *L. biglobosa*. Since *L. biglobosa* 'canadensis' was first isolated in 1957, it has been widespread in Canada on oilseed rape. *L. maculans* was first isolated in Canada in 1975 in Saskatchewan from oilseed rape and subsequently spread to Alberta and Manitoba (Gugel & Petrie, 1992). At present, only resistant cultivars are involved in production of all Canadian oilseed rape. *L. maculans* and *L. biglobosa* exist in the UK, France and Germany; however, the relative prevalence of the two species varies between locations (West *et al.*, 2001). Both species have recently been reported from the USA (Anon, 2004). In Poland, phoma stem canker was exclusively associated with *L. biglobosa* until the mid-1990s (Jedryczka *et al.*, 1994). Afterwards, *L. maculans* was widely distributed in western Poland by 2002 while only *L. biglobosa* was found in eastern Poland (Karolewski *et al.*, 2002). Severe phoma stem canker on oilseed rape in Kenya was reported from 1972 to 1974 and it was stated that it had been present since 1951 on vegetable brassicas (Piening *et al.*, 1975). Plummer *et al.* (1994) described the distribution of *L. maculans* in Australia. Only *L. biglobosa* has been investigated in China from oilseed rape crops (West *et al.*, 2000; Zhang *et al.*, 2014).



**Figure 1.3** Life cycle of *Leptosphaeria maculans* (Rouxel & Balesdent, 2005). [1] saprophytic phase; [2] sexual reproduction and development of primary ascospore inoculum; [3] primary infection is followed by a necrotrophic phase; [4] asexual reproduction; [5] symptomless endophytic phase; [6] switch to necrotrophic phase which destroys the stem base.



**Figure 1.4 World-wide occurrence of *L. maculans* and *L. biglobosa* (Fitt *et al.*, 2006).** Arrows show the directions of spread of *L. maculans* in Canada and eastern Europe where *L. biglobosa* ‘canadensis’ and *L. biglobosa* ‘brassicae’ were predominant, respectively. Patches indicate areas where populations were characterised as predominantly *L. maculans* (red), *L. biglobosa* (blue) or a mixture of the two species (green). Yellow dots describe areas where the pathogens were reported (sometimes only a single report) but the species has not been identified.

### 1.13 Economic importance of phoma stem canker

Phoma stem canker is a severe and globally important disease of oilseed rape. It causes losses of more than £1000M per cropping season through severe epidemics in Europe, Australia and North America and is dispersing globally, threatening the production in Africa, China and India (Barnes *et al.*, 2010). National (England and Wales) survey data estimated that yield loss from this disease have increased from c. £13M in the 1980s (Fitt *et al.*, 1997) to £50M per cropping season in 2000-2002 (Fitt *et al.*, 2006).

Oilseed rape is grown throughout the world and it is an essential source of fuel, a most important source of protein meal and an important source of vegetable oil. Oilseed rape production has been increasing internationally, with total yield of 46 Mt after the 2005/2006 growing season. However, it is predicted that stem canker may cause losses of oilseed rape up to £140M p.a. in the UK by the 2020s (Evans *et al.*, 2010).

Phoma leaf spotting is infrequently associated with extensive death of young plants in the UK, although such epidemics are widespread in autumn and winter. By contrast, it was reported to produce death of seedlings and damage to crops in Australia at any stage from seedling to maturity (Khangura & Barbetti, 2001).

It is important to control the phoma stem canker. The current methods of control of phoma stem canker involve the use of fungicides, exploitation of resistance genes in commercial cultivars and the use of cultural techniques.

Phoma stem canker is controlled chemically by using different combinations of fungicide treatments, soil fungicides or foliar fungicide sprays in different regions depending on the epidemiology of the disease (West *et al.*, 2001; Fitt *et al.*, 2006).

Use of resistant cultivars is the best approach to grow crops with limited use of pesticide and low production costs. Genetic resistance against *L. maculans* have been described in *Brassica* species. However, genetic resistance against *L. biglobosa* is not known yet. Two main types of resistance have been described. Quantitative resistance (QR) is usually controlled by multiple genetic factors (Lindhout, 2002; Stuthman *et al.*, 2007). QR protects plants against *L. maculans* by reducing symptom severity but does not prevent pathogens from colonisation of plants. While, qualitative resistance is

usually controlled by single, dominant resistance (*R*) genes and depends on gene-for-gene recognition mechanisms (Flor, 1956; Huang *et al.*, 2014). It is often effective in preventing pathogens from colonisation of plants.

Cultural practices involve stubble management and good crop rotation. It decreases the risks of infection by ascospores released from colonized residue (West *et al.*, 2001).

### **1.14 Rationale**

Several plant diseases of fungal etiology, such as ash dieback, phoma stem canker, needle blight and light leaf spot are important economically as well as ecologically in the UK, since they present major threats to arable crops or trees. The control of plant pathogenic fungi is a difficult task due to the absence of suitable strategies to control diseases. Furthermore, the use of fungicides has increased risks to the environment and human health. As a result, novel biocontrol measures are being investigated to combat fungal diseases (Ghabrial & Suzuki, 2009). Since, from an agricultural perspective, mycoviruses appear to be useful tools for biologically controlling plant diseases, the phytopathogenic fungi causing ash dieback, phoma stem canker, needle blight and light leaf spot were assessed for the presence of mycoviruses.

### **1.15 Aims and objectives**

The main aims of the project are to assess the incidence of mycoviruses in five different plant pathogenic fungi and to investigate their effects on the growth and pathogenicity of the fungi. The investigation has focused on *L. biglobosa* since mycoviruses were identified in it for the first time and appeared to cause alterations in growth and pathogenicity of the host fungus. These observations might have implications for control of diseases caused by these fungi.

Objectives:

1. To identify and characterise mycovirus dsRNA elements in plant pathogenic fungi.

2. To eradicate LbMV-1 from *L. biglobosa* and compare the phenotypes of virus-infected and virus-free *L. biglobosa* isolates.
3. To detect effects of LbMV-1 infection on pathogenicity of *L. biglobosa* to oilseed rape (*Brassica napus*).
4. To investigate virus-infected or virus-free *L. biglobosa* induced systemic resistance to *L. maculans* in oilseed rape.

## **Chapter 2**

### **General materials and methods**

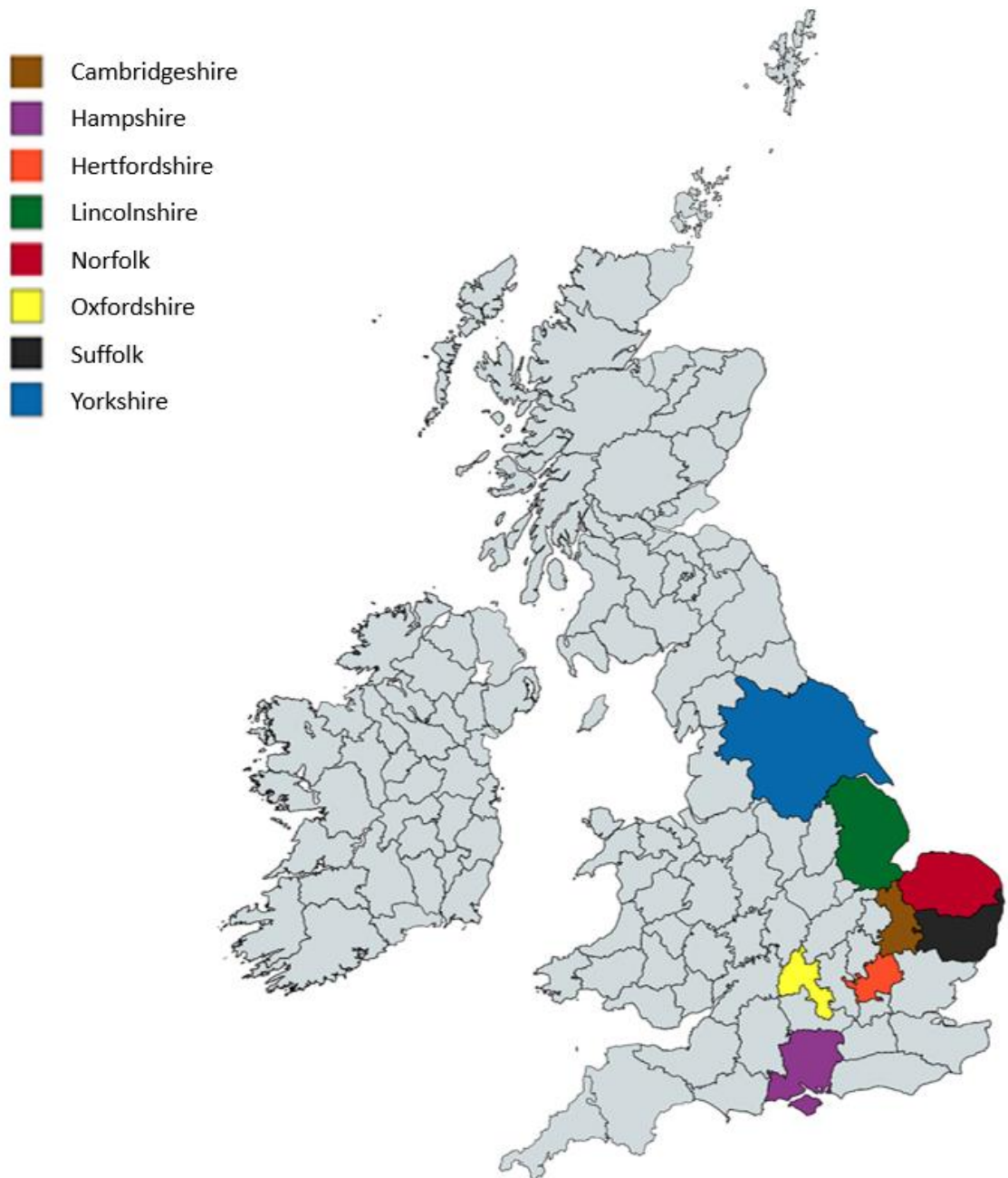


## 2.1 Source of fungal isolates

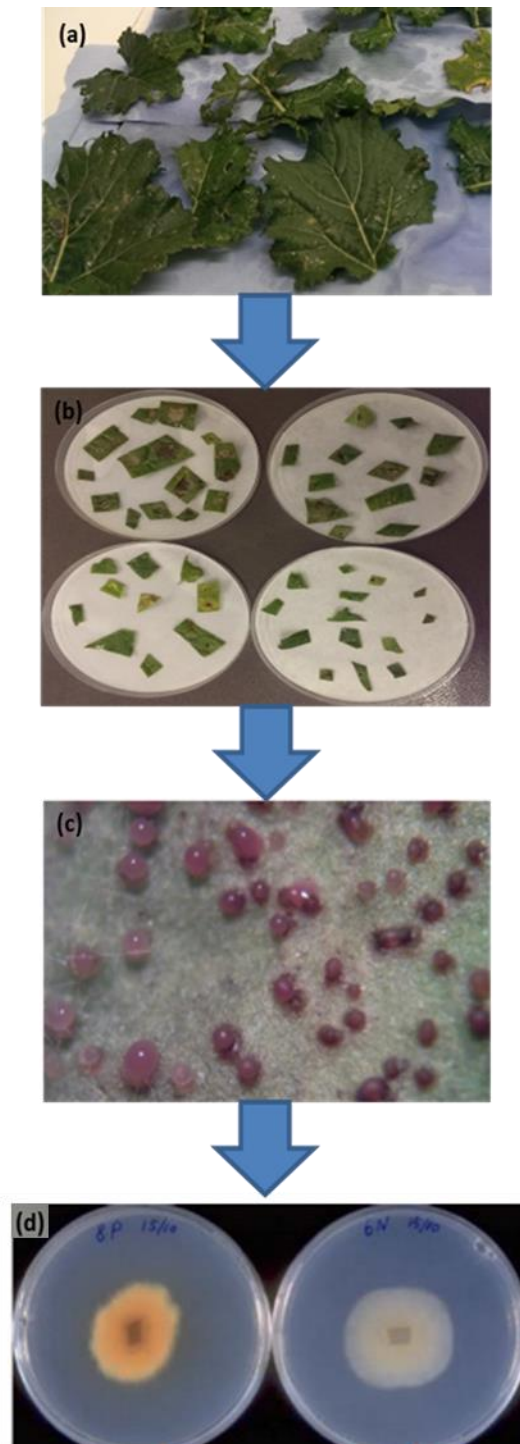
Isolates of *Hymenoscyphus fraxineus*, the causal agent of ash dieback, were obtained from Dr Tadeusz Kowalski (Poland) and isolates of *Dothistroma septosporum*, the causative agent of red band needle blight, were obtained from Dr Anna Brown (Forest Research, UK). *Leptosphaeria* (Table 3.2) and *Pyrenopeziza brassicae* isolates responsible for phoma stem canker and light leaf spot on oilseed rape cultivars, respectively, were obtained from the pathogen collection at the School of Life and Medical Sciences (University of Hertfordshire) to investigate the incidence of dsRNA mycoviruses. *Leptosphaeria* isolates provided were from different parts of UK (Fig. 2.1) and China. Moreover, some new isolates of *Leptosphaeria* were also collected from crops. In addition to this, glycerol stocks of purified *Aspergillus foetidus* viruses were obtained from the pathogen collection at the Department of Life Sciences (Imperial College London) for developing cloning and sequencing technologies. *H. fraxineus* isolates were provided in the form of small agar plugs containing mycelia in Eppendorf tubes, *Leptosphaeria* isolates were provided on potato dextrose agar (PDA) plates and *D. septosporum* isolates were provided on malt extract agar (MEA) plates.

## 2.2 Isolation and cultural identification of *Leptosphaeria* species

Fifty-three *Leptosphaeria* isolates were obtained from UH pathogen isolate collection. In addition to this, 20 isolates were collected personally from oilseed rape leaves with phoma spot symptoms collected from a field site near Cambridge (in autumn 2015). The isolates of *Leptosphaeria* spp. from the phoma leaf spots were obtained by the method of West *et al.* (2002). The leaves with phoma leaf spots were washed with water and dried (Fig. 2.2a). The phoma spots were cut from the leaves and leaf pieces were placed in Petri dishes on Whatman No.1 filter paper sprayed with distilled water (Fig. 2.2b). The leaves were then incubated at 20°C for 3-5 days under 12h light/12h darkness photoperiod to induce spore production. Pycnidia were observed using a dissection microscope and under sterile conditions the cirrhi from mature pycnidia (Fig. 2.2c) were collected using a fine needle to obtain single pycnidial isolates. Each cirrhus was mixed with a drop of sterilized water to make a spore suspension and then transferred onto a PDA plate. To produce confluent cultures, the plates were



**Figure 2.1 Map showing the different locations (indicated with different colours) in the UK from which *Leptosphaeria* isolates were obtained from School of Life and Medical Sciences (University of Hertfordshire) isolate collection and isolates collected by Unnati Shah (autumn 2015).**



**Figure 2.2 Isolation of *Leptosphaeria* species from oilseed rape cultivars.** (a) Leaves collected from crop samples for pathogen isolation. (b) Leaves with phoma spots were cut into small pieces and placed in Petri dishes on Whatman No. 1 filter paper that had been sprayed with distilled water. (c) Spores from mature pycnidia observed under a microscope were inoculated onto PDA plates. (d) Cultures of *Leptosphaeria* were grown on PDA at 20°C in darkness for 20 days.

incubated for 5 days at 20°C in darkness (Fig. 2.2d) and for the identification of pathogen species (whether *L. maculans* or *L. biglobosa*), the pathogens were cultured on PDA plates. The two species were distinguished on the basis of morphological characteristics on PDA plates and by PCR. All *L. biglobosa* isolates were confirmed as *L. biglobosa* 'brassicae' isolates by PCR using *L. biglobosa* 'brassicae' specific primers (Liu *et al.*, 2006). Cultures of *L. maculans* have no pigment and are white while *L. biglobosa* produces a yellow pigment with numerous pycnidia on PDA (Fitt *et al.*, 2006). All 20 isolates obtained from crops near Cambridge were found to be *L. maculans*, whereas 37 and 16 isolates obtained from the UH pathogen collection were classified as *L. maculans* and *L. biglobosa*, respectively.

## **2.3 Inoculation and growth of isolates**

*H. fraxineus*, *D. septosporum* and *P. brassicae* isolates were grown on MEA containing streptomycin while *Leptosphaeria* isolates provided on PDA plates were subcultured onto V8 agar plates containing penicillin and streptomycin (Appendix 1, 1). Prior to inoculation, the medium in the plate was covered with an autoclaved cellulose disc. Subsequently, a fungal mycelial plug was inoculated onto the plate and incubated at a specific temperature until mycelia proliferated. *H. fraxineus* isolates were grown at 25°C, whereas *Leptosphaeria*, *D. septosporum* and *P. brassicae* cultures were grown at 20°C.

## **2.4 Nucleic acid isolation and analysis**

### **2.4.1 Phenol and Sevag treatment**

Phenol solution was prepared by melting phenol at 68°C, adding 0.1% (w/v) parahydroxy-quinoline (Sigma) and finally equilibrating with 0.1 M Tris-HCl (pH 8.0). The solution was stored in a dark bottle at 4°C. Sevag solution was prepared by mixing chloroform and isoamyl alcohol (24:1 v/v). For nucleic acid extraction and protein denaturation, equal volumes of phenol and Sevag were added to an equivalent volume of solution containing nucleic acid. The suspension mixtures were vortexed and centrifuged at 13,000 rpm for 5 min, in order to separate the aqueous and organic phases. The upper aqueous phase was transferred into separate sterile Eppendorf

tubes and equal amounts of Sevag were added, vortexed and centrifuged to eliminate phenol. The aqueous supernatant containing nucleic acids was processed as described in Section 2.4.2.

#### **2.4.2 Precipitation of nucleic acids**

Nucleic acids were precipitated by the addition of 1/10 the volume of 3 M sodium acetate (pH 5.5) and 2.5 volumes of absolute ethanol (100%). The solution was mixed and kept for 16 h at -20°C for nucleic acid precipitation. The precipitated nucleic acids were collected following centrifugation (13,000 rpm, 10 min). The supernatant was discarded after centrifugation and the pellet was air dried for 15-20 min. The pellet was resuspended in water and left at 0°C for 30 min. The resuspended solutions were used for further enzymatic digestions, electrophoresis or storage at -80°C.

#### **2.4.3 DNase I treatment**

Nucleic acids were subjected to DNase 1 treatment to remove traces of fungal DNA. Pellets were resuspended in water (80 µl) followed by the addition of 10 µl DNase I buffer (10x) and 10 µl DNase I enzyme (1 U/µl; Promega). The mixture was incubated at 37°C for >1 h. After incubation, water (400 µl) was added and nucleic acids were extracted with an equal volume of phenol and Sevag to inactivate DNase I (Section 2.4.1).

#### **2.4.4 SI nuclease treatment**

Double stranded RNA (dsRNA) solutions were treated with SI nuclease to eradicate contaminating fungal single stranded (ss) RNA. Following DNase I treatment and ethanol precipitation for 16 h, dsRNA mixtures were centrifuged and the supernatant decanted prior to air drying the pellets. Pellets were resuspended in 88 µl of water followed by the addition of 10 µl SI nuclease buffer (10x) and 2 µl SI nuclease enzyme (1:10 enzyme dilution) (95 U/ µl; Promega). The mixture was incubated at 37°C for >1 h. Then the phenol and Sevag extraction method was used to inactivate SI (Section 2.4.1).

#### **2.4.5 Agarose gel electrophoresis**

Nucleic acids were separated and visualised by agarose gel electrophoresis. Agarose gels (1% w/v) were prepared by dissolving agarose powder by boiling in 1x TAE buffer (Appendix 2.12). When the temperature was ca. 50-60°C, GelRed nucleic acid stain (10,000x, Biotium) was added into the gel solution. The gel was then poured into a casting tray and left to solidify for at least 20 min before loading the samples that contained 5x loading buffer. The gel was electrophoresed in 1x TAE buffer for 2 h at 30-50 V. Gels were then observed under UV light.

#### **2.4.6 Gel extraction for recovery of dsRNA**

After gel electrophoresis, dsRNA bands of interest were isolated using the QIA MinElute gel extraction kit (Qiagen). A UV transilluminator was used to view the gels to identify dsRNA bands, which were excised using a scalpel blade. Gel pieces were transferred into 1.5 ml microcentrifuge tubes and weighed. Three volumes of buffer QG were added to each gel volume. The solution was incubated at 55°C for 10 min until the gel slices melted completely. This process can be facilitated by the inversion of the tube every 2 min during incubation. Following gel solubilisation and when the mixture turned to an orange or violet colour, 10 µl of sodium acetate (3 M) was added to adjust the pH, as indicated by a yellow colour. One gel volume of isopropanol was then added to the sample and the whole sample was mixed. The melted agarose solution was transferred to a silica membrane based MinElute spin column and centrifuged for 1 min to assist RNA binding to the membrane. The resulting flow-through was discarded and 500 µl of QG buffer was added to the column, which was centrifuged for 1 min, and the flow-through discarded once more. The same procedure was repeated following the addition of PE buffer (750 µl). The column was centrifuged again for 1 min to remove residual buffer and the column was placed in a 1.5 ml tube and 10 µl of elution buffer (EB) or water was added in the middle of the column to recover dsRNA. The tube was centrifuged for 1 min and pure dsRNA was collected for storage at -20°C until use.

### **2.4.7 Purification of amplicons generated by PCR amplification**

To purify amplicons generated by PCR amplification the QIAquick PCR Purification kit (Qiagen) was used, following the manufacturer's protocol. Amplicons in buffer were mixed with five volumes of binding buffer (PB) and mixed by inversion. Sodium acetate was added to optimise the pH to 5.5 and the mixture was placed onto the QIA quick column and centrifuged at 13,000 rpm for 1 min followed by washing with 700 µl of buffer PE. Flow-through was discarded on each occasion after centrifugation. Amplicon DNA adsorbed onto the membrane was eluted in 15 µl of nuclease-free water by centrifugation (13,000 rpm; 1 min).

## **2.5 Extraction of mycovirus dsRNA and purified mycovirus**

### **2.5.1 Small-scale dsRNA extraction**

This procedure is rapid and easy to perform and follows a protocol developed by Coenen *et al.* (1997) to identify dsRNA presence in small amounts of mycelia. Fungal mycelium (~0.2-0.5 g) was harvested and crushed using a mortar and pestle in liquid nitrogen prior to immediate transfer into a 2 ml Eppendorf tube containing 350 µl of extraction buffer (Appendix 1, 2.10). The mixture was then vortexed and incubated for 1 h at 70°C. After incubation, the mixture was mixed with 350 µl of phenol and equal volume of Sevag, vortexed and centrifuged (13,000 rpm for 5 min). After centrifugation, the supernatant was mixed with 350 µl of Sevag, vortexed and centrifuged (13,000 rpm for 5 min). The resulting supernatant was mixed with 800 µl of ethanol and 30 µl of sodium acetate and kept at -20°C for 16 h to precipitate dsRNA (Section 2.4.2).

### **2.5.2 Large-scale dsRNA purification using lithium chloride (LiCl)**

This method was adapted from Diaz-Ruiz and Kaper (1978). Fungal mycelia (~25-30 g) were crushed and homogenised to a fine powder in liquid nitrogen. The crushed powder was mixed with extraction buffer (60 g/L 4-aminosalicylic acid [sodium salt] and 50 ml/L Tris-HCl buffered phenol) and the mixture shaken on an ice plate for 60 min. This incubated mixture was centrifuged at 10,000 rpm for 40 min at 4°C and the supernatant containing total nucleic acid extract was transferred into a new sterile

centrifuge tube. The supernatant was treated with phenol (15 ml) followed by chloroform (15 ml) to deproteinise the nucleic acids and centrifuged on each occasion (10,000 rpm; 30 min). After centrifugation, the upper phase was transferred to a new tube and an equal amount of 4 M LiCl was added, mixed and incubated at 4°C for 16 h to precipitate nucleic acids. Then the solution was centrifuged (15,000 rpm; 30 min) to pellet the precipitated ssRNA and dsDNA. The supernatant was then transferred to a sterile tube and an equal volume of 8 M LiCl added and mixed to precipitate dsRNA at 4°C for 16 h. The precipitated nucleic acids (dsRNA) were collected by centrifugation at 10,000 rpm for 50 min and 1 ml of supernatant was collected as a negative control. The pellet, containing dsRNA, was resuspended in 2 ml of distilled water and stored at -20°C for further experiments. In order to remove contaminating DNA and single-stranded RNA, DNase I and SI nuclease treatments were performed, respectively.

### **2.5.3 Virus purification using polyethylene glycol (PEG)**

Virus purification was performed for the eventual purification of large quantities of dsRNA. To achieve this, fungi were grown in liquid media with aeration until sufficient amounts of mycelium (~40 g) had been produced, which was then collected in 50 ml Falcon tubes and kept at -80°C until required. Frozen mycelium was thawed, weighed and transferred into a blender with the addition of two volumes (w/v) of TE buffer (Appendix 1, 2.11). After homogenising the mycelium for 3 min, the homogenate was filtered through Miracloth, cell debris was discarded and flow-through was collected in sterilised Nalgene bottles. Centrifugation was carried out at 10,000 g for 20 min to pellet residual fungal debris. After centrifugation, the supernatant was transferred to a fresh sterilised bottle and PEG and NaCl were added to 10% (w/v) and 0.6 M, respectively, prior to stirring the mixture for 16 h at 4°C to precipitate the virus. The solution was then transferred to Nalgene tubes and centrifuged at 10,000 g for 20 min to collect the precipitated virus. The precipitated virus was then resuspended in 20 ml of TE buffer and recentrifuged at 10,000 g for 20 min. The clarified supernatant was subjected to ultracentrifugation at 105,000 g (30,000 rpm) for 90 min. The virus pellet was resuspended in 500 µl of TE buffer and transferred into a 1.5 ml microcentrifuge tube prior to centrifugation at 10,000 rpm for 20 min. Viral dsRNA was isolated from



purified virus following phenol and Sevag treatment and subsequently precipitated with ethanol and sodium acetate (Sections 2.4.1 and 2.4.2).

#### **2.5.4 RNeasy extraction of total RNA**

The RNeasy plant minikit (Qiagen) was used for total RNA extraction from fungal mycelium. A maximum amount of 100 mg of mycelium was homogenised in liquid nitrogen using a mortar and pestle without thawing the tissue. The tissue powder was mixed with 450 µl RLT lysis buffer and incubated for 1-3 min to disrupt the tissue. The lysate was transferred to a QIAshredder spin column and centrifuged for 2 min at 13,000 rpm to remove cell-debris. The supernatant of the flow-through fraction was carefully transferred to a microcentrifuge tube without disturbing the cell-debris pellet. The volume of cleared lysate was estimated and half that volume of absolute ethanol was added to it. This mixture was applied to an RNeasy spin column and centrifuged for 15 s at  $\geq 10,000$  rpm. The flow-through was discarded and 700 µl buffer RW1 was added to the RNeasy spin column followed by centrifugation for 15 s at  $\geq 10,000$  rpm. The flow-through was discarded and 500 µl RPE buffer was added and centrifuged for 15 s at  $\geq 10,000$  rpm to wash the spin column membrane. The flow-through was discarded again and the washing step with RPE repeated using centrifugation for 2 min at  $\geq 10,000$  rpm. Residual RPE buffer was removed by an additional centrifugation step at 13,000 rpm for 1 min. Total RNA was eluted in 30-50 µl water following centrifugation at 13,000 rpm for 1 min.

## **2.6 Sequencing mycovirus dsRNAs using genome walking**

A genome walking procedure was used to generate clones from the dsRNA elements constituting the genomes of mycoviruses discovered in both *D. septosporum* and *L. biglobosa*. Here, following the generation of recombinants produced using random cDNA cloning, new oligonucleotide primers were designed on the basis of known sequence and RT-PCR amplification was performed to generate further clones.

### **2.6.1 Oligonucleotide primer design for RT-PCR amplification**

For successful PCR amplification, it is essential to design oligonucleotide primers carefully with correct melting temperature characteristics and to avoid primer-dimer

formation. Many factors were considered when designing primers in these experiments. Primers used were 18-22 nucleotides in length with the 3'-terminal nucleotide ending in C or G to promote binding with a melting temperature between 58°C-65°C. The preferred GC content of the primer was between 40 and 60%. The properties of all oligonucleotide used in this study were also checked using the OligoCalc tool (Kibbe, 2007), which calculates the physical properties of a given oligonucleotide sequence, including melting temperature, molecular weight, %GC content and absorbance coefficient. In addition to this, it can also check the self-complementarity for potential hairpin formation.

### **2.6.2 Reverse transcription and polymerase chain reaction amplification**

DsRNA (8 µl) extracted from different sources was denatured by adding 2 µl 100 mM methyl mercury and 1 µl of sequence-specific oligonucleotide primer (100 µM) was added. This mixture was incubated for 20 min at 20°C and subsequently kept at 0°C for 2 min to allow the primer to anneal to the RNA. Instead of methyl mercury, heat denaturation (90°C for 10 min) could also be used to denature the secondary structure of RNA after incubation at 20°C for 20 min. Denatured dsRNA was mixed with pre-heated (50-55°C for 1 min) reaction mixture to synthesise the first strand of cDNA, then incubated at 55°C for 1 min. Superscript™ III RNase H-Reverse Transcriptase (Invitrogen) was then added to the mixture, followed by incubation for 1 h at 55°C to synthesise cDNA.

#### Reaction mixture for first strand cDNA synthesis:

|   |          |
|---|----------|
| Reverse transcription buffer (5x, Invitrogen) | 10 µl    |
| DTT (100 mM, Invitrogen)                      | 4 µl     |
| dNTP mix (20 mM, Promega)                     | 1 µl     |
| RNasin™ RNase inhibitor (40 U/µl, Promega)    | 1.25 µl  |
| Sterilised distilled water                    | 23.75 µl |

After incubation, 450 µl of sterilised distilled water was added to the mixture, which was transferred to a Nanosep 30K column (Pall Corporation) to assist the separation of cDNA from buffers and small molecules. Tubes were centrifuged at 5000 rpm for

10 min and the flow-through was discarded. The retained sample was recovered in 150  $\mu$ l of sterilised distilled water. The solution containing cDNA was moved into another sterile tube for further use in PCR amplification.

Reaction mixture for PCR amplification:

|  |              |
|--|--------------|
| First strand cDNA                            | 50 $\mu$ l   |
| PCR reaction buffer (5x, Bioline)            | 20 $\mu$ l   |
| dNTP mix (20 mM, Promega)                    | 1.2 $\mu$ l  |
| Sequence-specific primer (100 $\mu$ M)       | 1 $\mu$ l    |
| MgCl <sub>2</sub> (25 mM)                    | 8 $\mu$ l    |
| Random hexamer (100 $\mu$ M)                 | 1 $\mu$ l    |
| Sterilised distilled water                   | 18.3 $\mu$ l |
| GoTaq DNA polymerase (5 U/ $\mu$ l, Promega) | 0.5 $\mu$ l  |

The amplification reaction was run in a PCR thermocycler with specific conditions according to the oligonucleotide primers and the DNA polymerase used.

Thermocycling conditions for PCR reaction (GoTaq DNA polymerase)

|  |           |
|--|-----------|
| 94°C for 2 min                               | 1 cycle   |
| 94°C for 1 min/60°C for 1 min/72°C for 3 min | 30 cycles |
| 72°C for 5 min                               | 1 cycle   |

Store at 4°C

One tenth of each reaction mixture was used to analyse the amplicons following 1% (w/v) agarose gel electrophoresis (Section 2.4.5).

**2.6.2.1 Froussard procedure**

While the polymerase chain reaction can be used to amplify known DNA sequences present in genomic DNA samples, the Froussard procedure makes the amplification of unknown RNA sequences possible by the use of cDNA (Froussard, 1992). This

strategy may be the simplest way of producing representative cDNA libraries using Froussard primers. In this procedure, dsRNA was denatured in the presence of the forward Froussard primer for the synthesis of cDNA and the amplification of randomly synthesised cDNA population was then performed in the presence of reverse Froussard primer. Thus, this procedure was carried out using standard RT-PCR amplification but using the Froussard primers.

Froussard forward: 5'-GCCGGAGCTCTGCAGAATTCNNNNNN-3'

Froussard reverse: 5'-GAATTCTGCAGAGCTCCGGC-3'

### **2.6.2.2 Single primer amplification**

Normally, the specificity of the DNA amplification reaction is determined by two oligonucleotide primers and it is required to have the known nucleotide sequence at both ends of target DNA to select two suitable primers. The use of a single primer makes it possible to amplify unknown sequence that is adjacent to a known DNA sequence (Parks *et al.*, 1991). PCR amplification was performed using either forward or reverse sequence-specific primers to investigate flanking sequences. PCR amplicons generated by this method, once sequenced, were easily recognisable by the fact that the same primer was present at both ends of the clones.

### **2.6.3 RNA linker mediated rapid amplification of cDNA ends (RLM-RACE)**

RLM-RACE PCR procedure was used to determine the 5'- and 3'- terminal sequences of the dsRNAs. This procedure involves the modification of the 3'-end of dsRNA, by ligating LIG Rev primer (5'-kinated-PO<sub>4</sub>; 3'-OH-blocked; 5'-GATCCAAGTCTAGAGCGG-3') at the 3' termini of dsRNA (Coutts & Livieratos, 2003). RLM-RACE is divided into three phases.

First phase:

Firstly, 7 µl of dsRNA was mixed with LIG Rev primer (1 µl) and heated to 90°C for 2 min followed by snap cooling on ice. Secondly, the mixture of T4 RNA ligase buffer (10 µl), ATP (1 µl) and sterile water (62.5 µl) was incubated at 37°C for 10 min and at 20°C for 2 min, followed by the addition of the dsRNA and LIG Rev pre-heated mixture.

Subsequently DMSO (10 µl), RNase inhibitor (2.5 µl), T4 DNA ligase (1 µl) and T4 RNA ligase (5 µl) were added and the whole mixture was incubated at 17°C for 16 h.

Second phase:

The 16 h incubated ligation mixture (100 µl) was increased in volume to 500 µl using water and concentrated using a Nanosep 30K column (VWR) by centrifugation (5,000 g; 5-10 min), resulting in the recovery of ca. 100 µl of solution. The ligated dsRNA (100 µl) was then added into the mixture of Go Taq buffer (5x, 40 µl), dNTPs (10 mM, 4.8 µl), sterile water (54.2 µl) and GoTaq DNA polymerase (5 U/µl, 1 µl). The final volume of 200 µl was incubated at 68°C for 3 h, precipitated and incubated for 16 h at -20°C.

Third phase:

Then, ligated nucleic acid was pelleted and resuspended in 8 µl of sterile water and kept at 0°C for 30 min. Then, ligated nucleic acid was denatured by adding 2 µl of methyl mercury (CH<sub>3</sub>HgOH) together with 2 µl of LIG For primer (5'-CCGCTCTAGAACTAGTTGGATC-3'). The mixture was incubated at 20°C for 20 min and kept at 0°C for 2 min. To synthesise first strand cDNA, denatured dsRNA (12 µl) was added to the first strand cDNA synthesis reaction mixture, which was pre-heated at 50°C for 1 min, followed by addition of 1 µl of Superscript™ III RNase H-Reverse Transcriptase (200 U/µl; Invitrogen). The entire mixture was then incubated at 50°C for 1 h and 70°C for 15 min. This mixture could be stored at -20°C or used immediately for PCR amplification.

First strand cDNA synthesis reaction mix:

|   |          |
|---|----------|
| Reverse transcription buffer (5x, Invitrogen) | 10 µl    |
| DTT (100 mM, Invitrogen)                      | 4 µl     |
| RNasin™ RNase inhibitor (40 U/ µl, Promega)   | 1.25 µl  |
| dNTP mix (10 mM, Promega)                     | 2.4 µl   |
| Sterilised distilled water                    | 19.35 µl |

Prior to PCR amplification, the solution was size-fractionated and concentrated using a Nanosep 30K column. The volume of the first strand cDNA solution was increased to

500 µl with H<sub>2</sub>O and it was centrifuged (5000 g for 5-10 min), resulting in the recovery of ca. 100 µl of solution. PCR amplification was then performed as described below:

PCR mixture:

|                                    |        |
|------------------------------------|--------|
| First strand cDNA                  | 5 µl   |
| PCR reaction buffer (5x, Promega)  | 20 µl  |
| LIG For primer (100 mM)            | 1 µl   |
| Sequence-specific primer (100 mM)  | 1 µl   |
| dNTP mix (10 mM, Promega)          | 2.5 µl |
| Sterile distilled water            | 70 µl  |
| GoTaq polymerase (5 U/µl, Promega) | 0.5 µl |
| Total volume                       | 100 µl |

The reaction was run in a PCR thermocycler (Hybaid) using the thermocycling programme (Section 2.6.2). One tenth of each reaction mixture was electrophoresed in a 1% agarose gel to check the size of any amplicons produced.

## **2.7 Ligation of PCR products with plasmid vector**

PCR products (3 µl) were ligated with vector pGEM-T Easy (Promega) (1 µl) in the presence of 2x ligation buffer (5 µl) using T4 DNA ligase enzyme (1 µl). Ligation mixtures were kept at 4°C for minimum of 16 h.

## **2.8 Preparation of competent cells (*Escherichia coli* XL10-Gold Ultracompetent cells)**

Competent cells were prepared using either of the procedures described below:

### **2.8.1 Transformation storage solution (TSS) method**

The preparation of *E. coli* competent cells was carried out using a transformation storage solution (TSS, Appendix 1, 2.7 ) method (Chung *et al.*, 1989). Frozen *E. coli* XL10-Gold Ultracompetent cells were thawed and 1 µl was inoculated into sterile 2x

LB broth (5 ml, Appendix 1, 2.6). These cells were allowed to grow for 16 h at 37°C on a shaker (250 rpm). Then 500 µl of the culture was diluted into 1x LB broth (50 ml, Appendix 1, 2.5). This suspension was incubated at 37°C until the optical density (OD) of the cells reached 0.40-0.60 at 600 nm. The cells were then chilled on ice for 10 min in pre-cooled 50 ml centrifuge tubes and centrifuged (3000 g; 10 min) at 4°C. The pelleted cells were resuspended in 5 ml chilled TSS, aliquoted (100 µl) into Eppendorf tubes and stored at -80°C.

### **2.8.2 Calcium chloride (CaCl<sub>2</sub>) method**

A small aliquot of frozen *E. coli* XL10-Gold Ultracompetent cells was inoculated into sterile LB media (5 ml) and cells were grown at 37°C on a shaker (250 rpm) for 16 h. The culture (1 ml) was then added into a 250 ml flask containing sterile LB broth (100 ml) and again grown at 37°C until the OD (600 nm) reached 0.40-0.60. Then cells were kept at 0°C for 5 min and centrifuged at 3000 g for 10 min at 4°C. The supernatant was decanted and pellet was resuspended in cold 25 ml CaCl<sub>2</sub> (100 mM). This suspension was kept at 0°C for at least 20 min. Centrifugation was repeated and the supernatant was removed. The pellet was finally resuspended carefully in 1 ml storage buffer (300 µl of 100% glycerol (VWR) plus 700 µl of 100 mM CaCl<sub>2</sub>) and aliquots were stored at -80°C.

### **2.9 Transformation**

Transformation procedure was used to transfer recombinant plasmids into *E. coli* cells. To perform transformation, stored (-80°C) competent *E. coli* cells were thawed at 0°C. Ligation mixture (5 µl) was mixed with 40 µl of competent cells in a microcentrifuge tube for transformation. The solution was gently mixed by flicking the bottom of the tube a few times and the tube was kept at 0°C for 20 min. The transformation tube was heat-shocked by placing it into a 42°C water bath for 45 sec. Tubes were then immediately placed at 0°C for 2 min. Then, SOC solution (900 µl) (Appendix 1, 2.9) was added to the mixture and incubated at 37°C on a shaker for 1 h. After incubation, the solution was centrifuged at 13,000 rpm for 10 sec and 800 µl of supernatant was discarded and the pellet was resuspended gently by pipetting in the remaining solution. The transformation mixture was then spread on LB agar plates containing

ampicillin plus X-Gal (5-bromo-4-chloro-indolyl- $\beta$ -D-galactopyranoside) and IPTG (Isopropyl  $\beta$ -D-1-thiogalactopyranoside) for selection of recombinant colonies (Appendix 1, 2.8). Plates were incubated for 16-20 h at 37°C. After incubation, plates could be stored at 4°C. Blue-white screening was carried out for the detection of successful transformants containing recombinant plasmids. Successful recombinants were identified as being white colonies which were distinguished from blue, wild type colonies. White colonies were selected and transferred into 5 ml LB medium containing ampicillin using tooth picks and incubated for 16 h at 37°C on a shaker (200 rpm).

## **2.10 Plasmid extraction from *E. coli* cells using the alkaline lysis method and restriction digestion of recombinant clones**

The QIA Miniprep kit (Qiagen) was used for the isolation of plasmid DNA from *E. coli* cells. 5 ml bacterial cultures grown in LB from single colonies were centrifuged at 8,000 rpm for 5 min. The supernatants were removed by decanting the tubes and cell pellets were resuspended in P1 buffer (250  $\mu$ l), and were dissolved completely by vortexing and transferred into a 1.5 ml microcentrifuge tube. P2 buffer (250  $\mu$ l) (0.2 N NaOH and 1% SDS) was added and the tube was inverted six times to assist lysis of bacterial cells. When the solution became transparent, N3 buffer (350  $\mu$ l) was added for neutralisation and the tube was inverted gently to mix. Subsequently, the tubes were centrifuged at 13,000 rpm for 10 min and the supernatant carefully transferred into QIA Miniprep columns. Columns were centrifuged for 1 min at 13,000 rpm to allow binding of plasmid DNA to the columns and the flow-through was discarded. The columns were then washed using 500  $\mu$ l of PB buffer, centrifuged for 1 min (13,000 rpm) and the flow-through was discarded. Columns were again washed using 750  $\mu$ l of PE buffer followed by the centrifugation for 1 min (13,000 rpm), removal of flow-through and centrifugation for 1 min (13,000 rpm) for the removal of residual washing buffer. Finally, columns were placed into 1.5 ml microcentrifuge tubes and 50  $\mu$ l of EB buffer or nuclease-free water was added for the elution of plasmid DNA. The columns with the elution buffer were allowed to stand for 1 min and then they were centrifuged to collect the DNA.



Eluted plasmid DNA was digested with restriction endonuclease *EcoRI*, which released the insert, in order to assess size of any inserts.

|                                  |        |
|----------------------------------|--------|
| <i>EcoRI</i> buffer (10x)        | 2 µl   |
| Bovine serum albumin (BSA; 100x) | 0.2 µl |
| Plasmid DNA                      | 10 µl  |
| <i>EcoRI</i> enzyme              | 1 µl   |
| Sterilised distilled water       | 6.8 µl |
| Total volume                     | 20 µl  |

Tubes were incubated at 37°C for >1 h. The results of restriction digestion were visualised by agarose gel electrophoresis.

## 2.11 Sequencing of RT-PCR amplicons

The DNA concentrations of recombinant plasmids were quantified using a Nanodrop and then samples were sent for sequencing to the Genewiz Laboratory (Essex, UK). Recombinant plasmids comprising inserts were sequenced using either of the universal oligonucleotide primers described below which prime within the pGEM-T Easy vector.

M13 Forward: 5'-GTAAAACGACGGCCAGT-3'

M13 Reverse: 5'-AACAGCTATGACCATG-3'

Sequence data were analysed and identified for similarity with a library of sequences in databases using the NCBI, BLAST program (Altschul *et al.*, 1990). Sequence alignment and translation were performed using the MAFFT and ExPASy programs, respectively.

## 2.12 Northern blot hybridization

Northern blot is a method used to detect specific RNA molecules among total RNA preparations. The term 'Northern blot' actually refers to the transfer of RNA from electrophoresis gel to membrane (Trayhurn, 1996).

This procedure involves three steps:

### (1) RNA blotting and fixation

DsRNAs separated by 18 h of agarose gel electrophoresis were photographed, followed by treatment with 0.25 M HCl for 20 min. Nucleic acids were denatured by soaking the gel in 0.1 M NaOH for 30 min. Afterwards, the gel was neutralised by immersing twice in 0.1 M Tris-HCl for 20 min. Then, the gel was blotted by capillary transfer with 20x SSC for a minimum of 6 h. Once the RNA was transferred to the nylon membrane, it was briefly rinsed in 2x SSC and RNA was fixed to the membrane by UV-cross linking for 30 min or by baking (120°C for 30 min or 80°C for 2 h). This membrane could be either stored at 2-8°C or used immediately for pre-hybridization.

Nylon membranes were pre-hybridized with DIG-Easy Hybridization solution for 30 min with gentle agitation at 68°C. Then, DIG-labelled probes, with predetermined labelling efficiency, were denatured by boiling for 5 min and rapidly cooled at 0°C. These denatured probes were mixed with pre-warmed DIG-Easy Hybridization solution and the membrane was incubated for 6 h or 16 h at 68°C with gentle agitation. The pre-hybridized membrane was washed with 2x SSC (2 x 5 min, at 15-25°C) and 0.1x SSC (2 x 15 min, at 68°C) in combination with 0.1% SDS. Stringency washes were followed by immunological detection.

### (2) DNA template preparation

Plasmid DNA was used for template preparation and DNA template was linearized using a chosen restriction enzyme and linearized plasmid DNA was transcribed to create labelled probes. Recommended size of the labelled RNA is in the range of 200-1000 bases.

#### *In vitro* transcription and DIG-RNA labelling

|                                |       |
|--------------------------------|-------|
| Linearized plasmid DNA         | 5 µl  |
| Transcription buffer (5x)      | 2 µl  |
| Labelling mix (5x)             | 2 µl  |
| RNA polymerase (SP6, T7 or T3) | 1 µl  |
| Total                          | 10 µl |

The mixture was incubated at 42°C for 1 h and then incubated at 37°C for 1 h with 1 µl of DNase I to remove the template DNA.

### (3) Determination of labelling efficiency

Determination of labelling efficiency of probes is the direct detection method in comparison to control RNA probes (provided in the kit). A dilution series of labelled probes and control probes was prepared (Tubes 1-4, Table 2.1) and applied to nylon membrane (1  $\mu$ l). Nucleic acid was fixed to nylon membranes by UV-cross linking for 30 min or by baking (120°C for 30 min or 80°C for 2 h).

#### Immunological detection of hybridised RNA nylon membrane

Cross-linked nylon membrane was incubated with in turn, washing buffer (20 ml, 2 min; Appendix 1, 2.15), blocking solution (10 ml, 30 min; Appendix 1, 2.14), antibody solution (10 ml, 30 min), washing buffer (20 ml, 15 min x 2). Membrane was equilibrated with 10 ml detection buffer (Appendix 1, 2.16) for 2-5 min and membrane was transferred to a development folder. CDP-Star was applied on membrane and then exposed to an imaging device for 5-25 min for detection.

**Table 2.1 Preparation of a sequential dilution series of DIG-labelled probe and control probe, where tube 1 is either a DIG-labelled probe or the control RNA at a concentration of 10 ng/μl.**

| Tube No. | RNA (μl) | From tube            | RNA dilution buffer (μl) | Final concentration |
|----------|----------|----------------------|--------------------------|---------------------|
| 1        | -        | Sample to be diluted | -                        | 10 ng/μl            |
| 2        | 2        | 1                    | 18                       | 1 ng/μl             |
| 3        | 2        | 2                    | 198                      | 10 pg/μl            |
| 4        | 15       | 3                    | 35                       | 3 pg/μl             |

## **Chapter 3**

### **Identification and characterisation of mycovirus dsRNA elements in plant pathogenic fungi**

### 3.1 Introduction

The study of viruses that infect plant-pathogenic fungi is important from an agricultural perspective as mycoviruses may contribute to sustainable agriculture as biocontrol agents and reduce the use of fungicides. In addition to this, hypovirulent plant-pathogenic fungal strains that contain transmissible viruses have attracted much interest to investigate different fungal species for the presence of mycoviruses. Mycoviruses have been investigated in all major groups of plant pathogenic fungi. Comprehensive screening of plant-pathogenic fungi for the presence of dsRNA showed prevalence of fungal viruses in them, with incidence as high as >80% (Ghabrial & Suzuki, 2009).

In this study, five plant pathogenic fungi *Hymenoscyphus fraxineus*, *Dothistroma septosporum*, *Leptosphaeria maculans*, *Leptosphaeria biglobosa* and *Pyrenopeziza brassicae* species, previously unexamined for the presence of mycoviruses, were screened for viruses. Subsequently further research focused on *Leptosphaeria* species where a mycovirus was discovered for the first time in several isolates of *L. biglobosa*. Since *Leptosphaeria* species are responsible for causing economically important disease of oilseed rape these investigations are pertinent and timely as the effects of mycovirus infection on pathogenicity are unknown.

In addition to this, small projects were undertaken to characterise viruses from *Aspergillus foetidus* and *D. septosporum* to develop technology and standardise techniques that could be used to successfully complete the main objective of the PhD thesis.

#### 3.1.1 *Hymenoscyphus fraxineus*

*Hymenoscyphus fraxineus* (anamorph *Chalara fraxinea*) is the causal agent of ash dieback disease of ash trees (Kowalski & Holdenrieder, 2009). The European ash *Fraxinus excelsior* and narrow-leaved ash *F. angustifolia* are the most affected species in Europe (Gross *et al.*, 2014). European ash is common in Europe and is a valuable tree due to its ecological characteristics, high economic value and wood properties (Source: Woodland Trust, Ash (*Fraxinus excelsior*)).

In Europe, ash dieback was reported initially in Poland, afterwards in Germany, Sweden, Lithuania and Austria (Halmschlager & Kirisits, 2008). The first observations of the disease were made in the north-western regions of Poland and ash dieback subsequently spread through the whole country (Kowalski, 2006).

Infected plant parts exhibit discoloration (Halmschlager & Kirisits, 2008), wilting of leaves, stem canker and dieback of crowns (Fig. 3.1). Necrotic lesions reach towards the xylem in the active stage of the canker (Kowalski & Holdenrieder, 2009).

### **3.1.2 *Dothistroma septosporum***

*D. septosporum* is an ascomycete belonging to the class Dothideomycetes (Bradshaw, 2004). *D. septosporum* is an economically important hemibiotrophic pathogen that causes dothistroma needle blight of pine trees. It affects more than 70 species of pine, including *Picea* species (Bednářová *et al.*, 2006). Needle blight has been reported for many decades in pine plantations in the southern hemisphere and since the early 1990s has increased in the northern hemisphere. This fungus produces a toxin called dothistromin that accumulates and produces brick-red bands around the infected needles (Shain & Franich, 1981). Dothistromin has a structural similarity to a precursor of highly toxic and carcinogenic fungal metabolite, aflatoxin (Shaw *et al.*, 1978).

Several weeks after infection, *D. septosporum* produces conidia which can be spread to other pines by rain-splash. In later phases of the disease, black fruiting bodies (stromata) appear in the red band (Fig. 3.1). Eventually, the whole band becomes necrotic and drops prematurely (Edwards & Walker, 1978; Kershaw *et al.*, 1988). In severe epidemics of disease, complete defoliation causes retardation of growth and tree death (Gibson, 1974; Woods, 2003). Whilst *D. septosporum* has a predominantly asexual lifecycle, it is also sexually active in some parts of the world (Groenewald *et al.*, 2007). Dothistroma needle blight requires more than a year to complete its life cycle and a few years to become a serious problem within the tree canopy (Karadžič, 1989).

### **3.1.3 *L. maculans* and *L. biglobosa***

Phoma stem canker of oilseed rape is associated with two closely related fungal species, *L. maculans* and *L. biglobosa*. Of these, *L. maculans* is the most damaging pathogen (Section 1.11). In plant-pathogen systems, gene-for-gene interactions normally exist and the disease could be controlled with the use of resistance genes (*Rlm* genes). However, there is little information about resistance to *L. biglobosa* and resistance genes towards *L. maculans* do not affect *L. biglobosa* (Fitt *et al.*, 2006). Between 2005 and 2014, phoma stem canker caused annual yield losses in oilseed rape in England up to £87M (Fig. 3.2).

### **3.1.4 *Pyrenopeziza brassicae***

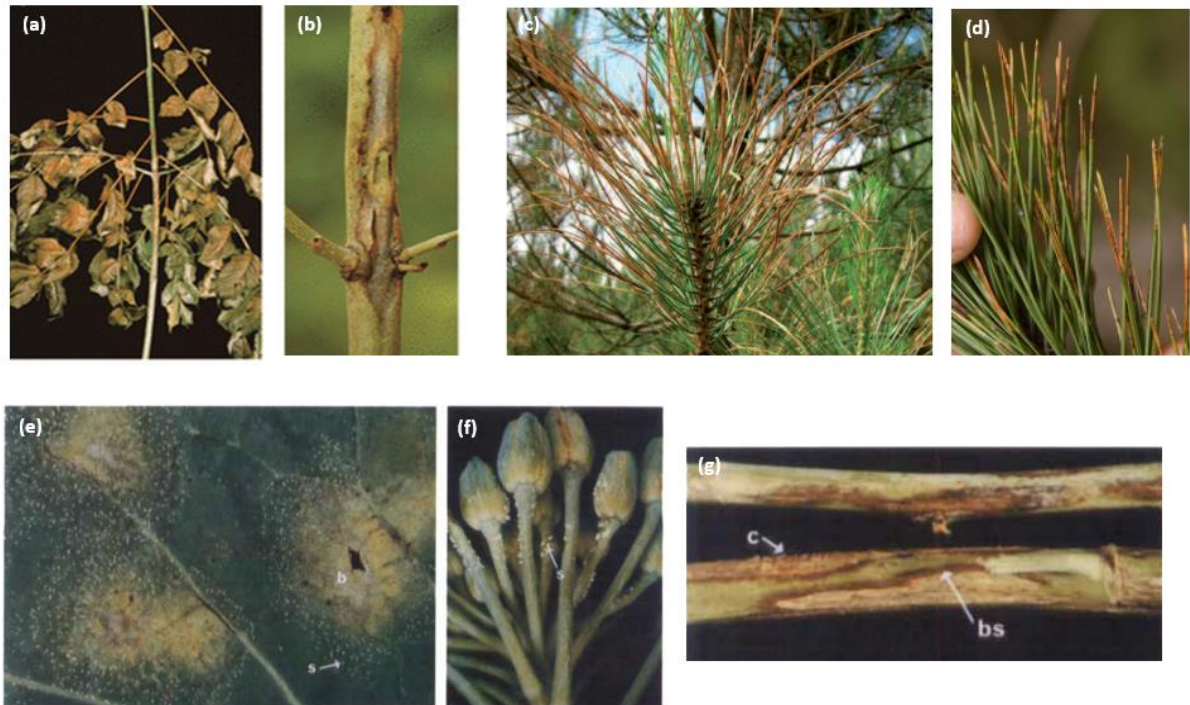
*Pyrenopeziza brassicae* (anamorph *Cylindrosporium concentricum*) produces light leaf spot on winter oilseed rape cultivars in the UK, specifically in the high-rainfall parts of northern England and Scotland (Figuroa *et al.*, 1995; Sutherland *et al.*, 1998). The disease was first described in France in 1977 and it has caused severe yield losses recently. In 2012, light leaf spot caused yield losses in oilseed rape in England of more than £159M and in 2014 the second highest loss was recorded, in excess of £140M (Fig. 3.2).

The affected leaf areas exhibit small white pustules which contain conidial spores. Green leaves of different ages and youngest unexpanded leaves exhibit sporulation which might occur on both upper and lower leaf surfaces. Light leaf spot lesions first appear as mottled light green areas that later convert to brittle and bleached lesions (Fig. 3.1; Fitt *et al.*, 1998).

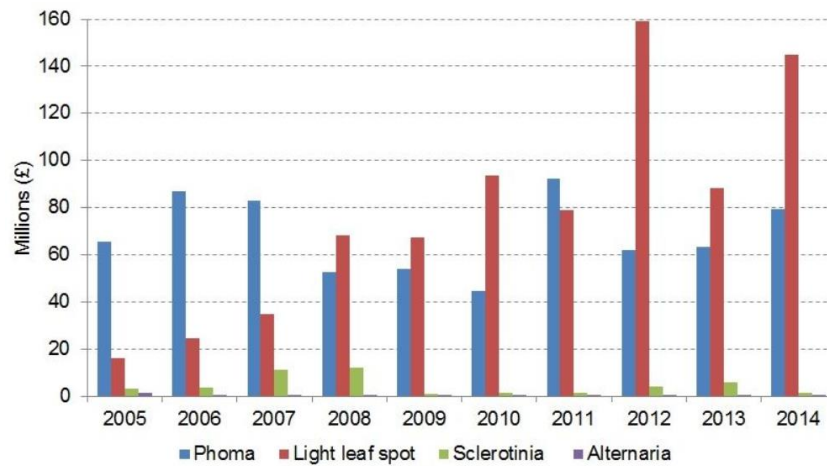
### **3.1.5 *Aspergillus foetidus* virus**

*Aspergillus foetidus* was reported as a biological agent which was used for decolourising liquid waste through bioadsorption (Sumathi & Phatak, 1999). The filamentous fungus *Aspergillus foetidus* was shown to contain viruses over 50 years ago and *Aspergillus foetidus* virus (AfV) was the first report of mycovirus infection in the genus *Aspergillus* (Banks *et al.*, 1970). *A. foetidus* strain IMI-41871 contains six





**Figure 3.1 Disease symptoms of ash dieback, dothistroma needle blight and light leaf spot.** (a, b) Symptoms of ash dieback: wilting of leaves 2 months after infection, extended stem lesion 12 months after infection, respectively (Kowalski & Holdenrieder, 2009). (c, d) Symptoms of dothistroma needle blight. (c) Needle tips turning brown on pine (Source: Forestry Commission, Dothistroma needle blight (*Dothistroma septosporum*)). (d) Dead spots or bands on the needle show black fruiting bodies. (e, f, g) Symptoms of light leaf spot on oilseed rape. (e) Pale, brittle, (b) bleached light leaf spot lesions on leaves showing white pustules (s) of spore masses of *P. brassicae*. (f) Sporulation (s) of *P. brassicae* on infected flower buds. (g) Fawn-coloured light leaf spot lesions on stems with black speckling (bs) at the indistinct edges and transverse cracking (c) (Fitt *et al.*, 1998).



**Figure 3.2 Annual yield losses in oilseed rape in England due to phoma stem canker, light leaf spot, sclerotinia stem rot and alternaria pod spot diseases for the harvest years 2005-2014** (Source: CropMonitor, FERA, based on DEFRA Winter Oilseed Rape Disease Survey).

dsRNAs which are apparently encapsidated in two different types of virions, designated *A. foetidus* virus-fast (AfV-F) and *A. foetidus* virus-slow (AfV-S), based on the relative electrophoretic mobilities of the dsRNAs (Fig. 3.3) (Ratti & Buck, 1972). Moreover, centrifugation in caesium chloride density gradients showed four components of both AfV-F (F1, F2, F3 and F4) and AfV-S (two major fractions S1, S2 and two minor fractions S3, S4; (Buck & Ratti, 1975).

AfV-F virions contain tetra-segmented, polyadenylated dsRNAs, which are similar in genetic organisation and sequence to *Alternaria alternata* virus 1 (Kozlakidis *et al.*, 2013a). All four dsRNAs of AfV-F were sequenced and characterised (Kozlakidis *et al.*, 2013b). The sizes of these dsRNA elements are ca. 3.6 kbp, 2.8 kbp, 2.5 kbp and 2.0 kbp. AfV-S comprises AfV-S1, a victorivirus; AfV-S2, an unclassified RNA; and AfV-S3 (Fig. 3.6a), a previously uncharacterized dsRNA element.

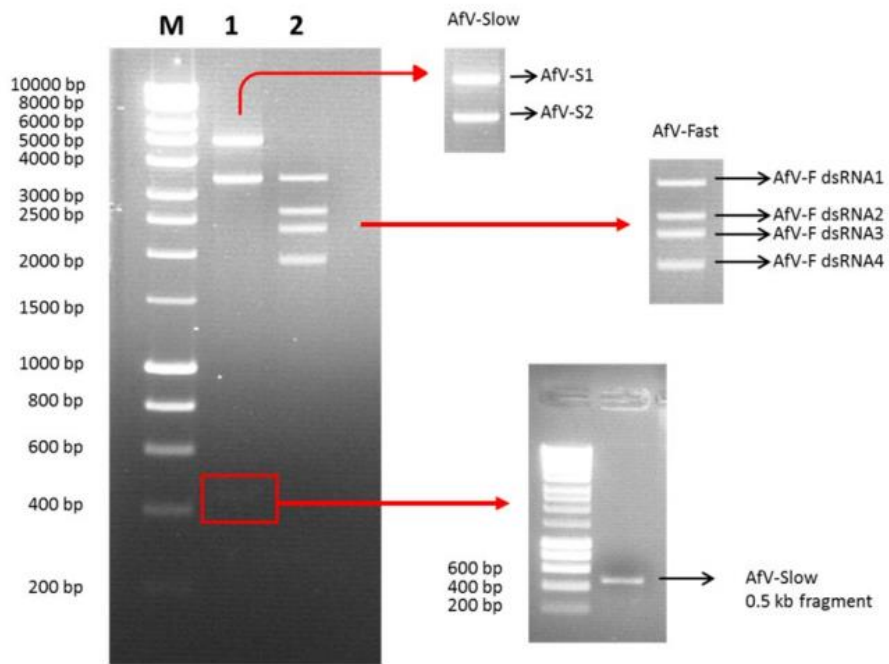
AfV-S1, a virus of 5.2 kbp encapsidated dsRNA, encodes an RdRP and a coat protein. AfV-S2, a non-segmented dsRNA of 3.6 kbp is considered to be a satellite virus (Kozlakidis *et al.*, 2013b). In this study, the complete nucleotide sequence of AfV-S3, the smallest dsRNA element, was determined (Shah *et al.*, 2015).

### 3.1.6 Aim and objectives

The main aim of the research reported in this chapter was to identify and characterise dsRNA mycovirus elements from plant pathogenic fungi including *L. biglobosa*. For this purpose, a small project was performed to identify and characterise dsRNA mycoviruses from *A. foetidus* and *D. septosporum*.

Objectives:

1. To screen different isolates of *H. fraxineus*, *D. septosporum*, *L. maculans*, *L. biglobosa* and *P. brassicae* species for mycoviruses.
2. To characterise *Aspergillus foetidus*-S3 virus.
3. To characterise *Dothistroma septosporum* chrysovirus-1 (DsCV-1).
4. To characterise *Leptosphaeria biglobosa* mycovirus-1 (LbMV-1).
5. To perform northern blot hybridization to analyse the presence of specific sequences of LbMV-1 dsRNAs 1 and 2.
6. To identify dsRNA elements present in all virus-infected *L. biglobosa* isolates.



**Figure 3.3 Agarose gel electrophoresis of dsRNAs present in *A. foetidus*.** Lane 1, *A. foetidus* slow virus (AfV-S). Lane 2, *A. foetidus* fast virus (AfV-F). Lane M contains Hyperladder, DNA marker (Kozlakidis *et al.*, 2013b).

## **3.2 Materials and methods**

### **3.2.1 Small-scale screening of fungal isolates for the presence of dsRNA mycoviruses**

*H. fraxineus* isolates exhibited slow growth, so they were allowed to grow for two months while isolates of *D. septosporum*, *Leptosphaeria* species and *P. brassicae* were grown on growth media (Section 2.3) for approximately 20 days. Isolates of *H. fraxineus*, *Leptosphaeria* and *P. brassicae* generally grow on the upper surface of the agar, while mycelia of *D. septosporum* isolates not only grow on the upper surface but also penetrate below the surface of the agar.

Fungal mycelium was harvested, placed in liquid nitrogen and crushed using a mortar and pestle. The homogenate was immediately transferred into a 2 ml Eppendorf tube, mixed with 350 µl of extraction buffer, vortexed and incubated for 1 h at 70°C. After incubation, the mixture was treated with 350 µl of phenol and an equal volume of Sevag, vortexed and centrifuged (13,000 rpm for 5 min). After centrifugation, the supernatant was mixed with 350 µl of Sevag, vortexed and centrifuged (13,000 rpm for 5 min). The resulting supernatant containing nucleic acid was incubated for 16 h with 800 µl of ethanol and 30 µl of sodium acetate at -20°C (Section 2.4.2). The precipitated samples were centrifuged to collect the nucleic acid. The pellet was dried and resuspended in 20 µl of water followed by DNase I and SI nuclease treatment (Sections 2.4.3 and 2.4.4). Then, the samples were checked for the presence or absence of dsRNAs by agarose gel electrophoresis.

### **3.2.2 Characterisation of *Aspergillus foetidus*-S3 virus**

Glycerol stocks of AfV-S were used to complete cDNA cloning and sequencing of the smallest dsRNA element associated with AfV-S mycoviruses. Initially dsRNA was purified from virus particles using the phenol and Sevag extraction procedure, which was followed by ethanol precipitation and gel electrophoresis. The desired dsRNA molecule was purified using gel extraction. The extracted dsRNA was used as a template in an improved RLM-RACE procedure (Coutts & Livieratos, 2003). The whole dsRNA element was amplified without requirement for sequence-specific primers, PCR amplicons were cloned into the pGEM-T Easy vector and transformed into

competent *E. coli* cells (XL10-Gold cells; Agilent). Gel electrophoresis was carried out to assess the presence of inserts in plasmids following *EcoRI* digestion. Two inserts of interest were found in recombinant plasmids, which were sent for sequencing. Afterwards, the origin of the clones was confirmed by northern blot hybridization performed according to the manufacturer's instructions (Section 2.12).

### **3.2.3 Characterisation of *Dothistroma septosporum* chrysovirus-1 (DsCV-1)**

Forty-five *D. septosporum* isolates were assessed for the presence of mycoviruses using a small-scale dsRNA extraction procedure. During this investigation, four dsRNAs were observed from isolate D752.1. Resistance to DNase I and SI nuclease treatment confirmed the dsRNA nature of the nucleic acid (Section 2.4.3 and 2.4.4). Purified dsRNAs were separated by agarose gel electrophoresis (Fig. 3.5a). Based on their molecular weight, it was suspected that these dsRNA elements may be closely related to the *Chrysoviridae* family, so on the basis of multiple alignment of RdRPs of different members of this family primers were designed (Appendix 2, Table 2.1) to perform RT-PCR and random cDNA clones of dsRNAs 1-4 were obtained. Afterwards for the synthesis of additional cDNAs, sequence-specific primers were designed (Appendix 2, Table 2.2) from the sequences of the initial clones.

### **3.2.4 Characterisation of *Leptosphaeria biglobosa* mycovirus-1 (LbMV-1)**

Initially, *Leptosphaeria* isolates were screened for the presence of dsRNA elements using a small-scale dsRNA extraction protocol. Later, dsRNAs were isolated from purified virus from *L. biglobosa* (isolate C-Rox 12.8.1) (Section 2.5.3) for use in cloning and sequencing. Extracts were treated with DNase I and SI nuclease to remove host contaminating nucleic acids. Agarose gel electrophoresis was performed to assess the presence, size and purity of dsRNAs.

Purified dsRNAs were separated by agarose gel electrophoresis and cDNA clones were produced using a random priming procedure with methyl mercuric hydroxide-denatured dsRNAs (Froussard, 1992). Alternatively, another procedure was also performed initially to obtain the cDNA clones using a random priming method (Kozlakidis *et al.*, 2006).

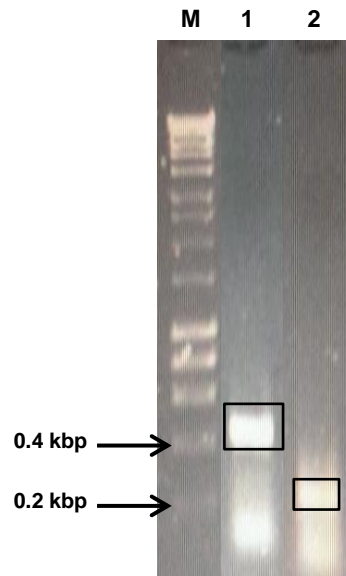
RT-PCR generated amplicons were extracted (Section 2.4.7), ligated into the pGEM-T Easy vector and transformed into *E. coli* competent cells. The resulting clones were sequenced using universal primers and analysed to identify homology with previously characterised sequences in the database using the BLAST programs (Altschul *et al.*, 1990). After obtaining the sequence data for dsRNA elements, sequence-specific primers (Appendix 2, Table 2.3) were designed upstream (5'-) and downstream (3'-) of the extant sequences for a RLM-RACE PCR and genome walking (Coutts *et al.*, 2004). The RLM-RACE PCR procedure was carried out to determine the 5'- and 3'-terminal sequences of dsRNAs and an example is shown in Fig. 3.4 (showing 5'- and 3'- amplicons; Coutts & Livieratos, 2003).

### **3.2.5 Northern hybridization analyses**

Northern blotting was performed using probes produced from cDNA clones of dsRNAs, generated by targeted PCR amplification and PCR products were cloned into pGEM-T Easy vector (Section 2.12). The resulting recombinant plasmids were linearized using the restriction enzyme and single-stranded RNA probes of defined length were generated by *in vitro* transcription of linearized template DNA in the presence of digoxigenin-UTP, using T7 RNA polymerase (DIG Northern Starter Kit; Roche), followed by immunological detection using alkaline phosphatase-conjugated, anti-digoxigenin antibody (Roche).

### **3.2.6 Identification of dsRNA elements present in virus-infected *Leptosphaeria biglobosa* isolates**

Total RNA extraction using the RNeasy kit (Section 2.5.4) followed by diagnostic RT-PCR was carried out to compare the sequence similarity between the different isolates of *L. biglobosa*. Sequence-specific forward and reverse primers were designed (Table 3.1) from the known sequence of the LbMV-1 dsRNA 2. Total RNA was used as template for cDNA synthesis and then PCR amplification was performed using sequence-specific primers. PCR products were subsequently cloned and sequenced (Sections 2.7, 2.9, 2.10 and 2.11).



**Figure 3.4** Agarose gel electrophoresis showing the amplicons produced by the RLM-RACE procedure to generate products corresponding to the 5'- and 3'-ends of LbMV-1 dsRNA 2. Lanes 1 and 2 contain amplicons corresponding to: 5' end of dsRNA; 3' end of dsRNA, respectively. Hyperladder 1 (M; 10 kbp) was used as a marker.



**Table 3.1 Primers used to identify specific sequence of the LbMV-1 dsRNA 2 ORF found in various *L. biglobosa* isolates.**

| <b>Name of primer</b> | <b>Sequence</b>                       |
|-----------------------|---------------------------------------|
| <b>LBFS1</b>          | <b>5'- CAAGTCCGAATCTCTGCATTCAG-3'</b> |
| <b>LBRS2</b>          | <b>5'- GATCACCTCTTGCTCGCATG-3'</b>    |

### 3.3 Results

#### 3.3.1 Small-scale screening of fungal isolates for the presence dsRNA mycoviruses

Fifty-three isolates of *L. maculans* (37 isolates) and *L. biglobosa* (16 isolates) obtained from the University of Hertfordshire pathogen collection (Table 3.2) and twenty isolates of *L. maculans* obtained from oilseed rape crops (73 in total) were screened for dsRNA mycoviruses. Eleven out of sixteen *L. biglobosa* isolates were found to contain dsRNA elements. In contrast, none of the fifty-seven *L. maculans* and ten *P. brassicae* isolates contained dsRNA elements. In addition to this, none of the 162 *H. fraxineus* and only one of the 45 *D. septosporum* isolates appeared to contain dsRNA elements.

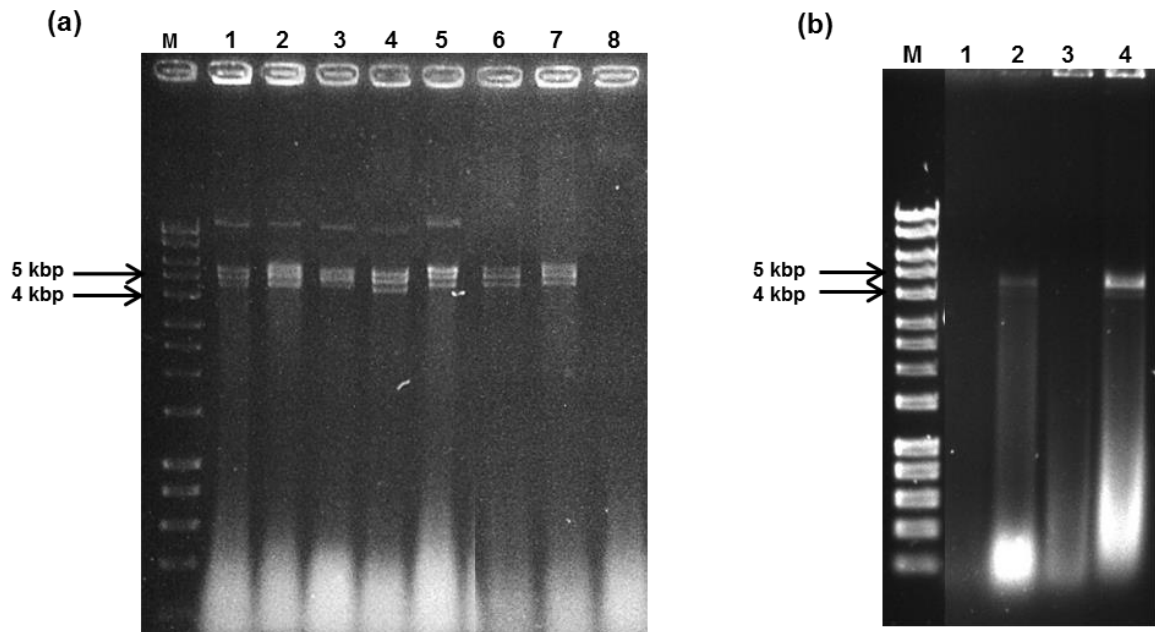
This study represents the first report of LbMV-1 (the genome made up of dsRNA components). However, this is a preliminary naming of the virus found from *L. biglobosa*. Small-scale dsRNA screening from eight representative *L. biglobosa* isolates is shown in Fig 3.5 (a), where C-Rox 12.8.1 and A-Exc 12.12.10 were used as positive and negative controls. These screens revealed that the six isolates of *L. biglobosa*, viz. K-Exc 12.10.21, D-Rox 12.10, H-Exc 12.10.3, H-Exc 12.12.3, F2-Exc 12.3.1, F2-Exc 12.6.1 contained at least three dsRNA elements ranging in size from 4.0-4.9 kbp (Fig. 3.5a).

The dsRNA nature of the LbMV-1 genome was confirmed following DNase I and SI nuclease digestion. RNase III treatment confirmed these observations as it specifically digests dsRNA. To determine whether LbMV-1 is encapsidated in virions the sensitivity of LbMV-1 dsRNA and purified LbMV-1 to RNase III was examined. In Fig. 3.5 (b) both dsRNA (lane 1) and purified virions (lane 3) were sensitive to RNase III digestion as compared to their respective controls (lanes 2 and 4) incubated in the same manner without enzyme, suggesting that LbMV-1 was not conventionally encapsidated. *D. septosporum* isolate D 752.1 was found to be infected with a mycovirus with a dsRNA profile comprising four segments ranging in size from 2.8 to 3.5 kbp (Fig. 3.7a).

**Table 3.2 List of *Leptosphaeria maculans* (Lm) and *Leptosphaeria biglobosa* (Lb) isolates, location of crop, county and infection with dsRNA. [-] indicates absence of dsRNA, [+] indicates presence of dsRNA.**

| #  | Isolate                     | Location of crop<br>(field isolates) | County             | dsRNA<br>presence |
|----|-----------------------------|--------------------------------------|--------------------|-------------------|
| 1  | E-Exc 12.10.21 (Lm)         | Bainton                              | Yorkshire (UK)     | -                 |
| 2  | E-Exc 12.2.21 (Lm)          | Bainton                              | Yorkshire          | -                 |
| 3  | E-Exc 12.3.1 (Lm)           | Bainton                              | Yorkshire          | -                 |
| 4  | E-Exc 12.7 (Lm)             | Bainton                              | Yorkshire          | -                 |
| 5  | E-Rox 12.1.2 (Lm)           | Bainton                              | Yorkshire          | -                 |
| 6  | E-Rox 12.4.1 (Lm)           | Bainton                              | Yorkshire          | -                 |
| 7  | E-Rox 12.9.3 (Lm)           | Bainton                              | Yorkshire          | -                 |
| 8  | A-Exc 12.9.1 (Lm)           | Banbury                              | Oxfordshire (UK)   | -                 |
| 9  | A-Exc 12.12.10 (Lb)         | Banbury                              | Oxfordshire        | -                 |
| 10 | A-Rox 12.1.5 (Lm )          | Banbury                              | Oxfordshire        | -                 |
| 11 | A-Rox 12.6.1 (Lm )          | Banbury                              | Oxfordshire        | -                 |
| 12 | I1-Exc 12.4.1 (Lm)          | Horncastle                           | Lincolnshire (UK)  | -                 |
| 13 | I1-Exc 12.8.1 (Lm)          | Horncastle                           | Lincolnshire       | -                 |
| 14 | I1-Exc 12.7.1 (Lm)          | Horncastle                           | Lincolnshire       | -                 |
| 15 | I1-Exc 12.7.2 (Lm)          | Horncastle                           | Lincolnshire       | -                 |
| 16 | K-Exc 12.11.21 (Lm)         | Harpenden                            | Hertfordshire (UK) | -                 |
| 17 | K-Exc 12.7.23 (Lm)          | Harpenden                            | Hertfordshire      | -                 |
| 18 | K-Exc 12.10.21 (Lb)         | Harpenden                            | Hertfordshire      | +                 |
| 19 | K-Rox 12.5.31 (Lm)          | Harpenden                            | Hertfordshire      | -                 |
| 20 | K-Rox 12.7.31 (Lm)          | Harpenden                            | Hertfordshire      | -                 |
| 21 | K-Rox 12.11.1 (Lb)          | Harpenden                            | Hertfordshire      | -                 |
| 22 | Mynickij 13-1 (Lm)          | Harpenden                            | Hertfordshire      | -                 |
| 23 | Shang U 13-2 (Lm)           | Harpenden                            | Hertfordshire      | -                 |
| 24 | State red russian 13-2 (Lm) | Harpenden                            | Hertfordshire      | -                 |
| 25 | State red russian 13-3 (Lm) | Harpenden                            | Hertfordshire      | -                 |
| 26 | Swede augus 1 (Lm)          | Harpenden                            | Hertfordshire      | -                 |
| 27 | Swede tina 13-2 (Lm)        | Harpenden                            | Hertfordshire      | -                 |
| 28 | Swede tina 13-3 (Lm)        | Harpenden                            | Hertfordshire      | -                 |
| 29 | Ning U 13-1 (Lm)            | Harpenden                            | Hertfordshire      | -                 |

|    |                     |             |                   |   |
|----|---------------------|-------------|-------------------|---|
| 30 | Ning U 13-2 (Lm)    | Harpenden   | Hertfordshire     | - |
| 31 | Samourai 1 (Lm)     | Harpenden   | Hertfordshire     | - |
| 32 | Shang U 13-3 (Lm)   | Harpenden   | Hertfordshire     | - |
| 33 | C-Rox 12.10.1 (Lm)  | Morley      | Norfolk (UK)      | - |
| 34 | C-Rox 12.8.2 (Lm)   | Morley      | Norfolk           | - |
| 35 | C-Rox 12.9.1 (Lm)   | Morley      | Norfolk           | - |
| 36 | C-Rox 12.8.1 (Lb)   | Morley      | Norfolk           | + |
| 37 | D-Rox 12.2 (Lm)     | Rothwell    | Lincolnshire (UK) | - |
| 38 | D-Rox 12.4 (Lm)     | Rothwell    | Lincolnshire      | - |
| 39 | D-Rox 12.5 (Lm)     | Rothwell    | Lincolnshire      | - |
| 40 | D-Rox 12.10 (Lb)    | Rothwell    | Lincolnshire      | + |
| 41 | H-Exc 12.10.3 (Lb)  | Cowlinge    | Suffolk (UK)      | + |
| 42 | H-Exc 12.12.1 (Lb ) | Cowlinge    | Suffolk           | - |
| 43 | H-Exc 12.12.3 (Lb)  | Cowlinge    | Suffolk           | + |
| 44 | H-Exc 12.3.2 (Lm)   | Cowlinge    | Suffolk           | - |
| 45 | F2-Exc 12.2.2 (Lm)  | Stockbridge | Hampshire (UK)    | - |
| 46 | F2-Exc 12.2.3 (Lb)  | Stockbridge | Hampshire         | - |
| 47 | F2-Exc 12.3.1 (Lb)  | Stockbridge | Hampshire         | + |
| 48 | F2-Exc 12.6.1 (Lb)  | Stockbridge | Hampshire         | + |
| 49 | F2-Excdm 11.5 (Lb)  | Stockbridge | Hampshire         | - |
| 50 | Noth2 (Lb)          | Hubei       | Hubei (China)     | + |
| 51 | N20.3 (Lb)          | Hubei       | Hubei             | + |
| 52 | W10 (Lb)            | Hubei       | Hubei             | + |
| 53 | N3.4 (Lb)           | Hubei       | Hubei             | + |



**Figure 3.5 (a) Agarose gel electrophoresis of DNase I and SI nuclease treated nucleic acid extracts of UK *Leptospaeria biglobosa* isolates.** Lane M contains Hyperladder I molecular weight marker. Lane 1 (isolate C-Rox 12.8.1) and 8 (isolate A-Exc 12.12.10) contain positive and negative controls, respectively. Lanes 2-7 contain dsRNAs isolated from *L. biglobosa* isolates K-Exc 12.10.21, D-Rox 12.10, H-Exc 12.10.3, H-Exc 12.12.3, F2-Exc 12.3.1, F2-Exc 12.6.1, respectively. Fungal genomic DNA contamination (>10 kbp) could not be removed after DNase I treatment, which can be seen in lanes 1-5. (b) RNase III sensitivity of dsRNA isolated from purified virus from isolate C-Rox 12.8.1 was investigated. Lane 1 contains dsRNA extract treated with RNase III (lane 1). Lane 2 contains control which was not treated with RNase III. Lane 3 contains purified virions treated with RNase III and lane 4 contains untreated virions as a control.

### 3.3.2 Characterisation of *Aspergillus foetidus*-S3 virus

Northern blot analysis showed strong signals for RNA with an AfV-S3 specific probe (Fig. 3.6b), confirming that the sequence obtained corresponded to the AfV-S3 RNA element and its size and sequence were also confirmed.

AfV-S3 is 439 bp in length with a GC content of 53% and contains no ORFs of any significant length. BLAST analysis (Altschul *et al.*, 1997) did not reveal any significant sequence similarity to any other protein in global databases. Moreover, it also did not exhibit homology with other AfV-S and AfV-F components. Short fragments of ORFs potentially encode polypeptides of <9 kDa in mass. The secondary structure of AfV-S3 dsRNA was modelled using the Mfold program (Zuker, 2003) and showed that ca. 54% of the ribonucleotides are involved in the formation of secondary structure and found to contain a stem-loop structure (Fig. 3.6c). Stem-loop structures are a common feature of mycoviruses and are considered to be associated with RdRP recognition and RNA replication (Ghabrial & Suzuki, 2009).

### 3.3.3 Characterisation of *Dothistroma septosporum* chrysovirus-1 (DsCV-1)

DsRNAs isolated from *D. septosporum* isolate D 752.1 were resolved into four bands by agarose gel electrophoresis with sizes that ranged from ~2.8-3.5 kbp. The genome organization of DsCV-1 is shown in Fig. 3.7 (a). Numbers 1 to 4 were assigned to the dsRNAs according to their decreasing size.

The nucleotide sequence of dsRNA 1 was partially completed and is >3550 bp in length (Daudu, unpublished data). It contains a single ORF of 1095 amino acids (aa) that encodes an RdRP. The analysis of the deduced amino acid sequence of dsRNA 1 revealed the presence of eight conserved motifs characteristic for dsRNA mycoviruses. Subsequent BLAST searches of the deduced amino acid sequence of this ORF showed similarity to RdRP encoded by *Isaria javanica* chrysovirus-1 (IjCV-1) RdRP (64% identity; Daudu, unpublished data). Sequence analysis of dsRNA 2 revealed that it is 3068 bp in size and contains an ORF that encodes the 938 aa capsid protein (CP), which showed high identity to IjCV-1 CP (56% identity and 70% similarity; Appendix 4, Fig. 4.1). The complete genome organisation of dsRNA 2 is shown in Fig. 3.7 (b). The sequence of dsRNA 3 is partially completed and is currently 2701 bp in length and includes a single ORF that encodes a hypothetical protein of unknown

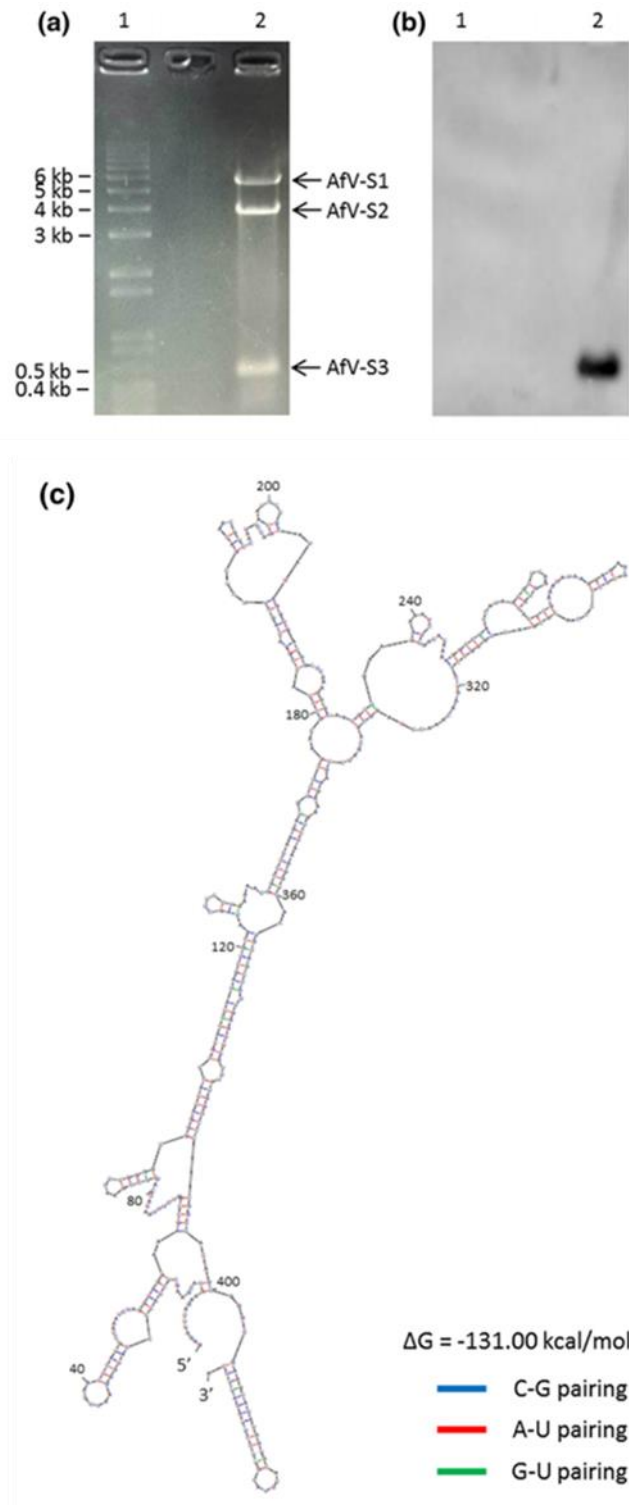
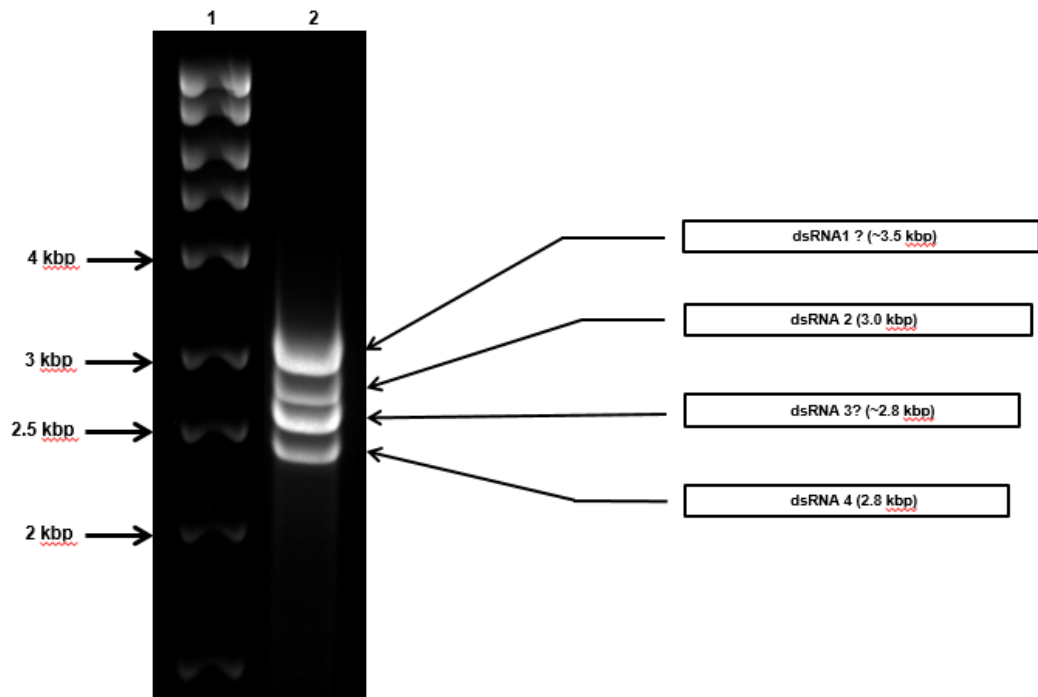


Figure 3.6 (a) Agarose gel electrophoresis of *A. foetidus*-slow viruses showing their RNA profile with their approximate sizes (lane 2). 1 kb Plus DNA ladder was used as a marker (lane 1) (b) Result of northern blot hybridization for AfV-S RNA3. (c) Schematic representation of the minimum free energy structure of the AfV-S3 dsRNA sequence. The  $\Delta G$  value was obtained using Mfold (Shah *et al.*, 2015).

(a)



(b)



**Figure 3.7 (a) Electrophoretic banding patterns of DsCV-1 dsRNA elements present in *D. septosporum* isolate D 752.1 (lane 2). Lane 1: Hyperladder 1. (b) Schematic representation of the genome organization of DsCV-1 dsRNA 2 and 3. The genome consists of four dsRNA segments but the work was focused on molecular characterisation of DsCV-1 dsRNAs 2 and 3. Sequencing of dsRNA 3 has not been completed. The rectangular box represents the open reading frames (ORF); ORF2 encodes the capsid protein (CP), and ORF3 encodes a hypothetical protein.**



function (Fig. 3.7b). BLAST searches of the amino acid sequence deduced from dsRNA 3 showed identity with a hypothetical protein encoded by the IjCV-1 dsRNA 3 (36% identity, 53% similarity; Appendix 4, Fig. 4.2). DsRNA 4 is 2816 bp in size and contains an ORF that encodes an 846 aa protease. BLAST searches of the amino acid sequence deduced from dsRNA 4 showed a high degree of identity to putative proteases from other members of the *Chrysoviridae* family (the highest identity was to Grapevine chrysovirus putative protease; 62% identity; Daudu, unpublished data).

The sequences of the 5'-UTRs of the four DsCV-1 dsRNAs are relatively long. They were 144 and 160 nt long for dsRNA 2 and dsRNA 4, respectively. The sequences of the 5'-UTRs of dsRNA 1 and dsRNA 3 are as yet unknown. The 3' UTRs were 109, 107, 102 and 117 nt long for dsRNAs 1, 2, 3 and 4 respectively.

The molecular characterisation of these four segments so far has shown the reiteration of the CAA triplet sequences upstream of the AUG initiator codon in the 5' UTRs of dsRNAs. The 5' sequences of dsRNAs 1 and 3 are incomplete. The (CAA) (n) repeats are translational enhancers which were also observed at the 5' UTRs of PcV, Hv145SV and tobamoviruses and identified as a characteristic feature of the 5' UTRs of chrysovirus dsRNAs (Jiang & Ghabrial, 2004; Gallie & Walbot, 1992; Ghabrial & Castón, 2004). In addition to this, the 3'-terminal UTR contained a conserved stretch of nucleotide sequence AATGAGTTATTTT which is conserved among the four segments of DsCV-1.

#### **3.3.4 Characterisation of *L. biglobosa* mycovirus-1 (LbMV-1)**

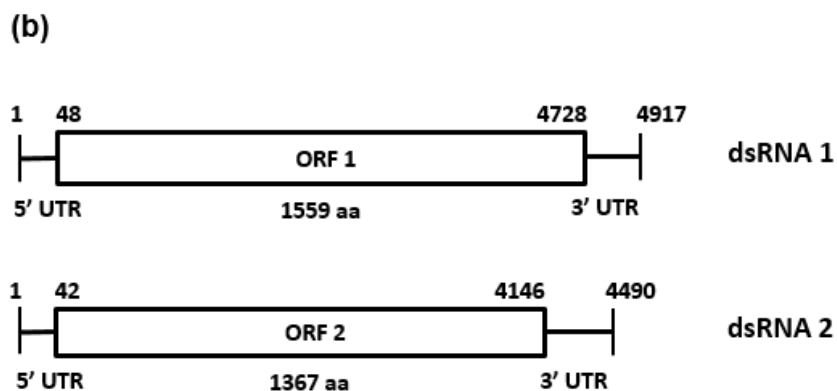
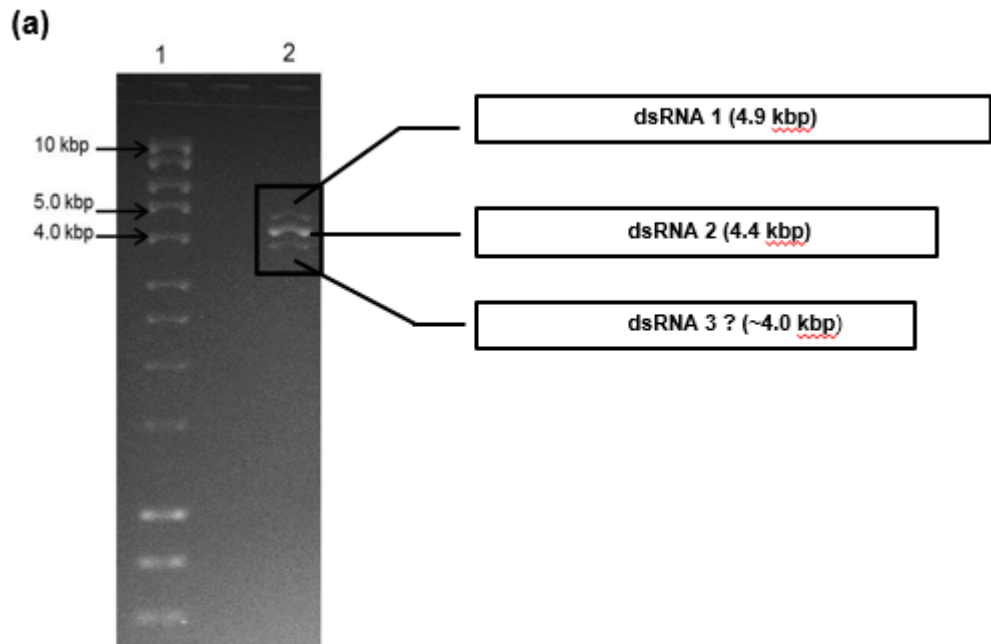
Initially, the analysis of the original dsRNA preparations on ethidium bromide stained agarose gels showed only one broad band. To check whether this apparently single dsRNA band from isolate C-Rox 12.8.1 was composed of several different segments of similar sizes, electrophoresis was performed on larger gels at 20 volts for 18 h. Examination of the stained gels revealed clear separation of at least three dsRNA elements, the size and profile of which is very similar to the members of the *Totiviridae* family (Fig. 3.8a). However, it is possible that the LbMV-1 virus genome might comprise four dsRNA elements rather than three elements as two dsRNA elements might co-migrate since dsRNA 2 appeared as a broad, dense band on stained gels. Similar results were described when *Penicillium chrysogenum* virus (PcV) was

sequenced. Initially PcV was also suspected to contain three components (Buck & Girvan, 1977; Wood & Bozarth, 1972) but cDNA cloning and sequence analysis demonstrated the presence of four distinct dsRNA components (Jiang & Ghabrial, 2004).

The three dsRNA elements were nominated L. biglobosa virus (LbMV-1) dsRNA 1, dsRNA 2 and dsRNA 3, respectively according to decreasing size (Fig. 3.8a). RT-PCR amplification using Froussard's and other random priming procedures were attempted for dsRNA 3 but no amplicons were generated in any of these experiments as the sequencing data confirmed that the clones obtained were the result of random priming on fungal DNA/RNA. However, successful amplicons were generated for dsRNA 1 and 2 using Froussard's procedure. A range of primers (Table 3.1) were designed from the known sequences of dsRNAs 1 and 2 to extend the sequences further using genome walking, single-primer amplification and RLM-RACE RT-PCR. Issues of obtaining the right clones of viral nucleic acid are discussed in Appendix 3. The complete sequences of dsRNAs 1 and 2 were respectively 4917, 4490 bp in length (Appendix 5). The sequence of dsRNA 3 is ca. 4000 bp. LbMV-1 dsRNAs 1 and 2 each contains a single ORF potentially encoding proteins of 1559 and 1367 amino acids with molecular masses of approximately 171 and 152 kDa, respectively (Fig. 3.8b).

BLAST searches of the deduced amino acid sequence of LbMV-1 dsRNA 1 ORF revealed that it has similarity to the hypothetical protein of unknown function encoded by Amasya cherry disease-large (ACD-L) dsRNA 1 (E-value 8e-86; 27% identity and 45% sequence similarity). BLAST searches of the deduced amino acid sequence of LbMV-1 dsRNA 2 ORF showed similarity to the RdRP encoded by ACD-L dsRNA 3 (E-value 0.0; 45% identity and 62% sequence similarity). High similarities of LbMV-1 dsRNA 2 were also found to the RdRP of ACD-L dsRNA 4 and members of the family *Totiviridae*, including the partially completed nucleotide sequence of Cherry chlorotic rusty spot-large (CCRS-L) associated totiviral-like dsRNA 4 and *Rosellinia necatrix quadrivirus* 1, a member of the family *Quadriviridae*.

It has been reported that the CCRS-associated L dsRNAs 3 and 4 are variants of ACD-L dsRNAs 3 and 4 as their partial sequences were 98% and 97% identical to the ACD-associated L dsRNAs, respectively.



**Figure 3.8 (a) Electrophoretic banding patterns of LbMV-1 dsRNA elements present in the *L. biglobosa* C-Rox 12.8.1 isolate.** Lane 1: Hyperladder 1 and lane 2: LbMV-1 dsRNAs were extracted from purified virus. (b) Schematic representation of the genome organization of LbMV-1 dsRNAs 1 and 2. The genome consists of three dsRNA segments but attempts to clone and sequence dsRNA 3 failed. Rectangular boxes represent the ORFs; ORF1 encodes a hypothetical protein and ORF2 encodes an RNA-dependent RNA polymerase (RdRP).

Examination of the deduced amino acid sequence of the LbMV-1 dsRNA 2 ORF demonstrated the presence of conserved motifs (Fig. 3.9) representative of RdRPs of dsRNA viruses of lower eukaryotes (Bruenn, 1993). Multiple alignments showed that motifs were also present in the three genera of the *Totiviridae* family: *Totivirus*: ACD-L 3, ACD-L 4, RnQV-1 (*Rosellinia necatrix* quadrivirus 1), HvV190S (*Helminthosporium victoriae* virus 190S), ScV-L1 (*S. cerevisiae* virus L-A), UmV-H1 (*U. maydis* virus H1); genus *Leishmaniavirus*: LRV1-1 (*Leishmania* RNA virus 1-1); genus *Giardiavirus*: TVV (*Trichomonas vaginalis* virus) and GLV (*Giardia lamblia* virus).

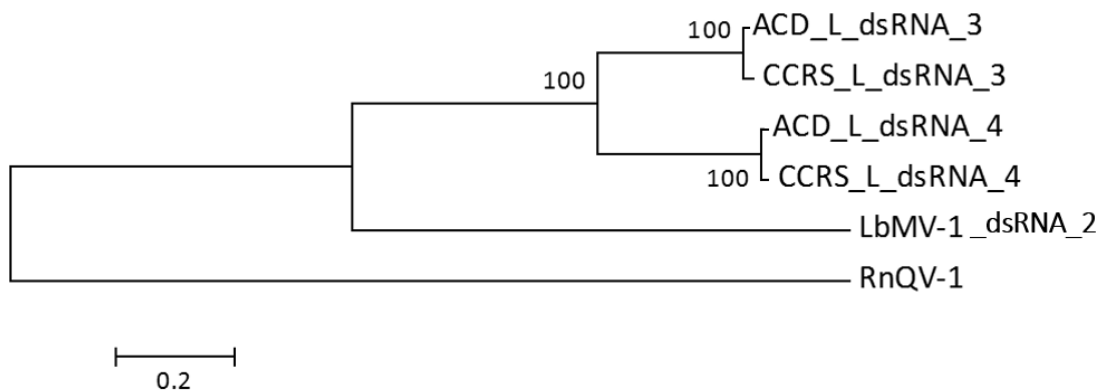
A phylogenetic tree indicates relationships among representative mycoviruses, based on the sequences of their RdRPs (Fig. 3.10). The high bootstrap values associated with these relationships support classification of LbMV-1 as a new putative member of the family *Totiviridae*.

The lengths of the 5'-UTRs flanking the single ORFs of LbMV-1 dsRNAs 1 and 2 were 48 and 42 nt, respectively. The lengths of the 3' UTRs of the same molecules were 189 and 344 nt, respectively. The 3'-UTRs are relatively long. The 5'- and 3'-UTRs of the two dsRNAs contained some sequence similarities in their internal regions. Specifically, this was the case with the 3'-UTRs, which contained a highly conserved stretch of nucleotides. (Fig. 3.11).

Viral UTRs contain the signals for RNA replication, transcription and packaging of virions. The 5' and 3' UTR sequences of both LbMV-1 dsRNAs 1 and 2 were examined for their potential secondary structures. Secondary structures were predicted using the Mfold program (Zuker, 2003). The 5' UTR regions are strongly conserved as compared to the 3' UTR regions at the nucleotide sequence level; however, both sets of termini contain distinct regions of nucleotide sequence identity. After the sequence alignment the similarity of both the sequences is reflected as the predicted secondary structures. 5' and 3' UTRs of both the dsRNAs contain numerous stem loop structures characteristic of mycovirus genomes (Fig. 3.12).

|           | 1          | 2              | 3            | 4               | 5                           | 6             | 7          | 8     |
|-----------|------------|----------------|--------------|-----------------|-----------------------------|---------------|------------|-------|
| LbMV-1    | LDRRD [52] | LTPSGSAFT [61] | SKVRALF [56] | DGKNFNIFHR [84] | GMFSGIRYTMFYNTIIFNRVYY [14] | HSGDDVFA [37] | EFLRI [12] | RSAAT |
| ACD-L-3   | LNHRA [67] | ATPAGSAFT [55] | SKLRALF [52] | DARNFNILHT [78] | GMFSGTRFTMLYNTIILNRAYY [14] | HSGDDVYA [33] | EFLRI [12] | RSAAT |
| ACD-L-4   | LNHRA [67] | TPAGSAFT [55]  | SKLRALF [52] | DAKNFNILHT [78] | GMFSGTRFTMLYNTIILNRAYY [14] | HSGDDVYS [33] | EFLRI [12] | RSAAT |
| RnQV-1    | RDRRD [49] | ETPAGSASS [55] | GKMRALF [52] | DAANFNILHT [73] | GLFSGHRLTMFINTVLRVYY [14]   | HSGDDVFA [33] | EFLRI [12] | RSCAT |
| HvV-190S  | LQGRY [61] | WCVNGSQNA [46] | GKDRALF [55] | DYDNFNSQHS [45] | TLMSGHRATTFINSVLNAYY [15]   | HAGDDVYL [34] | EFLRL [12] | RAIAS |
| LRV1-1    | LLGRG [59] | WAANGS.HS [49] | GKTRLLL [57] | DYDDFNSQHT [46] | TLMSGHRATTFINSVLNRAYI [15]  | HVGDDILM [33] | EFLRV [12] | RVISS |
| TVV-1     | LLGRG [58] | WSKSGS.HY [45] | GKERFIY [57] | DYDDFNSQHT [43] | TLMSGHRATTFINTVLRWCYT [15]  | CAGDDVIL [33] | EFLRK [12] | RAIAS |
| ScV-L-A   | LMNRG [57] | WVPGGSVHS [50] | GKQRAIY [52] | DYDDFNSQHS [52] | TLLSGWRLLTTRMNTVLRWAYM [15] | HNGDDVMI [33] | EFLRV [16] | RSCAT |
| UmV-H1    | LYGRG [66] | WLVSGSSAG [61] | GKARAIY [55] | DYPDFNSMHT [63] | GLYSGDRGTTLINTLLNIAYA [21]  | H.GDDIIT [34] | EYLRI [13] | RCVAT |
| GLV       | LLGKV [65] | WGTTGSGYI [41] | TKVRAVI [55] | DQSNFDRQPD [59] | GLPSGKWKTALLFALINVTQL [17]  | VQGGDIAL [33] | EFLRR [12] | RMMIK |
|           | * :        | **             | * * :        | * :*:           | ** : * : .::*               | ***:          | **:**      | *     |
| CONSENSUS | L..+.      | ....GS...      | .K.R&&&      | D..BFB....      | ...SG.+T.&..#&&N...&        | ..GDD&&.      | E&LR&      | R.&.# |

**Figure 3.9 Alignment of the region containing conserved motifs in RdRP of LbMV-1 dsRNA 2 with the RdRPs of some dsRNA viruses.** The numbers of amino acid residues separating individual motifs are indicated for each sequence. The numbers at the top refer to the numbers of eight conserved motifs (Bruenn, 1993). In the RdRP consensus line, symbol ‘#’ signifies S or T, the symbol ‘&’ signifies hydrophobic residues (I, L, V, M, F, Y, W, C, A), the symbol ‘+’ signifies positively charged residues and ‘B’ signifies asparagine or aspartic acid. In the sequence alignment, asterisks signify identical amino acid residues, colons signify highly conserved residues and single dots signify less conserved but related residues. Abbreviations and accession numbers of the viruses are shown in brackets: *Leptosphaeria biglobosa* virus, LbMV-1; *Amasya cherry disease large dsRNA 3*, ACD-L 3 (AM085134); *Amasya cherry disease large dsRNA 4*, ACD-L 4 (AM085135); *Rosellinia necatrix* quadrivirus 1, RnQV-1 (YP\_005097975); *Helminthosporium victoriae* virus 190S, HvV190S (U41345); *Leishmania* RNA virus 1-1, LRV1-1 (M92355); *Trichomonas vaginalis* virus, TVV (U57898); *Saccharomyces cerevisiae* virus L-A, ScV-L1 (J04692); *Ustilago maydis* virus H1, UmV-H1 (U01059) and *Giardia lamblia* virus, GLV (L13218).



**Figure 3.10 Phylogenetic analysis of LbMV-1 dsRNA 2.** Putative members of the same virus family (RdRP\_4, PF02123; Finn *et al.*, 2014) include the Amasya cherry disease-associated L dsRNAs 3 (CAJ29958) and L 4 (CAJ29959), together with the cherry chlorotic rusty spot associated totiviral-like dsRNAs L 3 (CAJ57273) and L 4 (CAJ57274). Rosellinia necatrix quadrivirus 1 (YP\_005097975), the most closely related virus (PSI-BLAST; Altschul *et al.*, 1997), was used as an outgroup. The RdRP sequences of the viruses were aligned with MUSCLE as implemented by MEGA 6 (Tamura *et al.*, 2013); the alignment was improved manually and all positions with <30% site coverage were eliminated. Maximum likelihood phylogenetic trees were constructed with MEGA 6 using the LG+G+I substitution model.

**5' UTR**

```

dsRNA1      UACGAAAAACACAAUAUCACCUACAGUACCACGCCGACCAGAGACAGC
dsRNA2      CACGAUUUACUGACAUAUACCAAUAGUACAUCACACAUUUGA-----
          ****      ** *      *** * ***** * * *      **

```

**3' UTR**

```

dsRNA1      UUAC-----
dsRNA2      CGAUACGACUGCUGAUUGCGAAUACGGGGUCAUGUCGAAGAGGCAAGGCCGAGUUGGCAA
          *

```

```

dsRNA1      -----ACACACCCGCAACAUGC-----
dsRNA2      UCAGCUUGCGUAGUGACAAACUCGGCAGGUGCACUGCGCAGACAAUAACGCAGGCUCAAC
          *** ** ** * .***

```

```

dsRNA1      -----AGCACGCUCACUAU
dsRNA2      GGAGCGGAGGUAGUACGAUAAAAACAAAAACAAAAUUUAGACAAUAAUUCAAUUUAAU
          * . * . * * **

```

```

dsRNA1      AACCGUGCGCAAACUAAUGCCGUAAACCAAGGGAAGGUUGCGGCAGAACGACAUUCGCAGG
dsRNA2      AAAAACAUUUAAA--AAUGCUGGAGACUAGGGCAGUCAAAGGCAGAACGACAUUCGCAGG
** . . *** ***** * * * ***** * . *****

```

```

dsRNA1      CCGUUGGCACGCCUGUAGUCUACAAAUCACUGCCAGUCCAUCUAUGAUGGUCCCCCAUG
dsRNA2      CACUUGACACGCCUGUAGUCUACAAUACGUCAGGCUGCUUC---GCAGUCCCCCAUG
* ***.***** ** * * * .*****

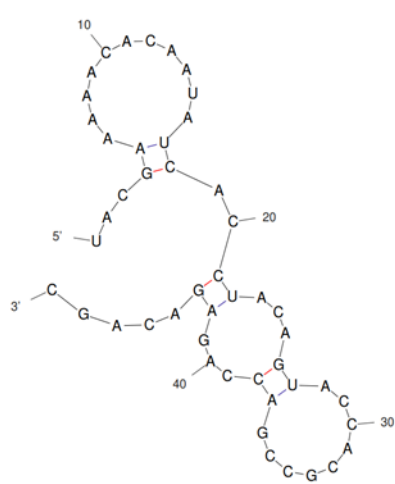
```

```

dsRNA1      GCUCCCAAGUGGUGGAACCCGAAAGGGUGAGAAC-----
dsRNA2      GCGCCAAGUGGUGGAGCUUUAUUAGCGAGAACGAGACUGUAUAUGCG
** ***** * ** * *****

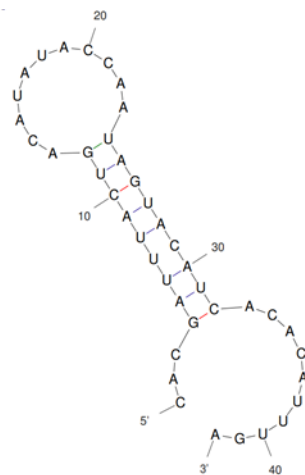
```

**Figure 3.11 Comparison of the 5'- and 3'- untranslated terminal regions of the coding strands of LbMV-1 dsRNAs 1 and 2.** Asterisks designate identical nucleotides while single dots specify less conserved, but related nucleotides.



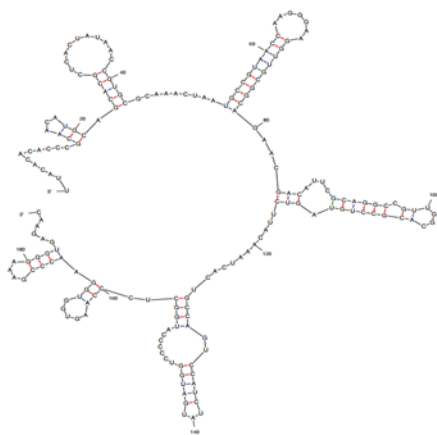
$\Delta G = -2.25$  kcal/mol

DsRNA 1 5' UTR



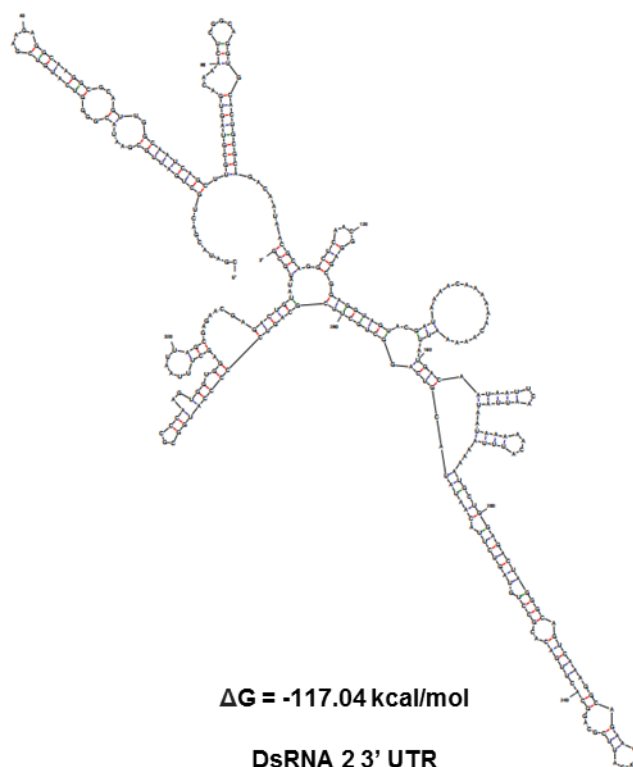
$\Delta G = -7.67$  kcal/mol

DsRNA 2 5' UTR



$\Delta G = -74.09$  kcal/mol

DsRNA 1 3' UTR



$\Delta G = -117.04$  kcal/mol

DsRNA 2 3' UTR

**Figure 3.12 Predicted folding of 5' and 3' -UTR regions of LbMV-1 dsRNA 1 and dsRNA 2.** The  $\Delta G$  values were calculated using the Mfold program.



### 3.3.5 Northern hybridization analyses

Purified viral LbMV-1 dsRNAs were fractionated by electrophoresis in a 0.8% agarose gel prepared in 1x TAE (Fig. 3.13a) for 18 h, denatured, neutralized, blotted onto a nylon membrane, and probed with clones specific for LbMV-1 dsRNAs 1 and 2. Hybridization of blotted dsRNAs with sequence-specific probes produced strong signals for both LbMV-1 dsRNAs 1 and 2, which confirmed that the sequences obtained corresponded to the two LbMV-1 dsRNA elements and that their classification according to their size and sequence was correct (Fig. 3.13b).

### 3.3.6 Identification of dsRNA elements present in virus-infected *Leptosphaeria biglobosa* isolates

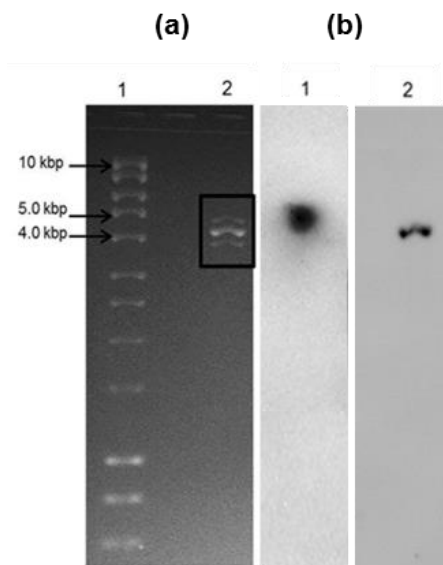
*L. biglobosa* sequence-specific amplification of internal fragment using total RNA template showed that all infected isolates harbour a single identical LbMV-1 dsRNA 2 element (Figs. 3.14, 4.2g). In addition to this, the clone fragments of seven UK *L. biglobosa* isolates were sequenced and found to be identical.

## 3.4 Discussion

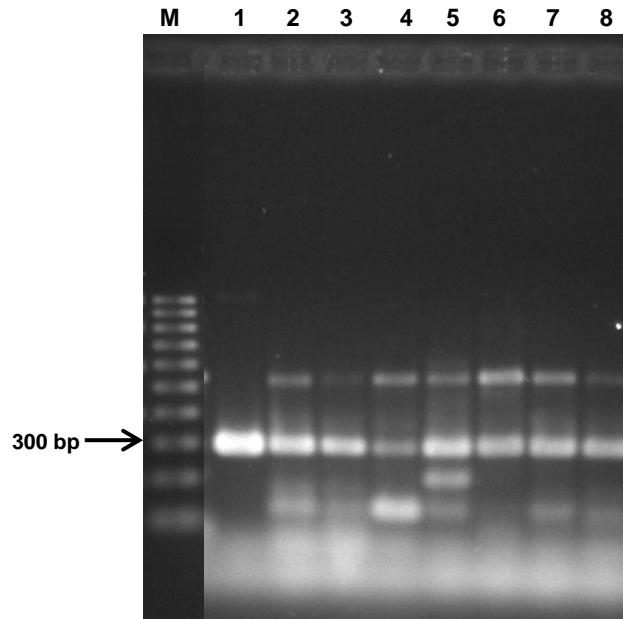
This study is the first to report the presence of dsRNA elements in isolates of *L. biglobosa* (Table 3.2). Fifty-seven *L. maculans* and sixteen *L. biglobosa* isolates were screened for the presence of dsRNA and 11/16 (incidence of 68.7%) *L. biglobosa* isolates contained dsRNAs. None of the 57 *L. maculans*, 162 *H. fraxineus* and 10 *Pyrenopeziza brassicae* isolates investigated harboured dsRNA elements.

Alignment of the nucleotide sequence of AfV-S3 with the AfV-S2 revealed that the first seven nucleotides at the AfV-S3 5'-terminus (5'-GGGATT-3') are identical to those of AfV-S2, suggesting that AfV-S3 might use the RdRP encoded by AfV-S2 for replication. It is also proposed that AfV-S3 might be a satellite component of AfV-S virus particle, as it depends on AfV-S2 for its replication and on AfV-S1 for its encapsidation (Shah *et al.*, 2015).

In the other study detailed in this chapter only one of 45 *D. septosporum* isolates examined was found to contain dsRNA elements. *D. septosporum* isolate D 752.1



**Figure 3.13 (a) Agarose gel electrophoresis of LbMV-1 viruses from *L. biglobosa* isolate C-Rox 12.8.1 (lane 2). (b) Northern blot hybridization of dsRNAs 1 and 2 using DIG-labelled probes derived from their cloned sequences. Lanes 1 and 2 show individual transfers hybridized with probes specific for dsRNAs 1 and 2, respectively.**



**Figure 3.14** Agarose gel electrophoresis of amplicons (~317 bp) generated by RT-PCR amplification using RdRP-specific primers to identify virus-infected *L. biglobosa* isolates. M- 100 bp ladder, 1- positive control (C-Rox 12.8.1), 2- K-Exc 12.10.21, 3- D-Rox 12.10, 4- H-Exc 12.12.3, 5- F2-Exc 12.6.1, 6- F2-Exc 12.3.1, 7- W10, 8- H-Exc 12.10.3.

harboured four dsRNA segments ranging in size from ca. 2.8-3.5 kbp (Fig. 3.7a). This work was focused on cloning and sequencing two of the four components of the genome, dsRNA 2 and dsRNA 3.

The larger segment, DsCV-1 dsRNA 1, encodes an RdRP which possesses conserved motifs, like other members of *Chrysoviridae* family and shows high identity to IjCV-1 RdRP (Daudu, unpublished data). DsCV-1 dsRNA 2 encodes a CP, which showed high identity to IjCV-1 CP. DsCV-1 dsRNA 3 encodes a hypothetical protein of unknown function with highest identity with IjCV-1 HP. DsCV-1 dsRNA 4 encodes a putative protease which is similar to Grapevine chrysovirus putative protease (Daudu, unpublished data).

The genome of DsCV-1 is comprised of four genome segments, similar to most other chrysoviruses described previously (Jiang & Ghabrial, 2004; Campo *et al.*, 2016; Covelli *et al.*, 2004). However, some non-quadrupartite chrysoviruses have also been described in fungi and plants. For example, *Fusarium graminearum* mycovirus-China 9 and *Magnaporthe oryzae* chrysoviruses 1-A and -B have genomes of five dsRNA segments, infecting *Fusarium graminearum* and rice blast fungus *Magnaporthe oryzae*, respectively. Moreover, *Raphanus sativus* mycovirus, a trisegmented chrysovirus was isolated from the radish, *Raphanus sativus* (Li *et al.*, 2013). These reports indicate that the numbers of genomic segments of the chrysoviruses were not always consistent. Only dsRNA1 encoded an RdRP, indicating that the other three dsRNAs might depend on this RdRP for replication. These findings established that these four dsRNA segments represent the genome of a novel virus.

The sequence analysis of the four dsRNAs thus far showed that 5'- and 3'- UTRs contained conserved termini. The molecular characteristics of DsCV-1 four dsRNAs are closely related to those of the genus *Chrysovirus* and on the basis of their size, sequence and structural features of their 5' UTRs, which are demarcation criteria in the genus (Ghabrial & Castón, 2004), it was suggested that they are the genomic components of a new species of this genus.

The three dsRNA elements found in *L. biglobosa* are approximately 4-4.9 kbp in size (Fig. 3.8a). The ds nature of the LbMV-1 dsRNAs was confirmed following the observation that purified virus itself was sensitive to RNase III and insensitive to S1 nuclease (Fig. 3.5), which signifies the absence of a conventional capsid protein.

Similar results were recently reported for *Aspergillus fumigatus* tetramycovirus-1 (AfuTmV-1) which contains four dsRNA elements and is regarded as an unconventional virus in that it is not capsidated but rather coated with a virus encoded protein and might also be enveloped in colloidal cellular components (Kozlakidis *et al.*, 2009; Spear *et al.*, 2010; Kanhayuwa *et al.*, 2015).

The molecular features of LbMV-1 dsRNAs 1 and 2 parallel those of the genus *Totivirus*. The larger segment, dsRNA 1, encodes for a hypothetical protein, whereas dsRNA 2 encodes for an RdRP which possesses conserved motifs, like other members of *Totiviridae* family. On the basis of size, sequence and structural features of their UTRs, both can therefore be regarded tentatively as novel species of *Totiviridae* family. In contrast, genomic dsRNAs of most of the members of this family comprise two ORFs- the first, ORF A, encoding the CP and the second, ORF B, encoding the RdRP, those from LbMV-1 dsRNA 2 are predicted to produce only a single protein, RdRP. Similarly, ACD-associated L dsRNAs 3 and 4 were also reported to contain only one ORF. In the genus *Totivirus*, UmV-H1, phylogenetically related to ACD-L dsRNAs 3 and 4, encode a polyprotein that is processed by a viral papain-like protease to generate RdRP and CP. Whilst a similar expression strategy was not identified in LbMV-1 dsRNA 2, BLASTP analysis of the amino acid sequences of LbMV-1 dsRNA 2 did not reveal any significant similarity with CPs of other totiviruses. Therefore, N-terminal region of the protein predicted from the sequence of LbMV-1 dsRNA 2 encodes CP is thus far unknown. Likewise, Kozlakidis *et al.* (2006) also reported the absence of CPs in ACD- associated totivirus.

It must be noted that the nature of the LbMV-1 dsRNA 3 is not known yet and it might represent a replicative intermediate of a ssRNA virus(es). In support of the second view, the size pattern of three dsRNAs is consistent with the previously reported ACD-associated dsRNAs (Kozlakidis *et al.*, 2006). Thus, it is more likely to encode another hypothetical protein or RdRP, as found in ACD-associated L dsRNAs. There is a significant identity in the 5'- and the 3'-UTRs of dsRNAs 1 and 2, and LbMV-1 dsRNA 1 does not apparently encode RdRP. Therefore, it may be replicated by the RdRP of LbMV-1 dsRNA 2, as previously advanced for other totiviruses (Kozlakidis *et al.*, 2006). Fungal infections with Totiviruses not only comprise genomic dsRNAs but may be accompanied by additional dsRNA elements known as satellite dsRNAs (Buck, 1988; Hillman *et al.*, 2000), which might be an explanation for the existence of dsRNA

3 in LbMV-1. However, without further molecular characterisation of LbMV-1 dsRNA 3, further speculation is difficult and this aspect will be the subject of further investigation.

Phylogenetic analysis illustrates that within the clusters, the RdRP gene of LbMV-1 is closely related to ACD-L 3 and distantly related to RnQV-1 (Fig. 3.10). The UTRs (5'- and 3'-) of both dsRNAs significantly vary in length. In spite of these variations, the UTRs comprise some conserved regions which indicate that the terminal sequences are correct.

The identities of the LbMV-1 dsRNAs 1 and 2 were established by northern blot hybridization, using purified preparations of the three dsRNAs as targets and cDNA clones of internal regions as probes (Fig. 3.13).

Since several isolates containing similar dsRNA elements were found in clusters regardless of different geographical locations, it is likely that some fungal isolates are clonal which was investigated by RT-PCR (Fig. 3.14) using sequence-specific primers and cloning of the amplicons.

### **3.5 Conclusions and future work**

The current study has revealed the presence of mycoviruses in the plant pathogenic fungi *L. biglobosa* and *D. septosporum*. Moreover, the complete nucleotide sequences of LbMV-1 dsRNAs 1 and 2, DsCV-1 dsRNAs 2 and 3 (dsRNA 3 partially complete), AfV-S3 found in *A. foetidus* were also reported. Characterisation of LbMV-1 revealed the presence of three dsRNA elements, which are closely related to ACD-associated L dsRNAs, while characterisation of DsCV-1 revealed the presence of four dsRNA elements which are closely related to IjCV-1.

It would be worthwhile to characterise the remaining dsRNA element/s of LbMV-1 and DsCV-1, so that their relationship can be studied in relation to other known viruses. It is also important to perform biological experiments to examine the effects of DsCV-1 infection on pathogenicity (if any) on pine by generating isogenic virus-free lines of *D. septosporum* isolate D752.1.

## Chapter 4

**Eradication of LbMV-1 from *Leptosphaeria biglobosa* and phenotypic comparison of virus-infected and virus-free *L. biglobosa* isolates**

## 4.1 Introduction

The biological consequences of mycoviral infection are poorly understood since most infections are cryptic or latent and do not cause any obvious phenotypic changes in the fungal host. It is difficult to determine the effects of these elements on fungal physiology and pathogenicity (Punja, 1995). However, there is evidence that mycoviruses can induce considerable morphological and physiological changes, including virulence and debilitation associated phenotypes (Castro *et al.*, 2003; Dawe & Nuss, 2001). This varies between isolates of the same fungus with different genetic backgrounds. Consequently, it is necessary to have identical genetic backgrounds for studies of fungus-mycovirus interactions and it is desirable to establish virus-infected and virus-free isogenic lines to explore these interactions. However, producing isogenic lines can be problematic. Since virus infection may be an unstable trait and the fungus may be recalcitrant to eradication of virus, these investigations are challenging (Aoki *et al.*, 2009; Carroll & Wickner, 1995; Herrero & Zabalgogea, 2011; Romo *et al.*, 2007; Souza *et al.*, 2000). Another problem concerns transfection of fungi with purified mycoviruses or their RNA, which is yet to be optimised for most mycovirus/fungus combinations, particularly as this is the most direct and simple way of studying horizontal virus transmission. Furthermore, virus transmission through hyphal anastomosis can be accompanied by the inheritance of a number of cytoplasmic factors, making it difficult to define whether any phenotypic alternations are caused by the mycovirus, other inherited cytological factors or both (Nuss, 2005). For these reasons, generating isogenic fungal lines that are virus-infected and virus-free is not an easy task. However, it can be aided by treatment with chemicals which interfere with transcription and translation.

Fink and Styles (1972) first reported the use of cycloheximide to eliminate mycovirus infection from yeast and also postulated that cycloheximide hindered cytoplasmic protein synthesis in eukaryotes, which is essential for replication of genetic elements. Cycloheximide has been successfully used to eradicate mycovirus infection (completely or partially) from different fungi and is the most consistent and commonly used method (Dalzoto *et al.*, 2006; Elias & Cotty, 1996; Robinson & Deacon, 2002; Yamada *et al.*, 1991). Sometimes cycloheximide treatment is accompanied by hyphal tip isolation to assist virus eradication (Aoki *et al.*, 2009; Marzano *et al.*, 2015).



#### **4.1.1 Aim and objectives**

The main of this chapter was the eradication of LbMV-1 infection from *Leptosphaeria biglobosa* to construct the isogenic lines of virus-infected and virus-free isolates and to assess the effects of virus infection on the fitness of the fungal host.

1. To eradicate LbMV-1 infection from *L. biglobosa* isolates using cycloheximide.
2. To investigate effects of virus infection on colony morphology, mycelial growth and biomass production of *L. biglobosa*.

#### **4.2 Materials and methods**

##### **4.2.1 Cycloheximide treatment for eradication of LbMV-1 infection from *L. biglobosa* isolates**

Attempts were made to eradicate LbMV-1 infection from *L. biglobosa* isolates C-Rox 12.8.1, K-Exc 12.10.21, Noth2, N20.3, W10 and N3.4 using cycloheximide treatment. Eradication with cycloheximide used a range of concentrations (0.01 mM to 150 mM; Table 4.1) of the drug in solid V8 media inoculated initially with *L. biglobosa* isolates C-Rox 12.8.1 and K-Exc 12.10.21. Cycloheximide solutions were filter-sterilised and incorporated into media at ~50°C. Inoculated plates were incubated at 20°C until confluent mycelial growth was obtained. In order to determine any effects of cycloheximide treatment on the eradication of mycoviral dsRNAs, total RNA extracts were isolated from the fungal mycelia using the RNeasy Plant Mini Kit (Qiagen). RT-PCR assays were then performed (Section 2.6.2), using sequence-specific oligonucleotide primers (Table 3.1) designed to generate amplicons representing a fragment of the coding region of the RdRP gene discovered on one of the dsRNA elements found in *L. biglobosa*.

##### **4.2.2 Colony morphology of isogenic lines of *L. biglobosa***

To compare the growth morphologies of virus-infected and virus-free *L. biglobosa* isolate W10, equal numbers of spores ( $n=10^5$ , 10 µl conidial suspension containing  $10^7$  spores/ml) were inoculated on the centre of the PDA plates and incubated at 20°C.

**Table 4.1 Dilution series and preparation of different cycloheximide concentrations for eradicating mycovirus infection from *L. biglobosa*.**

| Desired concentration (mM) | Amount of cycloheximide in 50 ml of V8A (mg) |
|----------------------------|--|
| 0.01                       | 0.14   |
| 0.1                        | 1.40   |
| 1                          | 14.06  |
| 5                          | 70.33  |
| 10                         | 140.67                                       |
| 15                         | 211.01                                       |
| 50                         | 703.37                                       |
| 75                         | 1054.46                                      |
| 100                        | 1407.08                                      |
| 150                        | 2110.12                                      |

#### **4.2.3 Colony diameter of isogenic lines of *L. biglobosa***

To assess radial growth, equal numbers of spores ( $n=10^5$ , 10  $\mu$ l conidial suspension containing  $10^7$  spores/ml) of isogenic lines of virus-infected *L. biglobosa* W10 and virus-free W10 were centrally inoculated onto PDA Petri plates and incubated at 20°C. Time periods were determined in such a way that fungal growth had been initiated before the first measurement and the last measurement was taken before the mycelium reached the Petri dish edge. Growth rate, as defined by the rate of colony diameter extension, was measured over a period of 34 days for 3 replicate plates.

#### **4.2.4 Biomass production of isogenic lines of *L. biglobosa***

To determine biomass production, equal numbers of spores ( $n = 2 \times 10^6$ , 200  $\mu$ l conidial suspension containing  $10^7$  spores/ml) of the virus-infected *L. biglobosa* W10 and virus-free W10 were inoculated into 250-mL flasks containing 150 mL of PDB (Potato Dextrose Broth) and incubated at 20°C on a rotary shaker (130 rpm) for 13 days. The mycelium from individual cultures was harvested by centrifugation at 15,000 g for 20 min. The pellets were lyophilized for dry weight determinations. This experiment was performed in triplicate.

### **4.3 Results**

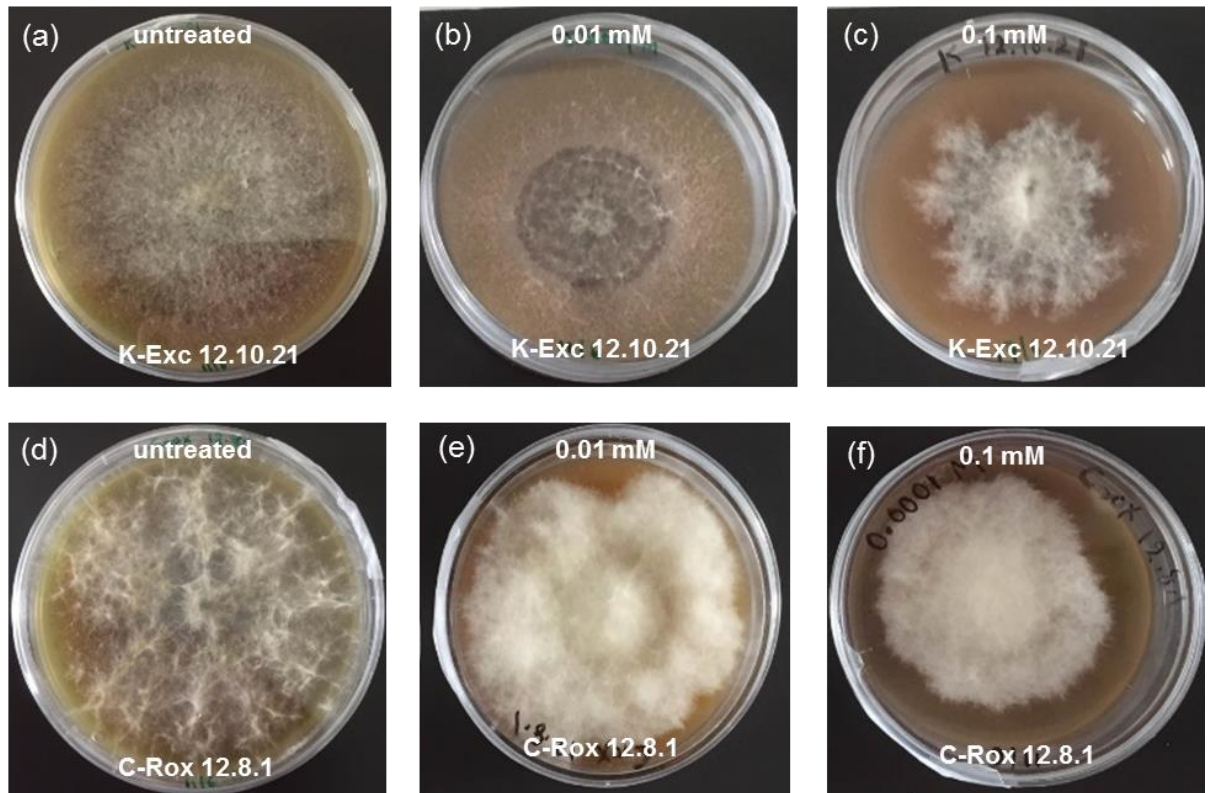
#### **4.3.1 Cycloheximide treatment for eradication of LbMV-1 infection from *L. biglobosa* isolates**

Several attempts were made to eradicate LbMV-1 dsRNA elements by cycloheximide treatment from *L. biglobosa* isolates C-Rox 12.8.1 and K-Exc 12.10.21. Attempts were also made to transfect virus-free isolates of *L. biglobosa* with purified virus but unfortunately optimal numbers of protoplasts of the virus-free isolate could not be produced for use in this procedure. In an effort to eliminate dsRNAs, V8A plates were amended with a limited range of cycloheximide concentrations (50 mM, 75 mM, 100 mM and 150 mM) and inoculated with the *L. biglobosa* isolates. All of these concentrations of cycloheximide completely inhibited fungal growth. Subsequently, experiments were done using lower concentrations of cycloheximide (5 mM-15 mM). However, similar results with no observable fungal growth were obtained, so the

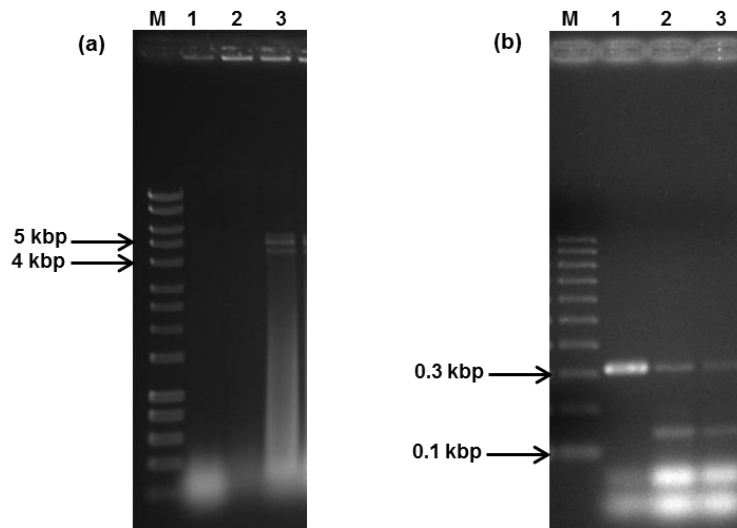
concentration of cycloheximide was decreased further. The media was thus amended with 0.01 mM and 0.1 mM cycloheximide and following inoculation with *L. biglobosa* and incubation at 20°C for 19 days and all isolates grew, albeit slowly.

Cultures treated with 0.01 mM cycloheximide did not show significant alterations in morphology or pigmentation (Fig. 4.1b, e). However, cultures treated with 0.1 mM cycloheximide demonstrated a reduced growth rate and reduced pigmentation (Fig. 4.1c, f). Mycelia from the margins of the cultures (C-Rox 12.8.1 and K-Exc 12.10.21) amended with 0.01 mM cycloheximide were inoculated onto V8 agar plates and incubated for 19 days prior to harvesting the mycelia for small-scale RNA extraction and agarose gel electrophoresis analysis. Comparisons of the electrophoretic profiles of dsRNA isolated between virus-infected *L. biglobosa* isolate C-Rox 12.8.1 (positive control) and the treated samples indicated that they were apparently virus-free (Fig. 4.2a). Therefore, virus-infected and putative virus-free isolates were subjected to RT-PCR amplification to check for infection with dsRNA. RT-PCR amplification of a fragment of the LbMV-1 RdRP gene revealed that curing was not successful (Fig. 4.2b) as the treatment only reduced dsRNA titre below the limits of resolution that could be detected by ethidium bromide staining of agarose gels.

Subsequently, a combined approach was devised by growing isolates on media amended with small concentrations of cycloheximide in order to reduce viral RNA concentrations and then conducting single conidium isolation. Thus spores of isolates K-Exc 12.10.21 and C-Rox 12.8.1 grown on agar amended with 0.01 mM cycloheximide were harvested and streaked on V8 agar (not amended with cycloheximide) to obtain single spore isolates. Six single spore colonies were selected randomly from each culture and a small-scale RNA extraction was performed to check the presence of dsRNA. Agarose gel electrophoresis analysis suggested that curing had been successful from K-Exc 12.10.21 (Fig. 4.2c). To confirm if these cultures (K-Exc 12.10.21 and C-Rox 12.8.1) were indeed virus-free, RT-PCR amplification was performed. An RdRP specific amplicon of 317 bp was generated from all single conidial isolates revealing that the sub-isolates were not cured of virus infection (Fig. 4.2d, e). From these data, it was anticipated that further treatment of the isolates by sub-culturing them again onto the medium containing cycloheximide might free them of dsRNA. In an attempt to achieve this, both the isolates were passaged a second time using 0.01 mM cycloheximide to 0.1 mM cycloheximide amended V8 agar media.



**Figure 4.1 Colony morphology of *L. biglobosa* isolates K-Exc 12.10.21 and C-Rox 12.8.1 grown on V8A amended with 0.01 mM and 0.1 mM concentrations of cycloheximide. *L. biglobosa* K-Exc 12.10.21 (a, b, c) and C-Rox 12.8.1 (d, e, f) grown at 20°C for 19 days on V8 agar amended with cycloheximide at 0.01 mM (b, e) and 0.1 mM (c, f) concentrations, along with untreated isolates (a, d) as positive controls.**



**Figure 4.2 Effect of 0.01 mM and 0.1 mM cycloheximide concentrations on LbMV-1 dsRNAs in different *L. biglobosa* isolates.**

(a) Small-scale extraction of RNA from *L. biglobosa* C-Rox 12.8.1 (lane 1) and *L. biglobosa* K-Exc 12.10.21 (lane 2) following treatment with 0.01 mM cycloheximide. Lane M contains Hyperladder 1. Lane 3; Nucleic acid extract of untreated isolate C-Rox 12.8.1- positive control.

(b) Agarose gel electrophoresis of RT-PCR amplicons generated from total nucleic acids extracted from isolates C-Rox 12.8.1 and K-Exc 12.10.21 (lanes 2 and 3 respectively). Lane M; 100 bp molecular weight marker. Lane 1; untreated positive control isolate C-Rox 12.8.1.

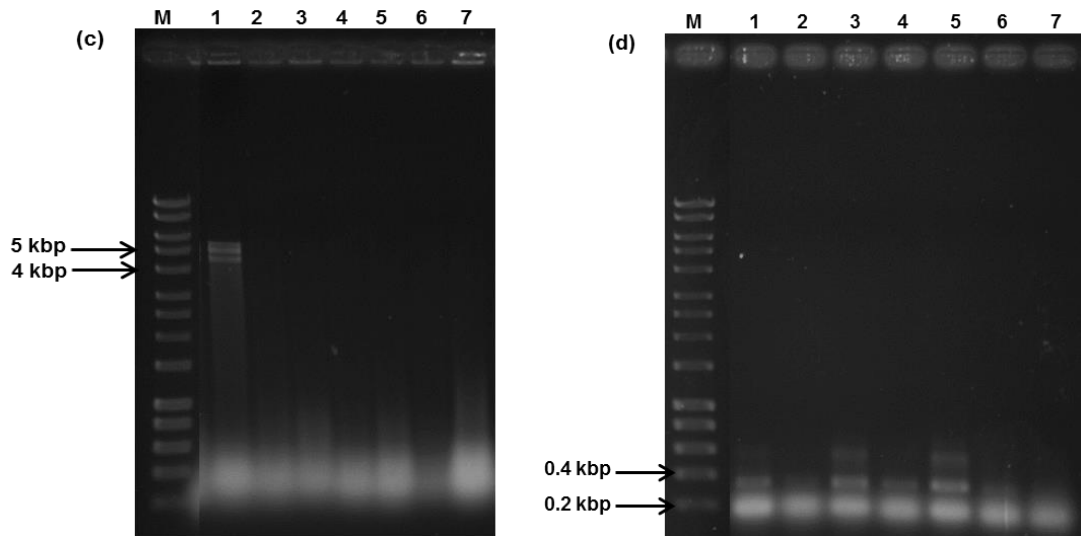


Figure 4.2 (c) Agarose gel electrophoresis of dsRNAs isolated after small-scale RNA extraction from control and sub-isolates of *L. biglobosa* K-Exc 12.10.21. Lane 1; untreated positive control isolate K-Exc 12.10.21. Lanes 2-7; six representative single colonies (sub-isolates K1-K6) of isolate *L. biglobosa* K-Exc 12.10.21 following treatment with 0.01 mM cycloheximide.

(d) Agarose gel electrophoresis of amplicons (~317 bp) generated by RT-PCR using RdRP-specific primers and total RNA template. Lane M; Hyperladder 1. Lane 1; untreated positive control isolate K-Exc 12.10.21. Lanes 2-7; six representative single colonies (sub-isolates K1-K6) of isolate *L. biglobosa* K-Exc 12.10.21 following treatment with 0.01 mM cycloheximide.

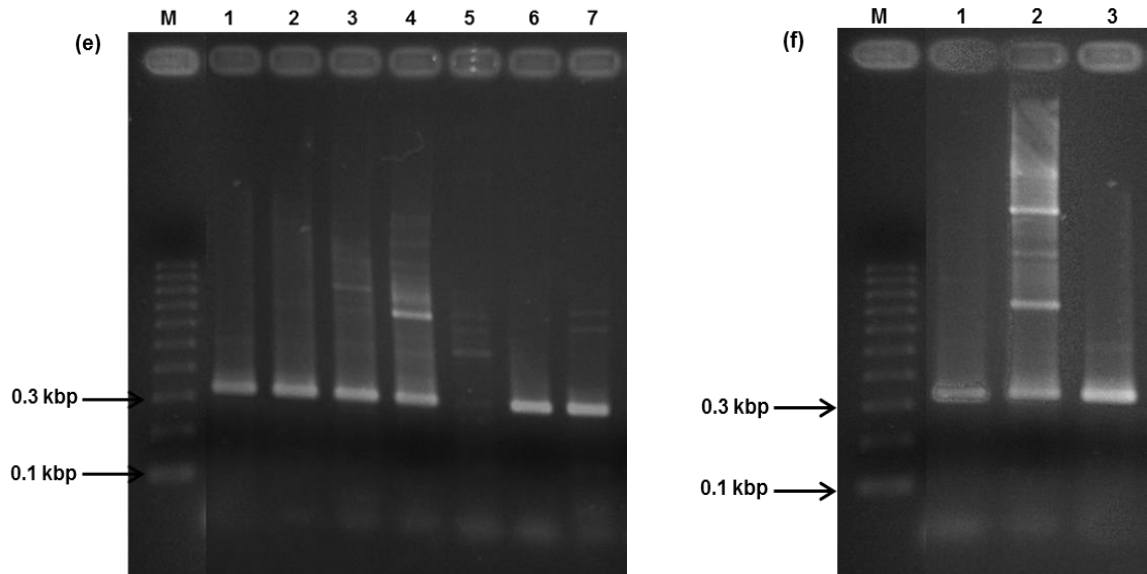


Figure 4.2 Agarose gel electrophoresis of amplicons (~317 bp) generated by RT-PCR using RdRP-specific primers and total RNA template.

(e) Lane M; 100 bp molecular weight marker. Lane 1; untreated positive control isolate *L. biglobosa* C-Rox 12.8.1. Lanes 2-7; six representative single colonies (sub-isolates C1-C6) of isolate *L. biglobosa* C-Rox 12.8.1 following treatment with 0.01 mM cycloheximide.

(f) Lane M; 100 bp molecular weight marker. Lane 1; untreated positive control isolate *L. biglobosa* C-Rox 12.8.1. Lanes 2 and 3; RNA extracts of C-Rox 12.8.1 and K-Exc 12.10.21, respectively which were sub-cultured from 0.01 mM to 0.1 mM cycloheximide amended V8 medium.



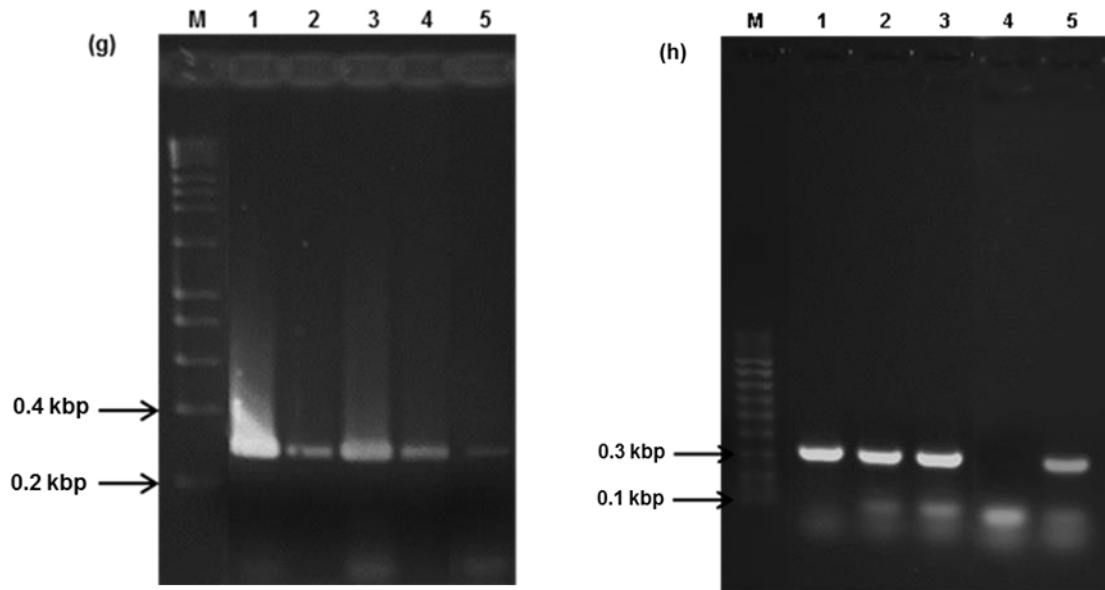


Figure 4.2 Agarose gel electrophoresis of amplicons (~317 bp) generated by RT-PCR using RdRP-specific primers and total RNA template.

(g) Lane M; Hyperladder 1. Lane 1; RNA extracts of virus-infected isolate C-Rox 12.8.1- positive control. Lanes 2-5, RNA extracts of untreated virus-infected Chinese isolates Noth2, N20.3, W10 and N3.4, respectively.

(h) Lane M; 100 bp molecular weight marker. Lane 1; untreated positive control isolate *L. biglobosa* C-Rox 12.8.1. Lanes 2-5, RNA extracts of 0.1 mM cycloheximide treated Chinese isolates Noth2, N20.3, W10 and N3.4, respectively.

Total RNA extracted from the second passage of K-Exc 12.10.21 and C-Rox 12.8.1 was used as a template for RT-PCR and generated sequence-specific amplicons confirming infection with LbMV-1 (Fig. 4.2f).

Finally, attempts were made to eradicate virus from four different virus-infected Chinese *L. biglobosa* isolates (Fig. 4.2g; Noth2, N20.3, W10 and N3.4) by growing them on V8A plates amended with 0.1 mM cycloheximide. Following RT-PCR amplification, LbMV-1 dsRNA 2 was absent from only one isolate, W10 (Fig. 4.2h) and this isolate was selected for additional testing with the aim of investigating the impact of virus infection on growth and virulence of the fungus.

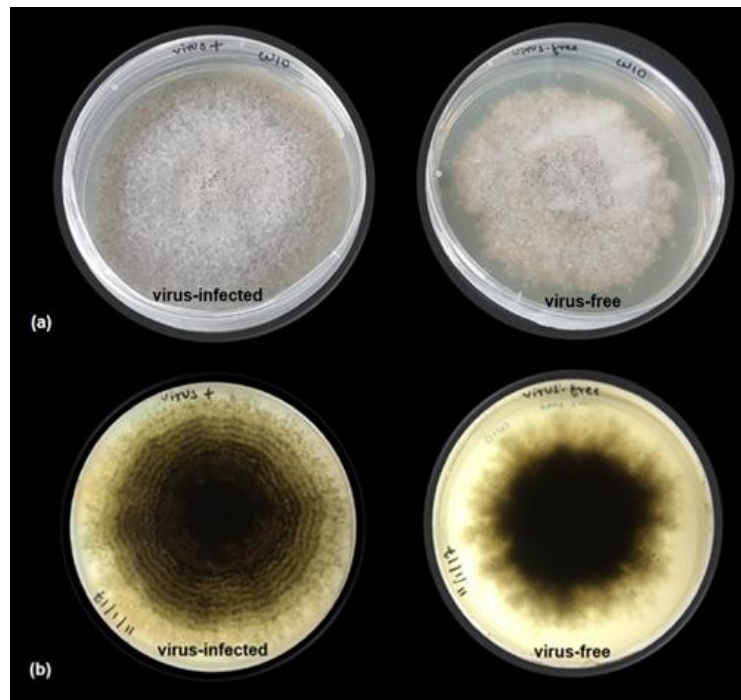
### **4.3.2 Effect of virus infection on colony morphology, mycelial growth rate and biomass production of *L. biglobosa***

#### **4.3.2.1 Colony morphology of isogenic lines of *L. biglobosa***

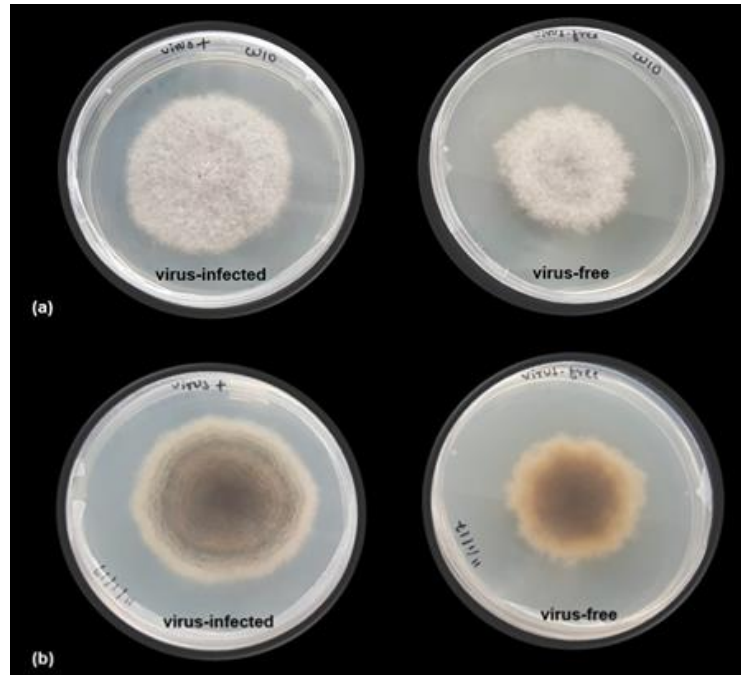
Visible differences were observed in colony morphology between virus-infected and virus-free strains after 26 days of incubation at 20°C. As compared to virus-infected cultures of the *L. biglobosa* W10 isolate, there were marked alterations in the phenotype associated with virus-free cultures (Fig. 4.3a, b). In the virus-infected strain, conidia formed a circular, distinct pattern of black pigmentation, which was even more evident when the plates were observed from the back. In contrast, the virus-free strain was pigmented with no distinct pattern of sporulation with an undulate colony margin. These observations suggest that LbMV-1 infection significantly alters fungal pigmentation and growth.

#### **4.3.2.2 Colony diameter of isogenic lines of *L. biglobosa***

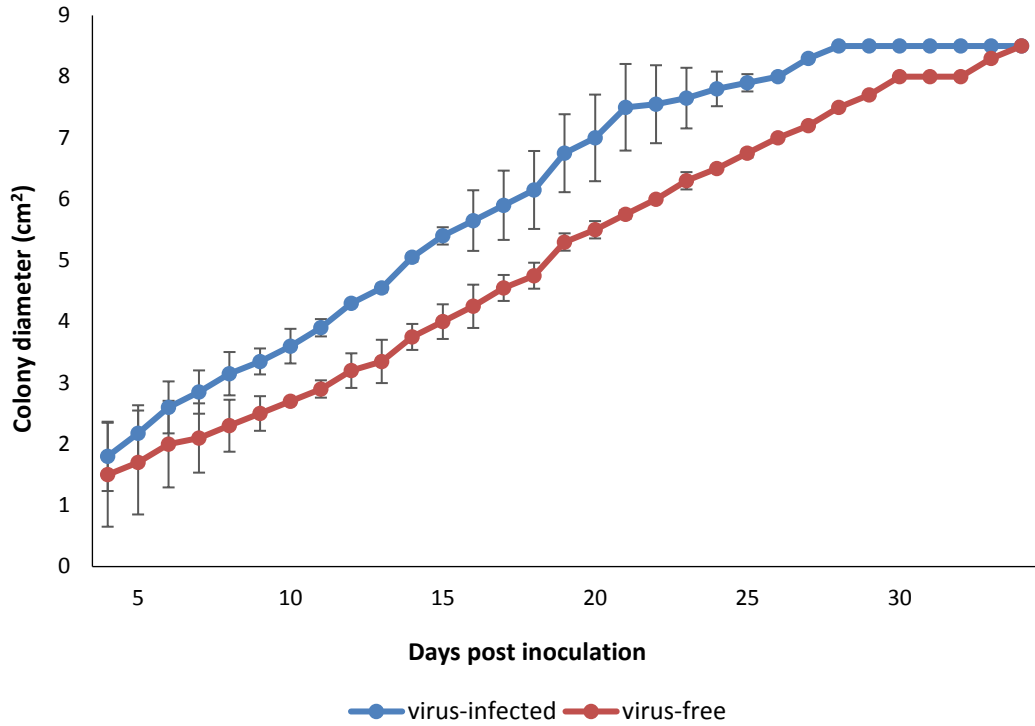
The radial growth assay showed that the virus-infected *L. biglobosa* isolate grew significantly faster than the virus-free isolate on PDA over 16 days after inoculation in three separate experiments ( $P < 0.0001$ , Fig. 4.4). This trend was more noticeable and maintained on PDA and occurred at an earlier time in a time course investigation (Fig. 4.5). Thus, this assay showed that virus infection resulted in increased growth of *L. biglobosa* isolate W10 on solid growth medium and this observation supports results obtained *in vivo* with *Brassica napus* following inoculation with virus-infected and



**Figure 4.3** Colony morphology of virus-infected and virus-free *L. biglobosa* isolate W10 on PDA plates following incubation at 20°C for 26 days. The photograph shows the appearance of the fungal colonies from the front (a) and the back (b) of the plates.



**Figure 4.4 Mycelial growth rates of the virus-infected and virus-free isogenic lines of *L. biglobosa* W10 on PDA plates following incubation at 20°C for 16 days. Plates were viewed from the front (a) and the back (b).**



**Figure 4.5 Colony diameter of isogenic lines of virus-infected and virus-free *L. biglobosa* W10 on PDA plates over a 34 day incubation period.** The means of the colony diameters measured were plotted to observe any differences between the isolates. Radial expansion was measured and compared in three replicate experiments.

virus-free isogenic lines of the *L. biglobosa* W10 isolate (Chapter 5).

#### **4.3.2.3 Biomass production of isogenic lines of *L. biglobosa***

A significant increase in biomass production was observed for the virus-infected strain as compared to the virus-free strain ( $P < 0.05$ , Fig. 4.6). Student's *t* test was used to analyse the data for significant differences in biomass production (Table 4.2).

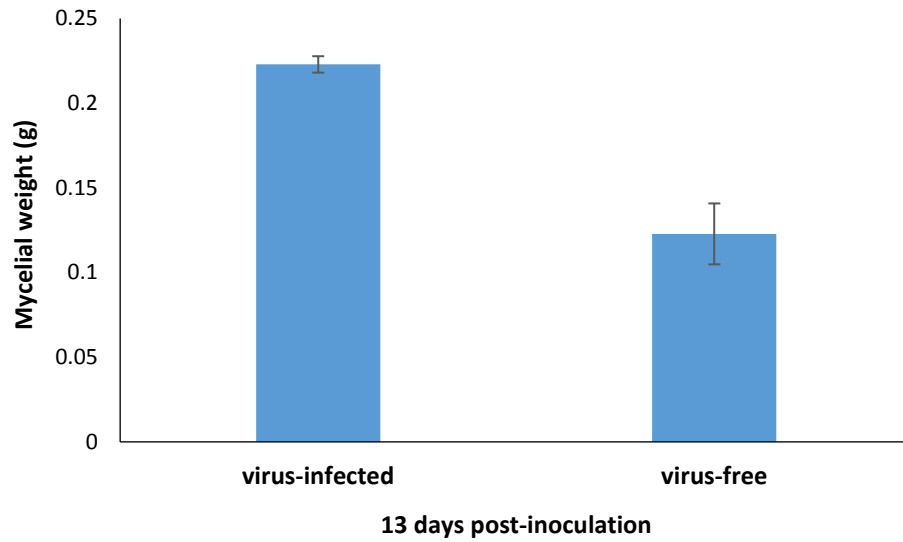
### **4.4 Discussion**

Previously, the efficiency of eradicating mycovirus infection from fungi was assessed following dsRNA extraction and examination of the extracts by agarose gel electrophoresis. In some cases, samples containing low concentrations of dsRNA could not be visually detected in stained gels, so a more sensitive and effective method such as RT-PCR amplification using sequence-specific primers was used to confirm the presence or absence of dsRNA (Section 2.6.2). Park *et al.* (2006) demonstrated the efficiency of such assays whilst eradicating *Chalara elegans* mitovirus infection from *Chalara elegans*. Efforts to eradicate dsRNA from *C. elegans* strains were made using various concentrations of cycloheximide and ribavirin but following extended periods of growth and repeated transfers, fungus was detected with the virus infection. During sequential transfers of mycelium at 35-37°C, loss of a 2.8 kbp dsRNA element was observed as assessed on agarose gels and also following northern blot hybridization. However, RT-PCR amplification with specific primers revealed the presence of the mitovirus and failure to generate a virus-free isolate.

Several techniques for eliminating mycovirus infection from fungi have been investigated including PEG-induced stress on the matric potential of the medium for the growth of fungi (Thapa *et al.*, 2016), mycelial fragmentation (Kim *et al.*, 2013), elevated temperature (Golubev *et al.*, 2003), exposure to UV light (Treton *et al.*, 1987), desiccation and freeze-thawing (Márquez *et al.*, 2007), treatment with a variety of chemicals including emetine (Ahn & Lee, 2001), acridine orange (Cansado *et al.*, 1989) and cycloheximide (Dalzoto *et al.*, 2006; Elias & Cotty, 1996; Robinson & Deacon, 2002; Yamada *et al.*, 1991). These methods achieved various degrees of success. Fulbright (1984) first observed the changes in the sporulation, pigmentation

**Table 4.2 Biomass production after a 13 day incubation period in PDB broth as assessed by dry weight of mycelia produced by virus-infected and virus-free *L. biglobosa* isolate W10.** The weights were analysed using the Student's *t* test. Biomass was assessed in three replicate experiments (mean  $\pm$  SD).

| <b>Isolate name</b> | <b>Mycelial weight (g)</b> |
|---------------------|----------------------------|
| <b>W10</b>          | <b>13 dpi</b>              |
| Virus-infected      | 0.222 $\pm$ 0.004          |
| Virus-free          | 0.122 $\pm$ 0.017          |



**Figure 4.6 Biomass production of the virus-infected and virus-free *L. biglobosa* isolate W10 following incubation at 20°C for 13 days in PDB broth. Vertical bars represent standard deviation errors (data presented in Table 4.2).**



and phenotype with cycloheximide treatment where removal of virus infection from *E. parasitica* strain restored its normal morphology.

Cycloheximide, an antifungal antibiotic, restricts a translocation step and blocks translational elongation for protein synthesis (Siegel & Sisler, 1963). Cycloheximide treatment has been successfully used to eradicate mycovirus infection from different fungal species. For example, in *Beauveria bassiana* strain CG25 (Dalzoto *et al.*, 2006), *Endothia parasitica* (Fulbright, 1984) and *Aspergillus fumigatus* (Bhatti *et al.*, 2011). On the other hand, there are also reports of unsuccessful curing in several fungal species such as *Aspergillus* section *flavi* (Elias & Cotty, 1996), *M. anisopliae* (Martins *et al.*, 1999), *Fusarium oxysporum* (Sharzehei *et al.*, 2007) and *Cytospora sacchari* (Peyambari *et al.*, 2014). These failures could be due to the use of low concentrations of cycloheximide and the presence of high titres of dsRNA elements. In addition to unsuccessful curing, partial curing of virus-infected fungi was also recognized in many cases such as *M. pinodes*, which changed pigmentation from dark brown to light pink and coincidentally restored the normal phenotype (Yamada *et al.*, 1991). Also a putatively-cured strain of *C. elegans* exhibited similar colony morphology to the wild-type strain with a slightly greater growth rate (Park *et al.*, 2006). *Rhizoctonia solani* containing several dsRNA elements (1.0 to 12.5 kbp in size) could not be completely cured as only the smaller segments were eliminated and larger segments 9.0 to 12.5 kbp were always retained after treatment (Robinson & Deacon, 2002). This indicates that partial eradication of dsRNA components might not affect the replication of other dsRNAs and they continue to replicate.

Here initial attempts were made to eliminate virus infection from the *L. biglobosa* isolates K-Exc 12.10.21 and C-Rox 12.8.1 using various concentrations of cycloheximide. Elevated cycloheximide concentrations ( $\geq 5$  mM) inhibited the growth of the fungus completely. Moreover, treatment with 0.01 mM cycloheximide or 0.1 mM cycloheximide and single spore culture did not affect mycovirus infection and dsRNAs were retained in both the isolates. As the antibiotic stress was removed, the dsRNA titre amplified and the fungus remained infected with virus genome components, indicating failure to eradicate the dsRNA elements. However, only the W10 isolate, treated with 0.1 mM cycloheximide, was successfully freed from infection with LbMV-1 as confirmed by dsRNA extraction and RT-PCR amplification analyses. The use of monoconidial culture and hyphal tip sub-culturing techniques give variable results for

virus exclusion, depending on the fungal species and strain. Santos *et al.* (2017) were unable to cure *M. anisopliae* ESALQ 26 through hyphal tip sub-culturing as colonies regenerated from hyphal tip subcultures remained infected with dsRNA. While the cured colony of *M. anisopliae* ESALQ 1256 was achieved by single conidium isolation.

Moreover, a combination of a chemical treatment and single conidium isolation or hyphal tip sub-culturing technique led to the successful elimination of viral elements in certain fungal species (e.g. Aminian *et al.*, 2011; Aoki *et al.*, 2009; Kotta-Loizou & Coutts, 2017; Van Diepeningen *et al.*, 2006).

The generation of isogenic lines of *L. biglobosa*, with or without mycovirus infection, obtained by curing with cycloheximide, facilitated comparisons of phenotype and quantification of fitness effects. In this study, differences in colony morphology were observed between the virus-infected and virus-free isolates of *L. biglobosa* W10 when they were grown on PDA media. Uniform patterns of sporulation were found for the virus-infected strain as compared to the cured, virus-free strain. Abnormal phenotypes associated with virus-infection were also reported in some fungi, e.g. *Nectria radicola* (Ahn & Lee, 2001), *M. anisopliae* (Giménez-Pecci *et al.*, 2002; Melzer & Bidochka, 1998) and *D. ambigua* (Preisig *et al.*, 2000). Bhatti *et al.* (2011) observed that *Aspergillus fumigatus* chrysovirus (AfuCV) infection of *A. fumigatus* isolate 56 resulted in an abnormal formation of aconidial sectors and darker green pigmentation as compared to the cured isolate which was pigmented light green, uniform and non-sectored while *Aspergillus fumigatus* partitivirus-1 (AfuPV-1) infection of the yellow *A. fumigatus* isolate 88 produced aconidial sectors with light pigmentation as compared to the virus-free isolate which was more pigmented and uniform. In contrast to the *Penicillium* and *Cryphonectria* chrysoviruses (Jiang & Ghabrial, 2004; Liu *et al.*, 2007), which are associated with latent infections of their hosts (Ghabrial, 2008), *Magnaporthe oryzae* chrysovirus 1 and *Aspergillus* virus 1816, AfuCV (Urayama *et al.*, 2010; Van Diepeningen *et al.*, 1998, respectively) are both responsible for disease phenotypes of their hosts, reduced growth and altered pigmentation.

A significant increase in radial growth of the virus-infected strain W10 as compared to the cured strain W10 was observed on PDA media. These results are similar to the hypervirulent effects noted in *A. fumigatus* strain A78 following infection with

*Aspergillus fumigatus* tetramycovirus (AfuTmV-1; Kanhayuwa *et al.*, 2015; Özkan & Coutts, 2015) and *B. bassiana* isolate EABb 92/11-Dm infected with a polymycovirus (Kotta-Loizou & Coutts, 2017).

Additionally, similar observations were made on growth performance when the biomass produced by the virus-infected W10 was compared with that produced by the virus-free W10 where significantly larger amounts of mycelium was produced by virus-infected isolate. In agreement with these results, previous studies showed that *B. bassiana* isolate EABb 92/11 infected with a polymycovirus produced more mycelia than the virus-free isogenic line (Kotta-Loizou & Coutts, 2017). By contrast, Wu *et al.* (2007) found debilitated mycelial growth of *Botrytis cinerea* isolate CanBC-1 was associated with mycovirus infection. Mycovirus infection of *A. fumigatus* with either the chrysovirus AfuCV or AfuPV-1 also resulted in significant attenuation of growth of the fungus (Bhatti *et al.*, 2011). These effects indicate that the virus infection and dsRNA replication hinders the normal growth pattern of uninfected fungi.

#### **4.5 Conclusions and future work**

To investigate the effects of mycoviruses on *L. biglobosa*, attempts were made to eradicate the virus from a number of different isolates of *L. biglobosa* using cycloheximide and once a suitable concentration of the drug was established that did not impair growth of the fungus, one isolate W10 was found to be virus-free. Establishment of isogenic lines of virus-infected and virus-free *L. biglobosa* W10 facilitated a direct comparison of the effects of virus infection with LbMV-1 on growth, phenotype and pathogenicity of the fungus. A comparison of virus-infected and virus-free isogenic lines of *L. biglobosa* isolate W10 revealed a hypervirulent effect of LbMV-1 on *L. biglobosa* growth as determined by growth rate on solid media and hyphal biomass in broth.

Other alternative methods were also considered to generate isogenic lines, such as transfection of virus-free isolate with purified virus particles. This procedure involves the inoculation of purified virus into protoplasts of the virus-free isolate. Apart from protoplast transfection, protoplast fusion has also been used effectively to transfer mycoviruses intra- and inter-specifically to overcome the apparent obstacles of vegetative incompatibility in mycovirus transmission and facilitate broadening the host

range of mycoviruses. Protoplast transfection was successfully used to infect virus-free isolates of *A. fumigatus* prior to quantifying the effects of virus infection on the host following a comparison of the growth of virus-infected and virus-free isogenic lines (Bhatti *et al.*, 2011; Kanhayuwa *et al.*, 2015). Successful protoplast fusion has also been reported between *A. niger* and *A. nidulans* (Van Diepeningen *et al.*, 1998). Furthermore, the transmission of *Fusarium boothi* dsRNA virus to other *Fusarium* species and into *C. parasitica* was not only successful but also reduced the virulence of these fungi (Lee *et al.*, 2011).

## **Chapter 5**

### **Effects of LbMV-1 infection on pathogenicity of *Leptosphaeria biglobosa* to oilseed rape (*Brassica napus*)**

## 5.1 Introduction

Double-stranded RNAs (dsRNAs) are common in all classes of plant-pathogenic fungi (Buck, 1986). The majority of mycovirus infections are asymptomatic and persistent. The mycoviruses of families *Totiviridae* and *Partitiviridae* normally cause latent infections in their host fungi (Ghabrial, 1998). However, in some fungal hosts mycovirus infection exhibits virulence-associated traits, such as alterations in growth rate (Boland, 1992), sporulation (Bottacin *et al.*, 1994), pigmentation (Anagnostakis & Day, 1979), enzymatic activities (Rigling & Van Alfen, 1993), distinctive morphological and physiological alterations, including toxin production (Magliani *et al.*, 1997, Varga *et al.*, 1994) and cytological changes in cellular organelles (Newhouse *et al.*, 1983). For example, infection with hypoviruses (family *Hypoviridae*) can cause significant virulence-attenuated phenotypes (Anagnostakis, 1984; Buck, 1986; Cole *et al.*, 1998; Hillman *et al.*, 1990; Jian *et al.*, 1997; Lemke, 1979; Nuss & Koltin, 1990). Hypovirus-mediated hypovirulence of *Cryphonectria parasitica*, chestnut blight fungal pathogen, was first discovered in 1964 in Italy (Grente, 1965) and then it was successfully used to control chestnut blight in Europe (Anagnostakis, 1982; Nuss, 1992).

To determine if mycoviruses affect the virulence of *L. biglobosa*, conidial suspensions of virus-infected and virus-free isolates of *L. biglobosa* were inoculated onto oilseed rape plants and symptoms were compared.

### 5.1.1 Aim and objectives

To assess effects of LbMV-1 infection of *L. biglobosa* on its pathogenicity to oilseed rape (*Brassica napus*).

Objectives:

1. To perform a pathogenicity assay using non-isogenic lines of *L. biglobosa*.
2. To perform a pathogenicity assay using isogenic lines of *L. biglobosa*.

## **5.2 Materials and methods**

### **5.2.1 Pathogenicity assays using non-isogenic and isogenic isolates of *L. biglobosa***

The pathogenicity of six different *L. biglobosa* isolates (two virus-containing and four virus-free, details of isolates are given in Table 5.1) was examined on cotyledons of the cultivars Drakkar and Excel. Afterwards, the effects of LbMV-1 on the pathogenicity of isogenic lines of W10 isolate were examined on cotyledons of oilseed rape.

#### **5.2.1.1 Preparation of spore suspensions of *L. biglobosa* isolates**

*L. biglobosa* cultures were grown on V8 agar plates under alternating 12h light and 12h darkness to assist sporulation. The spore plates were covered with sterile distilled water and the agar surface was scraped with a Lazy-L spreader to release the conidia into the water. The spores were collected after filtering the spore suspension through Miracloth. The spore numbers were counted using a haemocytometer and spores were re-suspended in water to adjust the spore concentration to  $10^6$  or  $10^7$  spores/ml.

#### **5.2.1.2 Growth of plants**

Seeds of cultivars Drakkar and Excel were placed in Petri dishes on Whatman filter paper, which was sprayed with distilled water and incubated at 20°C to induce germination. The seeds were sown in seed trays containing 50:50 Miracle Gro: John Innes No.3 compost. Seed trays were kept in large plastic trays filled with water (1 cm depth) during the experiments and placed inside a controlled environment chamber (20°C day/20°C night, 12 h light/12 h darkness, light intensity  $210 \mu\text{E}/\text{m}^2\text{s m}^{-2}\text{s}^{-1}$ , 80-85% relative humidity). To maintain the moisture and humidity, water was sprayed daily.

#### **5.2.1.3 Inoculation of spore suspensions on cotyledons**

1. 14 days after sowing, any true leaves were removed and the plants were supported using sticks (Fig. 5.1a). At the edge of one of the cotyledon lobes, a small hole was made for identification purposes. Each cotyledon lobe was wounded gently at the centre with a sharp pin.



**Figure 5.1 (a) Tray with the cultivars of *Brassica* seedlings before inoculation with *L. biglobosa* spore suspensions, (b) schematic representation showing inoculation plan on cotyledons with the four inoculation sites (1-4) two on each lobe.**



2. *L. biglobosa* conidial suspensions (10 µl) were inoculated onto the wounded areas (Fig. 5.1b). After inoculation, the cotyledons and the lids used to cover the trays were gently sprayed with water. Inoculated seedlings were then covered with a lid and black polythene sheets and moved into plant growth chambers. Plants were then incubated in the dark under conditions of high humidity (80-85% relative humidity) for 48 h. After 24 h of incubation, the black polythene sheets were removed and 48 h later the lids were removed. Then the plants were incubated uncovered in the controlled-environment cabinet at 20°C until the end of the experiments.

#### **5.2.1.4 Disease assessment and statistical analysis**

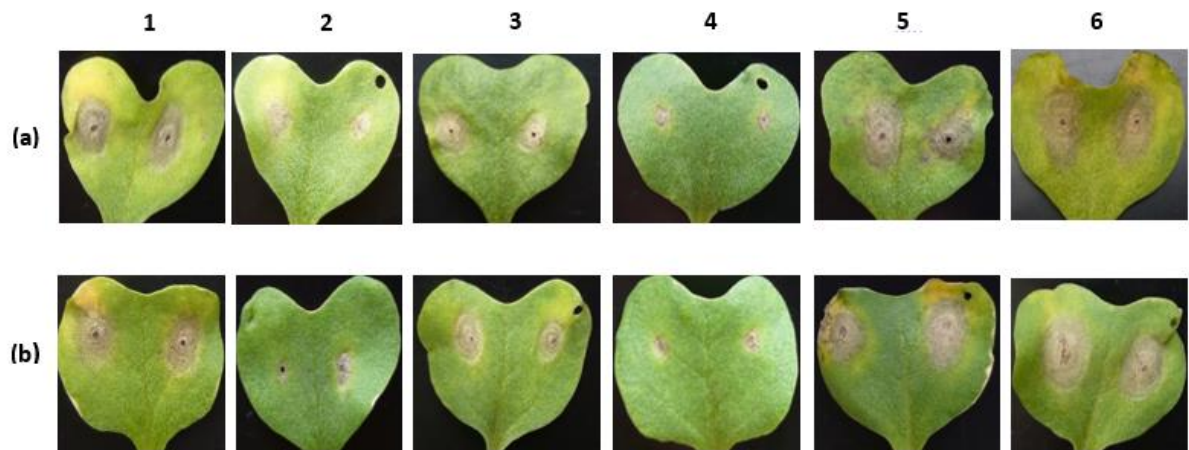
Disease incidence was assessed by measuring the size of lesions on the two inoculation sites on each cotyledon. The incubation period was estimated as the time from inoculation until the first lesions were observed. Lesion sizes were measured and recorded at different days post inoculation (dpi) until the plants died. Approximate lesion areas (length x width) were calculated and these values were plotted against time. Significant differences in lesion area were calculated using two-way ANOVA tests.

### **5.3 Results**

#### **5.3.1 Pathogenicity assay using non-isogenic lines of *L. biglobosa***

Four virus-free and two virus-containing isolates were tested on two different cultivars of oilseed rape, Drakkar and Excel. Virus-free isolates were nominated as isolates 1, 2, 3, 4 and virus-infected isolates were nominated as isolates 5 and 6.

Phoma leaf spot symptoms were observed on all plants inoculated with either virus-infected or virus-free isolates. There were no differences in the sizes of lesions produced by the isolates between cultivars Drakkar and Excel (Table 5.1a, b). However, there were noticeable differences in the lesion size produced between virus-containing isolates and virus-free isolates (Fig. 5.2 & 5.3). Following inoculation onto oilseed rape plants, differences in lesion size were noted for the different isolates 7



**Figure 5.2 Phenotypic variation of phoma leaf spots caused by virus-free (1 to 4) and virus-infected (5, 6) *L. biglobosa* isolates on representative cotyledons of the cultivars Drakkar (a) and Excel (b), at 7 dpi. 1-6 represent the isolates used for inoculation. Details of isolates are given in Table 5.1**



**Figure 5.3 Phenotypic variation of phoma leaf spots caused by virus-free (1 to 4) and virus-infected (5, 6) *L. biglobosa* isolates on representative cotyledons of the cultivars Drakkar (a) and Excel (b), at 12 dpi. 1-6 represent the isolates used for inoculation. Details of isolates are given in Table 5.1**

**Table 5.1 Mean lesion area on cotyledons of *B. napus* cvs. Drakkar (a) and Excel (b) post inoculation with virus-free (1 to 4) and virus-infected (5 and 6) isolates, 4-12 dpi. The lesion areas were assessed for up to 10 lesions of each cultivar for each isolate (mean  $\pm$  SD).**

**(a)**

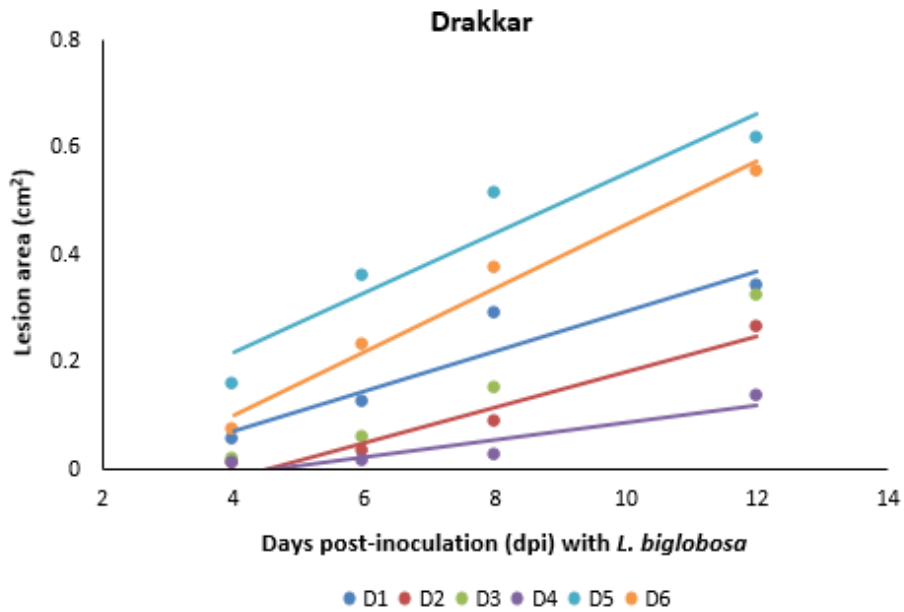
| No. | Isolate name  | Lesion area (cm <sup>2</sup> ) on cultivar Drakkar |                   |                   |                   | Group | Intercept | Slope |
|-----|---------------|--|-------------------|-------------------|-------------------|-------|-----------|-------|
|     |               | 4 dpi  | 6 dpi             | 8 dpi             | 12 dpi            |       |           |       |
| 1   | K-Rox 12.11.1 | 0.056 $\pm$ 0.025                                  | 0.125 $\pm$ 0.085 | 0.287 $\pm$ 0.131 | 0.341 $\pm$ 0.094 | G1    | -0.074    | 0.037 |
| 2   | H-Exc 12.12.1 | 0.011 $\pm$ 0.004                                  | 0.033 $\pm$ 0.034 | 0.087 $\pm$ 0.175 | 0.265 $\pm$ 0.156 | G2    | -0.152    | 0.036 |
| 3   | F2-Excdm 11.5 | 0.018 $\pm$ 0.015                                  | 0.058 $\pm$ 0.048 | 0.149 $\pm$ 0.114 | 0.322 $\pm$ 0.184 | G2    | -0.152    | 0.036 |
| 4   | F2-Exc 12.2.3 | 0.010 $\pm$ 0.003                                  | 0.015 $\pm$ 0.009 | 0.027 $\pm$ 0.013 | 0.136 $\pm$ 0.033 | G3    | -0.074    | 0.016 |
| 5   | C-Rox 12.8.1  | 0.158 $\pm$ 0.096                                  | 0.358 $\pm$ 0.095 | 0.513 $\pm$ 0.219 | 0.617 $\pm$ 0.112 | G4    | -0.004    | 0.055 |
| 6   | D-Rox 12.10   | 0.073 $\pm$ 0.024                                  | 0.231 $\pm$ 0.114 | 0.376 $\pm$ 0.124 | 0.553 $\pm$ 0.123 | G5    | -0.136    | 0.059 |

**(b)**

| No. | Isolate name  | Lesion area (cm <sup>2</sup> ) on cultivar Excel |                   |                   |                   | Group | Intercept | Slope |
|-----|---------------|--|-------------------|-------------------|-------------------|-------|-----------|-------|
|     |               | 4 dpi  | 6 dpi             | 8 dpi             | 12 dpi            |       |           |       |
| 1   | K-Rox 12.11.1 | 0.061 $\pm$ 0.037                                | 0.180 $\pm$ 0.057 | 0.373 $\pm$ 0.118 | 0.453 $\pm$ 0.256 | G1    | -0.041    | 0.038 |
| 2   | H-Exc 12.12.1 | 0.007 $\pm$ 0.006                                | 0.021 $\pm$ 0.009 | 0.076 $\pm$ 0.050 | 0.203 $\pm$ 0.043 | G2    | -0.091    | 0.021 |
| 3   | F2-Excdm 11.5 | 0.026 $\pm$ 0.016                                | 0.069 $\pm$ 0.035 | 0.182 $\pm$ 0.089 | 0.370 $\pm$ 0.158 | G3    | -0.172    | 0.044 |
| 4   | F2-Exc 12.2.3 | 0.009 $\pm$ 0.002                                | 0.027 $\pm$ 0.008 | 0.075 $\pm$ 0.102 | 0.143 $\pm$ 0.103 | G2    | -0.091    | 0.021 |
| 5   | C-Rox 12.8.1  | 0.175 $\pm$ 0.015                                | 0.309 $\pm$ 0.047 | 0.539 $\pm$ 0.038 | 0.548 $\pm$ 0.091 | G4    | 0.038     | 0.047 |
| 6   | D-Rox 12.10   | 0.053 $\pm$ 0.057                                | 0.360 $\pm$ 0.023 | 0.623 $\pm$ 0.049 | 0.672 $\pm$ 0.074 | G5    | -0.089    | 0.069 |

Parameters were estimated from the linear regression equations  $y=a+bt$ , where  $y$  is average lesion area,  $t$  is days (dpi), and  $a$  and  $b$  are the intercept and slope parameters of the lines, respectively. Values of the intercept and slope of regression lines (Fig. 5.4a, b) and groups of isolates were determined by analysis of position and parallelism. On Drakkar (a) isolates 2 and 3 were combined in group G2 and on Excel (b) isolates 2 and 4 were combined in group G2 as there were no significant differences between intercept and slope values.

(a)



(b)

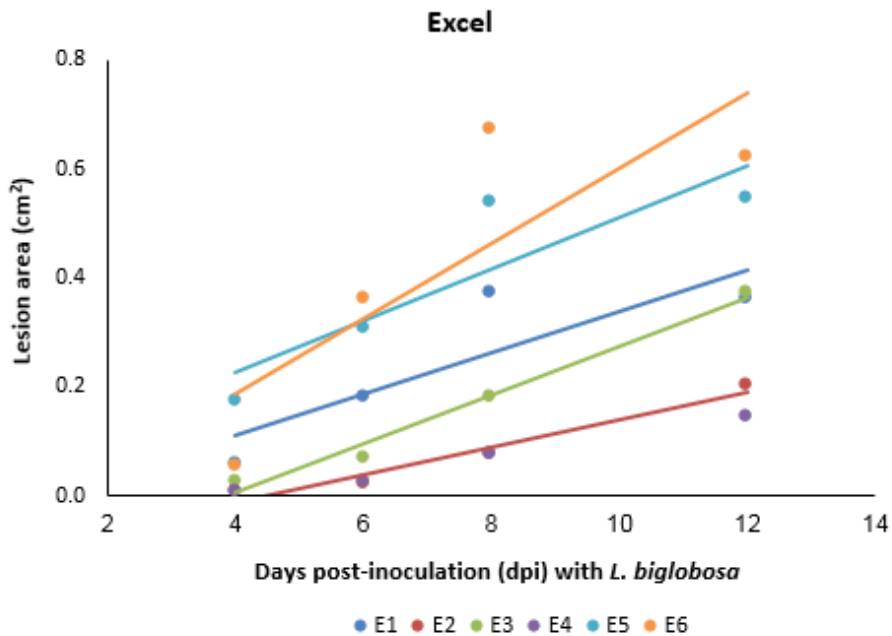


Figure 5.4 Linear regressions of the data presented in Table 5.1 (a, b) showing phoma lesion area caused by virus-free (1 to 4) and virus-infected (5 and 6) isolates of *L. biglobosa* on cotyledons of *B. napus* cvs. On Drakkar (a) isolates 2 and 3 and on Excel (b) isolates 2 and 4 contained similar intercept and slope values and showed with a common line.

days post inoculation on cotyledons of both cultivars. The largest lesions were observed on cotyledons inoculated with isolates 5 and 6 and the smallest with isolates 2 and 4 (Fig. 5.2a, b).

In virus-free isolates, a small difference in the lesion area was observed on cotyledons inoculated with isolates 2 and 4 at 4 to 8 dpi on both the cultivars (Fig. 5.4a, b). They produced smaller lesions than virus-free isolates 1 and 3. Isolate 1 produced the greatest lesions among the other virus-free isolates 2, 3 and 4 on both the cultivars (Fig. 5.4a, b). In virus-infected isolates, isolate 5 produced bigger lesions than isolate 6 on cultivar Drakkar and isolate 6 produced bigger lesions than isolate 5 on cultivar Excel (Fig. 5.4a, b).

No symptoms were visible on any plants at 1 dpi with *L. biglobosa* spores. However, at 4 dpi, average lesion area increased slowly in cotyledons inoculated with the virus-free isolates, whereas it increased rapidly in cotyledons inoculated with the virus-infected isolates. There was a significant increase in lesion area produced by virus-infected isolates compared to virus-free isolates inoculated leaves at 6-8 dpi ( $P < 0.05$ ). However, by 12 dpi the average lesion area on inoculated cotyledons had increased rapidly for virus-free isolates 1, 2, 3 and 4 due to hyphal growth of the pathogen and there were less significant differences in lesion area between virus-infected and virus-free isolates (Fig. 5.3a, b).

### **5.3.2 Pathogenicity assay using isogenic isolates of *L. biglobosa***

Initially, different virus-infected and virus-free *L. biglobosa* isolates were tested on cotyledons. However, for actual comparisons of virulence, isogenic lines of virus-infected and virus-free W10 isolate were created. These virus-infected and virus-free W10 isolates were examined on cotyledons of cultivar Excel.

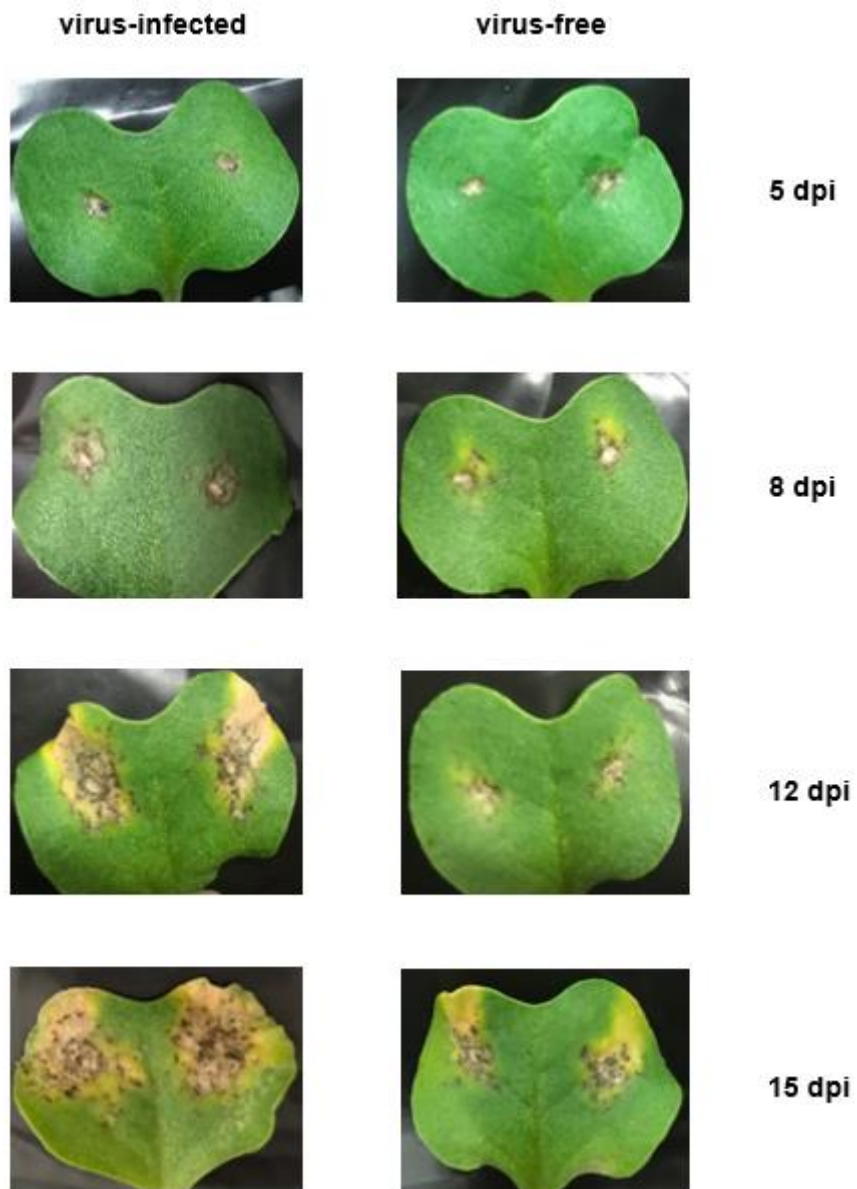
Pathogenicity was assessed up to 15 days dpi by checking phoma leaf spot appearance. There were significant differences between virus-infected and virus-free inoculum in incubation period for phoma leaf lesion development, with the incubation period shorter for virus-infected isolates as compared to virus-free isolates. Phoma leaf spot lesions were first observed at 4 dpi on plants inoculated with virus-infected conidia but phoma lesions were not observed on plants inoculated with virus-free conidia until 5 dpi.

There were no statistically significant differences in the lesion sizes produced between virus-infected and virus-free isolates at 5 dpi. However, after 5 dpi average lesion area increased slowly in cotyledons inoculated with the virus-free isolates, whereas lesion area increased rapidly in cotyledons inoculated with the virus-infected isolates (Fig. 5.5). The lesion area was greater on cotyledons inoculated with virus-infected isolates rather than virus-free isolates at 8 dpi (Fig. 5.6).

There were statistically significant differences in the lesion sizes produced by virus-infected and virus-free isogenic lines at 12 and 15 dpi ( $P < 0.0001$ , Table 5.2). This suggested that virus-infected *L. biglobosa* isolate W10 was more pathogenic than the corresponding virus-free isogenic line on oilseed rape cotyledons.

## 5.4 Discussion

*L. biglobosa* causes stem canker in oilseed rape following premature lodging of adult plants and restricts crop production worldwide. Furthermore, many studies demonstrated that conidia can infect cotyledons (Hall, 1992) and the fungus penetrates the stem *via* the petiole (Hammond *et al.*, 1985). Comparison of different methods to assess the growth of pathogen from phoma leaf lesions along the main leaf vein and petiole to the stem in controlled environments suggested that leaf lesion area could be a good indicator of pathogenicity which might be used in field experiments. In addition to this, assessment of leaf lesions (size and appearance) is quicker and reliable method which can assess differences in virulence between pathogens. This method has been used to assess quantitative resistance against different fungal pathogens (Chartrain *et al.*, 2004; Silva *et al.*, 2012; Talukder *et al.*, 2004). More recently, quantitative (q) PCR amplification was also used for detecting symptomless growth of the pathogen in leaf or stem tissues (Huang *et al.*, 2014). For development of phoma leaf lesions, conidial suspensions of the pathogen were deposited on cotyledons wounded with a needle. This procedure was adopted to develop stem canker in oilseed rape (Travadon *et al.*, 2009). Another study showed that stem canker symptoms can be developed on hypocotyls following cotyledon inoculation if the inoculated plants are maintained under controlled environment



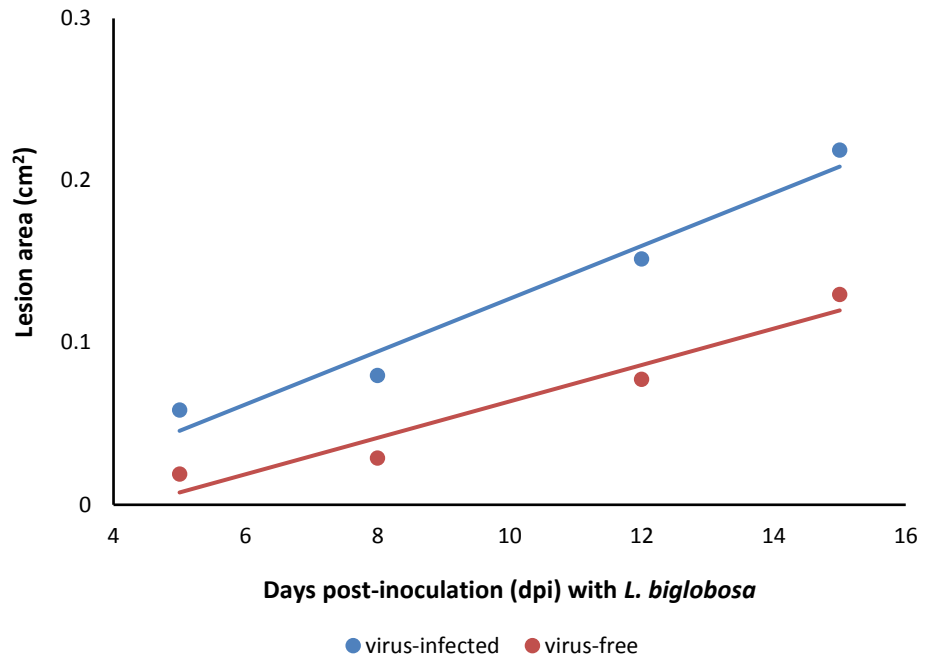
**Figure 5.5** Phenotypic variation of phoma leaf spots caused by virus-infected and virus-free isogenic lines of *L. biglobosa* isolate W10 on representative cotyledons of the cultivar Excel at 5, 8, 12 and 15 dpi.

**Table 5.2 Mean lesion area on cotyledons of *B. napus* cv. Excel, post inoculation with virus-infected and virus-free isogenic lines of *L. biglobosa* isolate W10, 5-15 dpi.** The lesion areas were assessed for up to 32 lesions for each isolate (mean  $\pm$  SD).

| Isolate name   | Lesion area (cm <sup>2</sup> ) |                   |                   |                   | Intercept         | Slope  |       |
|----------------|--------------------------------|-------------------|-------------------|-------------------|-------------------|--------|-------|
|                | W10                            | 5 dpi             | 8 dpi             | 12 dpi            | 15 dpi            | a      | b     |
| Virus-infected |                                | 0.058 $\pm$ 0.023 | 0.079 $\pm$ 0.033 | 0.151 $\pm$ 0.076 | 0.218 $\pm$ 0.132 | -0.036 | 0.016 |
| Virus-free     |                                | 0.018 $\pm$ 0.011 | 0.028 $\pm$ 0.012 | 0.077 $\pm$ 0.071 | 0.129 $\pm$ 0.078 | -0.049 | 0.011 |

Parameters were estimated from the linear regression equations  $y=a+bt$ , where  $y$  is average lesion area,  $t$  is days (dpi), and  $a$  and  $b$  are the intercept and slope parameters of the lines, respectively (shown in Fig. 5.6).





**Figure 5.6** Linear regressions of the data presented in Table 5.2 showing phoma lesion area caused by virus-infected and virus-free isogenic lines of *L. biglobosa* isolate W10 on cotyledons of *B. napus* cv. Excel at 5, 8, 12 and 15 dpi.

conditions (Travadon *et al.*, 2009). However, production of canker symptoms requires approximately 10 weeks and hypocotyl infection is not representative of what happens in field conditions in the UK. Moreover, hypocotyls are different in structure from stems (Sprague *et al.*, 2007). Disease severity response measured by the cotyledon infection assay as used in this study has been shown to correlate well with the adult plant response and consequently has been commonly used to evaluate disease progression (Kazan *et al.*, 1998; Stringam *et al.*, 1995; Wang *et al.*, 1999). The experiments conducted in this study confirmed that uniformly grown seedlings can be infected under controlled conditions using identical inocula to produce a uniform data set.

Six *L. biglobosa* isolates (F2-Excdm 11-5, K-Rox 12.11.1, H-Exc 12.12.1, F2-Exc 12.2.3, C-Rox 12.8.1 and D-Rox 12.10) obtained from winter oilseed rape, whose identities had been confirmed by both morphology/ pigment production and PCR tests, were chosen for disease severity testing. To investigate the virulence of these isolates on cultivars, cotyledons of two different cultivars (Drakkar and Excel) were inoculated with the conidial suspensions of these six isolates using standard cotyledon test methods and lesions were measured.

Virus-infected isolates (C-Rox 12.8.1 and D-Rox 12.10) consistently produced larger lesions on both cultivars as compared to virus-free isolates (F2-Excdm 11-5, K-Rox 12.11.1, H-Exc 12.12.1, F2-Exc 12.2.3). Thus disease severity of the virus-containing isolates was apparently greater than that of the virus-free isolates. In summary, it was apparent that from four separate measurements on all four occasions at 4, 6, 8 and 12 dpi, Drakkar and Excel did not differ in response to any of the six different isolates. The interaction between cultivar and isolate was not significantly different on any occasion. This observation suggests that any effect of any of the six isolates was the same on both Drakkar and Excel (Fig. 5.4 a, b). However, in all cases and in all experiments from 4 dpi or later the isolates differed significantly with respect to lesion size. Isolates 5 and 6 appeared to be most pathogenic while isolates 2 and 4 were the least pathogenic.

These results were further supported when isogenic, virus-infected and virus-free lines of *L. biglobosa* isolate W10 were inoculated on the cotyledons of the Excel cultivar, where mycovirus infection enhanced the virulence of their fungal host (Fig. 5.6). From

the data obtained, it might be stated that the infection with LbMV-1 does indeed increase the pathogenicity of *L. biglobosa* on oilseed rape cotyledons. The molecular mechanisms of this phenomena are, however, unknown.

Natural infection of oilseed rape crops is achieved by ascospores affecting unwounded leaves while in this study cotyledons were wounded and inoculated with conidia (West *et al.*, 2001). Therefore experiments with ascospores need to be pursued.

Mycovirus infection often attenuates fungal pathogenicity and there are many reports in the literature of this hypovirulent effect *viz.* *Fusarium virguliforme* (Marvelli *et al.*, 2014), *S. sclerotiorum* (Boland, 1992), *Botrytis cinerea* (Castro *et al.*, 2003; Potgieter *et al.*, 2013), *C. parasitica* (Choi & Nuss, 1992), *Aspergillus* spp. (Elias & Cotty, 1996; Van Diepeningen *et al.*, 2008). Conversely, few mycoviruses confer hypervirulence (e.g. enhanced sporulation, aggressiveness, growth and pathogenicity). For instance, in *Nectria radicicola*, the presence of a 6.0 kbp dsRNA was associated with high level of virulence where increase in pathogenicity was detected on ginseng plants grown in *N. radicicola*-infested soil (Ahn & Lee, 2001). Hypervirulence was also observed in A78 mycovirus-infected *Aspergillus fumigatus* (Özkan & Coutts, 2015) and polymycovirus-infected *B. bassiana* (Kotta-Loizou & Coutts, 2017) where increases in pathogenicity were detected in the moth model. This hypervirulent effect is somewhat similar to that demonstrated here for virus-infected *L. biglobosa*.

Both hypo- and hyper-virulence are of great interest to plant pathologists due to their potential for biocontrol of fungal diseases.

Presently, the exact mode of action of mycoviruses on phytopathogenic fungi is unknown, although biochemical analysis suggest that mycoviruses might control fungal virulence through signal transduction pathways (Ahn & Lee, 2001). It has also been shown that RNA silencing of mycoviruses might be involved in the phenotypic alternations in virus-infected *Aspergilli* (Hammond *et al.*, 2008) and in other fungi.

There are no commercially grown oilseed rape cultivars which demonstrate complete resistance to *Leptosphaeria* species. However, the ability of mycoviruses to regulate *L. biglobosa* pathogenicity might potentially offer alternative methods of regulating disease progression at the early stages of infection.

## 5.5 Conclusions and future work

Pathogenicity experiments clarified that there appeared to be no real differences in the behaviour of cotyledons inoculated with non-isogenic isolates of virus-infected and virus-free as compared to isogenic lines of virus-infected and virus-free isolate. Results of oilseed rape cotyledon infection experiments (using isogenic lines) are consistent with the measurements of growth rate and biomass production in terms of mycovirus mediated hypervirulence in *L. biglobosa*.

However, evaluating any disease resistance response of isogenic lines under field conditions and on different cultivars will now be necessary to establish any biological effects of mycoviruses on their host. Cotyledon inoculation experiments need to be further confirmed by assessing canker severity on stems. In addition to this, it is also important to shed light on mycovirus-fungus-plant interactions by transcriptome analysis.

## Chapter 6

**Investigation of virus-infected or virus-free *Leptosphaeria biglobosa* induced systemic resistance to *L. maculans* in oilseed rape (*Brassica napus*)**

## 6.1 Introduction

*L. maculans* and *L. biglobosa* possess similar life cycles and they often cause coincident infections on oilseed rape crops in Europe and North America (West *et al.*, 2001; 2002). However, their interactions with one another on plants are poorly understood. Previous work showed the local and systemic resistance of *B. napus* to *L. maculans* (Canadian isolate) induced by *L. biglobosa* 'canadensis' (Canadian isolate) conidia under natural environmental conditions (Mahuku *et al.*, 1996). Afterwards, Liu *et al.* (2006 & 2007) demonstrated that local and systemic resistance of *B. napus* to *L. maculans* 'brassicae' could also be induced by *L. biglobosa* 'brassicae'.

Major gene resistance depends on a "gene-for-gene" interaction in which the host R protein interacts with corresponding pathogen avirulence (Avr) protein to begin plant disease responses and resistance (Flor, 1956). Normally, the cultivars with the resistance gene *Rlm7* against *L. maculans* are widely used (Clarke *et al.*, 2011). However, the use of cultivars with *Rlm7* gene has increased the risk that *L. maculans* populations could change from avirulent to virulent against this cultivar. Severe phoma stem canker epidemics were observed in France in field experiments (Brun *et al.*, 2000; Daverdin *et al.*, 2012) and in France and Australia in commercial oilseed rape crops (Li *et al.*, 2003; Sprague *et al.*, 2006) when host resistance mediated by *Rlm* genes was rendered ineffective. *L. maculans* isolate H-Rox 12.2.1 was investigated to be virulent against *Rlm7* cultivar in the UK (Mitrousia *et al.*, 2018).

Induced resistance may occur locally near the point of infection or systemically in uninfected parts of the plant distant from the point of infection by the invading pathogen (Liu *et al.*, 2006; 2007). Thus, induced resistance can protect plants from subsequent infection by a wide variety of pathogens (Lucas, 1999). In many host-pathogen systems, biologically or chemically induced resistance to fungal infection has been described (Sticher *et al.*, 1997). Chemical inducers involve natural products such as salicylic acid (SA), synthetic compounds such as isonicotinic acid (Sticher *et al.*, 1997) and several inorganic compounds. Resistance induced by Acibenzolar-S-Methyl (ASM), an analogue of SA, has been described in many crops, including oilseed rape (Borges *et al.*, 2003; Cools & Ishii, 2003; Jensen *et al.*, 1998; Latunde-Dada & Lucas, 2001). Menadione sodium bisulphite, a water-soluble compound of vitamin K3, was

also shown to induce resistance in oilseed rape against *L. maculans* (Borges *et al.*, 2003). Induced resistance in plants against pathogens following inoculation with the same or other micro-organisms is well recognised but the mechanisms responsible for its occurrence are poorly understood.

This study investigates the ability of virus-infected and virus-free isolates of *L. biglobosa* to induce resistance in oilseed rape (*B. napus*) against phoma leaf spot caused by *L. maculans* under controlled environment conditions. The investigation used isogenic virus-infected and virus-free isolates of *L. biglobosa* generated as described in Section 4.2.1.

The hypothesis for this work is that *L. biglobosa*-induced host resistance response to infection by *L. maculans* might operate under natural conditions and play an important role in the control of phoma stem canker.

### **6.1.1 Aim and objective**

The main aim of this work was to investigate the systemic induced resistance of oilseed rape to *L. maculans* infection following prior inoculation with *L. biglobosa*.

Objective:

1. To detect phenotypes of resistance induced by virus-infected or virus-free *L. biglobosa* against *L. maculans* under controlled environment conditions.

## **6.2 Materials and methods**

### **6.2.1 Phenotypes of resistance induced by *L. biglobosa* against *L. maculans* under controlled environment conditions**

#### **6.2.1.1 Experimental design**

A controlled environment experiment was performed using nine plants for each pre-treatment. At 96 h after pre-treatment, the leaf immediately above the pre-treated leaf was challenge-inoculated with *L. maculans* conidia. This experiment examined effects of pre-treatment on development of phoma leaf lesions over time. Pre-treatments involved inoculation with *L. biglobosa* conidia or sterile water (control).

### **6.2.1.2 Plant material**

Winter oilseed rape (*Brassica napus*) cv. Excel was sown and plants were grown in a glasshouse at 20-24°C. This cultivar possesses the resistance gene *Rlm7* for resistance to *L. maculans*. Seven days old seedlings were transplanted into one plant/pot (9 cm in diameter) containing a mixture of Miracle Gro: John Innes No.3 compost (50:50 volume). Twenty-four h prior to inoculation, four-week-old plants were transferred into a controlled environment cabinet (20°C day/20°C night, 12 h light/12 h darkness, light intensity 210  $\mu\text{E}/\text{m}^2\text{s}$   $\text{m}^{-2}\text{s}^{-1}$ , 80-85% relative humidity). Following inoculation, all plants were maintained in the cabinet until the end of the experiments.

### **6.2.1.3 Preparation of inoculum**

Sporulating cultures of virus-infected *L. biglobosa* W10, virus-free *L. biglobosa* W10 and *L. maculans* H-Rox 12.2.1 were flooded with 10 to 15 ml of sterile distilled water and gently scraped with a flamed glass spreader to release conidia from pycnidia (Section 5.2.1.1). The spores were collected by filtering through sterilized Miracloth. Each spore suspension was prepared at a concentration of  $10^7$  spores/ml for inoculation. Spore suspensions were centrifuged at 9000 rpm for 20 min. The supernatant was discarded and the spore pellet was collected in approximately 1 ml of sterile distilled water for storage as a concentrate at -20°C.

### **6.2.1.4 Pre-treatment and challenge inoculation**

Plants were arranged in a randomised, complete block design with two replicates and nine plants per treatment. Pots were then placed in two large plastic trays which were kept filled with water (depth 1 cm) throughout the experiment. For pre-treatment with *L. biglobosa* (virus-infected or virus-free), small regions (7 mm diameter) of the first true leaves were abraded gently with a cylindrical eraser so that they could hold droplets of the spore suspensions (Huang *et al.*, 2001). Then each abraded area was wounded with a sterile needle and a 12  $\mu\text{l}$  droplet of spores ( $10^7$  spores/ml) was pipetted onto the wounded area. Inoculated plants were then covered with a lid and black polythene sheets and moved into plant growth chambers. Plants were then incubated in the dark under conditions of high humidity (80-85% relative humidity) for 48 h. After 24 h of incubation, the black polythene sheets were removed and 48 h later the lids were removed. Control pre-treatment used sterile distilled water inoculation of



abraded leaves. At 96 h after inoculation, the second true leaves of these plants were challenge-inoculated with two 12 µl drops of *L. maculans* isolate (H-Rox 12.2.1) conidial suspension with  $10^7$  spores/ml, one on each side of the midrib. All challenge-inoculated plants were maintained under the same conditions for 48 h and subsequently incubated uncovered in the controlled-environment cabinet.

#### **6.2.1.5 Disease assessment and statistical analysis**

Disease symptoms were observed every 12 h after challenge inoculation until small necrotic lesions (brown to grey in colour) were visible. The times from challenge inoculation to development of the first lesions were recorded for each plant. Afterwards, lengths and widths of lesions were measured at different time points until 20 days after inoculation (dpi). The approximate lesion areas (length x width) were evaluated. Lesion area was plotted against time to show the effects of different prior inoculations on *L. maculans* lesion development on the second true leaves. For statistical analysis, two-way ANOVA as implemented by GraphPad PRISM 6 was used.

### **6.3 Results**

#### **6.3.1 Phenotypes of resistance induced by *L. biglobosa* against *L. maculans* under controlled environment conditions**

When the first true leaves of plants were pre-inoculated with virus-infected *L. biglobosa* spores or water, the average lesion area elicited following *L. maculans* challenge inoculation on the second true leaves of the same plants increased slowly, while in the plants pre-inoculated with virus-free *L. biglobosa* spores, average lesion area increased rapidly 13 days after challenge inoculation (Fig. 6.1, Table 6.1).

As compared to control plants pre-inoculated with water, pre-inoculation with virus-infected *L. biglobosa* decreased the area of phoma leaf spot lesions caused by *L. maculans* on second leaves while pre-inoculation with virus-free *L. biglobosa* increased the size of phoma lesions. Statistically significant differences in leaf lesion area between virus-infected and virus-free *L. biglobosa* pre-inoculations from 15 days after challenge inoculation were noted ( $P < 0.0001$ ).

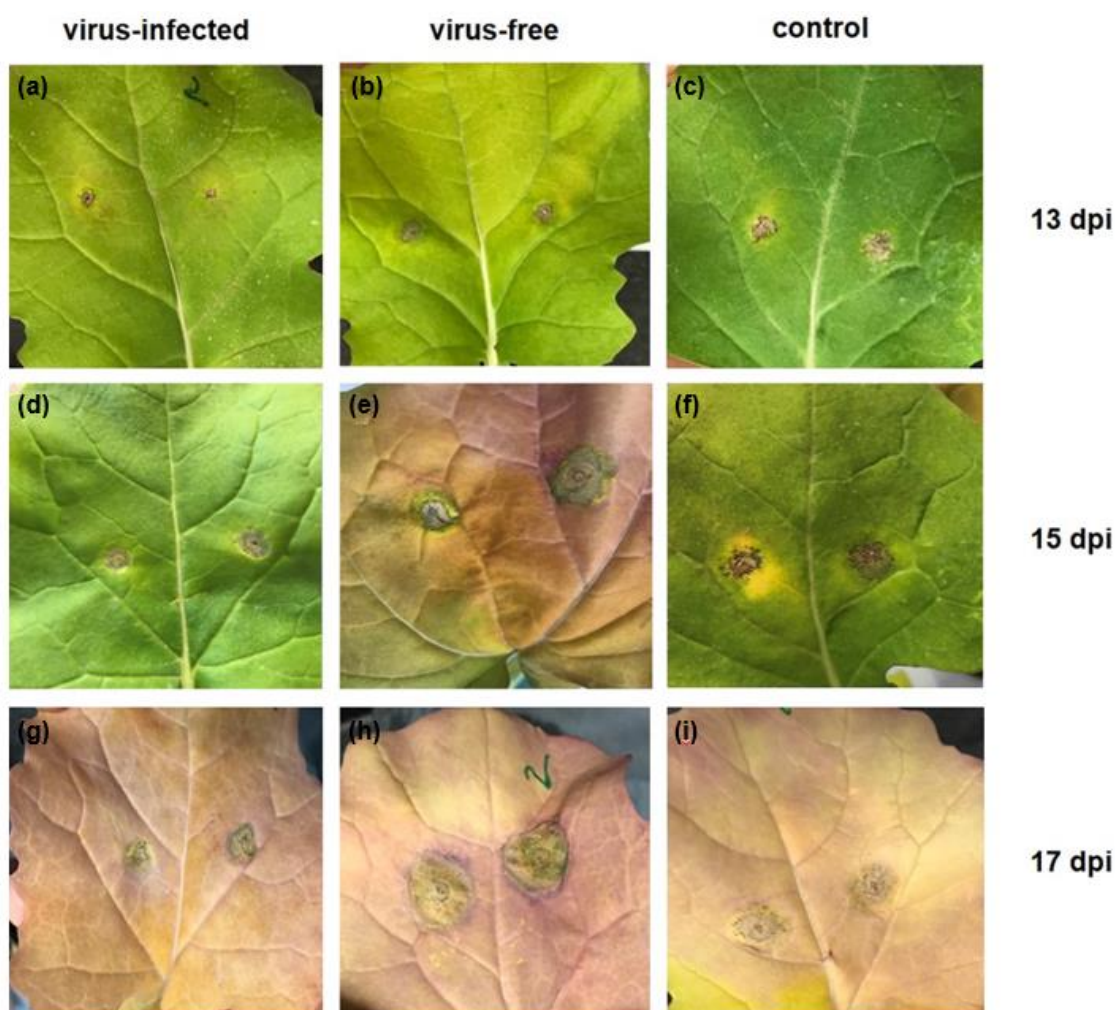
The rate of increase in leaf lesion area elicited by *L. maculans* challenge inoculation on the second true leaves differed between all pre-inoculations, being slowest with virus-infected *L. biglobosa*, fastest with virus-free *L. biglobosa* and intermediate in control, water pre-inoculated plants (Fig. 6.2).

Seventeen days after challenge inoculation, the average lesion area on leaf two (systemic effect) was significantly decreased ( $P < 0.0001$ , Table 6.1) on plants pre-inoculated with virus-infected *L. biglobosa* (0.31 cm<sup>2</sup>) as compared to control (0.66 cm<sup>2</sup>) and virus-free *L. biglobosa* (0.75 cm<sup>2</sup>).

## 6.4 Discussion

These results demonstrate that pre-inoculation of oilseed rape leaves with virus-infected *L. biglobosa* spores can enhance systemic resistance against infection caused by *L. maculans*. These observations confirm and extend previous investigations which reported a similar phenomenon where the size and development rate of *L. maculans* phoma leaf spots were decreased by pre-treatment with *L. biglobosa* (Liu *et al.*, 2006; 2007; Mahuku *et al.*, 1996). Mahuku *et al.* (1996) indicated that this was an example of systemic acquired resistance (SAR) which is defined as acquired resistance, a defence response in plants to a virulent pathogen, induced by pre-inoculation with either virulent, avirulent or non-pathogenic microbes or treatment with chemicals such as SA, or SA mimics such as ASM (Ryals *et al.*, 1996). SAR is characterized by the accumulation of SA in local and systemic tissues and the coordinate expression of genes which encode pathogenesis-related (PR) proteins (Durrant & Dong, 2004). SAR has been reported in many plants such as red clover, soybean, cucumber, muskmelon, watermelon, rice, pearl millet, potato and tomato (Kessmann *et al.*, 1994). It was demonstrated first in oilseed rape by Mahuku *et al.* (1996).

Moreover, pathogens closely related to the challenge isolate have been described as better inducers of resistance than those which are non-pathogens or pathogens of unrelated hosts (Mahuku *et al.*, 1996). For instance *L. biglobosa*, which is not economically destructive, appeared to be effective in inducing resistance to *L. maculans* in oilseed rape (Liu *et al.*, 2006; 2007). A single lesion of *L. biglobosa* on the leaf was able to induce resistance throughout the leaf and other parts of the plant.



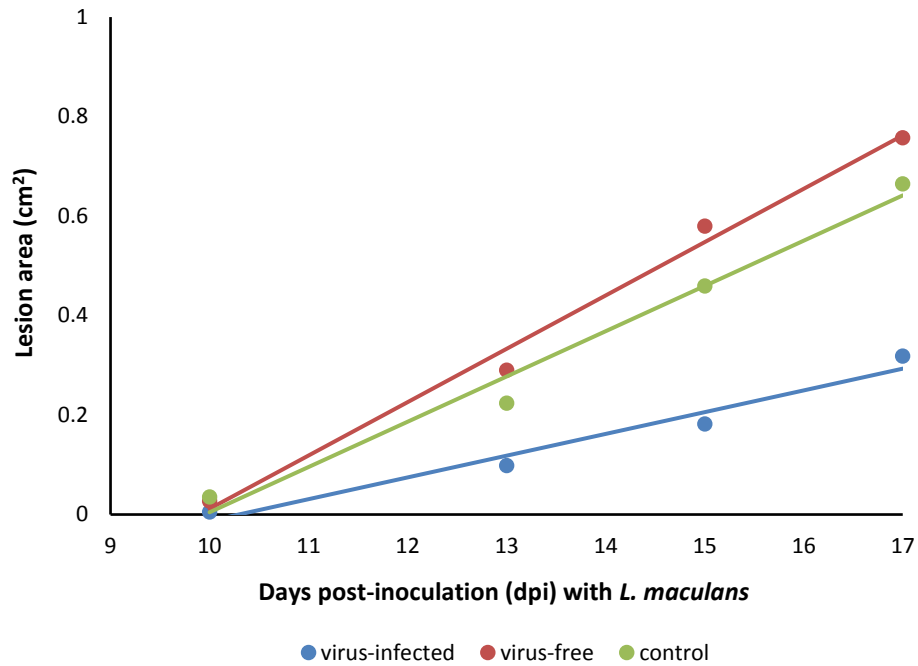
**Figure 6.1 Phenotypes of resistance induced by *L. biglobosa* against *L. maculans* under controlled environment conditions.** Effect of pre-inoculation of oilseed rape (cv. Excel) with virus-infected (a, d, g) and virus-free (b, e, h) *L. biglobosa* (isolate W10) on development of phoma leaf spot lesions on second true leaves following challenge inoculation with conidia of *L. maculans* (isolate H-Rox 12.2.1), assessed at 13 (a, b, c), 15 (d, e, f) and 17 (g, h, i) dpi. Leaf 1 was pre-inoculated with conidia of virus-infected *L. biglobosa*, virus-free *L. biglobosa* or sterile water (control: c, f, i).

**Table 6.1 Mean lesion area on oilseed rape second true leaves challenge inoculated with conidia of *L. maculans* following pre-inoculation of leaf one with spores of virus-infected *L. biglobosa*, virus-free *L. biglobosa* or water (control).**

Lesion areas were assessed over time up to 17 dpi for up to 18 lesions for each pre-treatment (mean  $\pm$  SD).

| Pre-treatment      | Lesion area (cm <sup>2</sup> ) |                   |                   |                   | Intercept | Slope |
|--------------------|--------------------------------|-------------------|-------------------|-------------------|-----------|-------|
|                    | 10 dpi                         | 13 dpi            | 15 dpi            | 17 dpi            | a         | b     |
| Virus-infected W10 | 0.005 $\pm$ 0.009              | 0.098 $\pm$ 0.062 | 0.182 $\pm$ 0.076 | 0.318 $\pm$ 0.117 | -0.450    | 0.044 |
| Virus-free W10     | 0.026 $\pm$ 0.033              | 0.289 $\pm$ 0.131 | 0.579 $\pm$ 0.200 | 0.757 $\pm$ 0.184 | -1.060    | 0.107 |
| Control            | 0.035 $\pm$ 0.038              | 0.223 $\pm$ 0.042 | 0.459 $\pm$ 0.194 | 0.664 $\pm$ 0.359 | -0.905    | 0.091 |

Parameters were estimated from the linear regression equations  $y=a+bt$ , where  $y$  is average lesion area,  $t$  is days (dpi), and  $a$  and  $b$  are the intercept and slope parameters of the lines, respectively (shown in Fig. 6.2).



**Figure 6.2** Increases in phoma leaf spot lesion area with time following challenge inoculation of second true leaves with *L. maculans* following different pre-inoculations of leaf one. Pre-inoculations were with spores of virus-infected *L. biglobosa*, virus-free *L. biglobosa* or water (control). Each pre-treatment comprised nine plants and eighteen replicates. Values of parameters for these linear regressions are given in Table 6.1.

Induction of SAR has also been demonstrated in other host/pathogen interactions following single leaf inoculations (Hammerschmidt *et al.*, 1982; Kuc & Richmond, 1977; Strömberg, 1995). This practice could be significant when inducing resistance in the field on a larger scale.

Rasmussen *et al.* (1992) observed a strong induction of the PR protein chitinase mRNA following *L. maculans* infection. Moreover, 1 day after inoculation of *L. maculans*, levels of chitinase mRNA were 3-fold higher in resistant as compared to susceptible *Brassica* cultivars. Differential expression of PR proteins in *B. napus* and *B. nigra* was also observed following inoculation of *L. biglobosa* or *L. maculans* (Dixelius, 1994). Significant accumulation of PR proteins was observed in resistant plants 2-4 h after infection by *L. biglobosa*, while in susceptible plants, significant accumulation was observed 8 h after infection. Both *L. maculans* and *L. biglobosa* are able to induce gene expression associated with SAR and these genes are activated soon after inoculation.

In previous work which investigated induced resistance in *B. napus*, ascospore suspensions were used as inoculum as these inoculations do not require wounding and avoid interference by wound-induced signalling (Liu *et al.*, 2006; 2007). Moreover, primary infection of winter oilseed rape crops arises from ascospores (West *et al.*, 2001). In the current study, conidial inocula, which do require wounding for successful invasion were used for both pre- and challenge inoculations. This method has been used successfully before to induce resistance in plants (Fristensky *et al.*, 1999; Mahuku *et al.*, 1996; Rasmussen *et al.*, 1992). Wounding might be a potential causal factor of a hypersensitive reaction. However, Felton *et al.* (1999) demonstrated that different signalling pathways are involved in systemic response to wounding and insect herbivory. Peltonen (1998) also reported that wounding itself did not induce defence related enzymes.

*L. maculans* and *L. biglobosa* not only co-exist in oilseed rape crops (West *et al.*, 2001; 2002), but also co-infect the same site on plants (Mahuku *et al.*, 1996); therefore, it is reasonable to propose that induction of host resistance against *L. maculans* also occurs naturally following simultaneous infection with, for instance virus-infected *L. biglobosa*.

When virus-free *L. biglobosa* was used for pre-inoculation, greater lesion sizes were noticed on the second true leaves following challenge inoculation with *L. maculans*. Decrease in lesion size was noticeable only in treatments when virus-infected *L. biglobosa* was used for pre-inoculation. This feature might have resulted from virulent *L. biglobosa* isolate competing with *L. maculans* for nutrients and space on their common host. Alternatively or additionally virulent *L. biglobosa* induced host defences against *L. maculans* through active synthesis and accumulation of structural and chemical defence components which enhance the plant defence capacity, resulting in plants being more resistant to direct penetration and colonization by an invading pathogen.

Inoculation of *B. napus* cotyledons with virus-infected *L. biglobosa* itself produced larger lesions than those of virus-free *L. biglobosa* (Section 5.3.2). So, virus-infected *L. biglobosa* might greatly enhance the activity of defence related enzymes (e.g. chitinase,  $\beta$ -1, 3-glucanase, peroxidase, and phenylalanine ammonia lyase) through the SA signal pathway.

This investigation indicates that inoculation with virus-infected *L. biglobosa* appears to trigger defence reactions against *L. maculans*. More research on the interactions of *L. maculans* and *L. biglobosa* with their host and their impact on biological control is required. How the presence and absence of mycoviruses in *L. biglobosa* affect the induction of resistance against *L. maculans* in oilseed rape is not understood but it may well involve gene silencing and this too requires further work.

## **6.5 Conclusions and future work**

Results of this study suggest that pre-treatment of *Brassica napus* leaves with virulent biological activator can induce systemic resistance to *L. maculans* on the development of phoma leaf spots under controlled environment conditions. Pre-treatment of oilseed rape leaves (cv. Excel) with virus-infected *L. biglobosa* not only delayed the appearance of *L. maculans* phoma leaf spot lesions but also decreased the phoma leaf spot lesion area on untreated leaves (systemic effect) while the rate of increase in phoma leaf spot area after challenge inoculation was significantly greater on virus-free *L. biglobosa* pre-treated plants. Results of this study will improve the understanding of resistance against *L. maculans*, which can be used to guide forward

planning for effective control of phoma stem canker. However, the following ideas could be implemented for the future work:

1. Use of ascospores for pre-treatment and challenge inoculation of *Brassica napus* leaves.
2. Use of different plant cultivars and different pre-inoculation methods to analyse any difference in systemic resistance to *L. maculans*.
3. Perform field experiments to determine whether pre-treatment can induce local and systemic resistance to *L. maculans* on development of phoma leaf spotting and stem canker.
4. Moreover, future work concerns quantification of transcript levels of genes involved in signalling pathways after *L. maculans* challenge inoculation for evaluation of defence-associated responses during pathogenesis.

New approaches in this direction can be prompted using techniques described in this study and previous work (e.g. Liu *et al.*, 2006; 2007).



## **Chapter 7**

### **General discussion**

This study is the first to report the presence and molecular characterisation of mycoviruses LbMV-1 and DsCV-1 infecting *L. biglobosa* and *D. septosporum*, respectively. Moreover, the molecular features of *Aspergillus foetidus* virus AfV-S3 were also characterised.

In current investigation 11 out of 16 *L. biglobosa* isolates were found to contain LbMV-1 while none of the 57 isolates of *L. maculans* were found to contain dsRNAs. In addition to this, one out of 45 *D. septosporum* isolates was found to contain DsCV-1. In contrast, none of the 162 *H. fraxineus* and 10 *Pyrenopeziza brassicae* isolates appeared to contain dsRNA elements.

Complete cDNA clones of LbMV-1, DsCV-1 and AfV-S3 were obtained using different RT-PCR methods such as Froussard procedure, single-primer method (Isogai *et al.*, 1998; Vreede *et al.*, 1998) and RLM-RACE (to determine 5' and 3' terminal sequences) for cloning dsRNAs of unknown sequence.

The three dsRNAs of the LbMV-1 genome, isolated from *L. biglobosa* strain C-Rox 12.8.1, with lengths 4917, 4490 and *ca.* 4000 bp (dsRNAs 1-3) were found to contain a single open reading frame (ORF) encoding a hypothetical protein (HP), an RNA-dependent RNA polymerase (RdRP) and possibly HP or RdRP, respectively, in which dsRNAs 1 and 2 exhibited some degree of sequence similarity to the comparable putative proteins encoded by ACD-L associated dsRNAs. Several attempts failed to produce any clones of LbMV-1 dsRNA 3.

The four dsRNA segments, ranging in size from *ca.* 2.8-3.5 kbp, of the DsCV-1 genome were isolated from *D. septosporum* strain D 752.1. Although DsCV-1 contains four dsRNA segments, this study was only focused on molecular characterisation of two dsRNA elements (DsCV-1 dsRNAs 2 and 3). Sequence analysis provided evidence that each of the identified dsRNA segments contained one ORF, putatively encoding an RdRP, CP, hypothetical protein and protease, respectively, all of which showed sequence similarity with members of the family *Chrysoviridae*.

Molecular characterisation of one of the slow migrating components of *A. foetidus* dsRNA AfV-S3 was completed using the RLM-RACE technique, which is used for sequencing 5' and 3' terminal sequences of dsRNAs. Here this technique was successfully employed to sequence the whole 439 bp fragment. The potential satellite

RNA of AfV-S investigated here might depend on the other helper viruses for its replication.

Based on the nucleotide sequence, sequence properties at the 5' and 3' termini, genome organization and phylogenetic analysis, it was confirmed that a new dsRNA mycovirus (LbMV-1) identified in *L. biglobosa* constitutes a new putative member of the *Totiviridae* family while a new dsRNA mycovirus (DsCV-1) identified in *D. septosporum* constitutes a new member of the *Chrysoviridae* family. In addition to this, AfV-S3 identified in *A. foetidus* has been presumed as a satellite component of AfV-S virus particle.

It is important to characterise the remaining dsRNAs of LbMV-1 and DsCV-1 so that the relationship of these viruses can be studied in relation to origin and function of other known viruses. The potential pathogenic effects of DsCV-1 in *D. septosporum* were not evaluated in the presented work. Further studies will help to elucidate how DsCV-1 affects the performance of *D. septosporum* as biocontrol agent.

The incidence of mycoviruses was normally assessed using common nucleic acid extraction methods (Bhatti *et al.*, 2012; Refos *et al.*, 2013). However, this small-scale dsRNA extraction method can be laborious to screen large number of isolates and when the virus is low in titre. So, in this study for the diagnosis of virus infection, a sensitive RT-PCR method was used accompanying the other advantages such as being easy to perform, rapid and cost-efficient (Dai *et al.*, 2012). This method uses the total RNA template obtained from a rapid kit RNeasy extraction which also makes the procedure reproducible and quicker. This method is only appropriate for characterising viruses with known genomic sequence.

The effects of mycovirus infection were determined by producing isogenic lines of *L. biglobosa* isolate W10, obtained by eradicating LbMV-1 from virus containing W10 using cycloheximide. A different range of cycloheximide concentrations (0.01 mM to 150 mM) were used for this purpose. Attempts were made to eradicate LbMV-1 from C-Rox 12.8.1, K-Exc 12.10.21, Noth2, N20.3, W10 and N3.4 isolates of *L. biglobosa*. However, virus infection could successfully be eradicated only from 0.1 mM cycloheximide treated W10 isolate. The isogenic lines were then investigated in terms of biomass production and mycelial growth.

Results showed that LbMV-1 has the potential to confer increased virulence (hypervirulence) to *L. biglobosa* as the infected isolate had a faster growth rate on PDA and greater biomass production in PDB as compared to the isolate that had been cured. Results exhibited in this study support the hypothesis that mycoviruses can alter host behaviour. As virus-infected and virus-free isogenic lines exhibited differences in phenotypes such as pigmentation and growth rate, it was assumed that *L. biglobosa* mycoviruses can affect gene expression via RNA silencing and can also affect pathogenicity of host. Moreover, a faster growth rate of the pathogen might develop from gene regulation by mycovirus-derived small (s) RNAs. Further investigations such as microarray or mRNA sequencing should also be considered in order to identify any differentially expressed genes during virus infection regardless of RNA silencing.

Assessment of virulence in the presence and absence of mycoviruses in *L. biglobosa* is pivotal to understand its pathogenicity. Here, for the first time, the effect of mycoviruses on the pathogenicity of *L. biglobosa* was investigated on cotyledons of oilseed rape. A statistically significant increase in phoma leaf lesion areas on cotyledons infected with virus-containing W10 isolate in comparison to virus-free W10 isolate was noted.

Thus, pathogenicity tests with isogenic lines of virus-infected and virus-free isolates of *L. biglobosa* indicated that the presence of mycoviruses confers hypervirulence to the fungal host. A detailed study should also be conducted to assess canker severity on stems using these isogenic lines of virus-infected and virus-free isolates of *L. biglobosa*. Moreover, transcriptome analysis will be essential to understand the mycovirus-fungus-plant interactions. Recently, genome-transcriptome analysis of *Fusarium graminearum* revealed the effect of mycoviruses on host gene expression and attempts were made to identify the genes linked to the phenotypic changes in the host. Lee *et al.* (2014) found that mycovirus infection resulted in down-regulation of host genes required for cellular transport systems, RNA processing and ribosome assembly. This study provides the first evidence towards characterisation and explaining the effects of mycoviruses on biological responses to *L. biglobosa*.

This work provides a reliable methodology to study the effect of pre-treatment with *L. biglobosa* on systemic resistance of oilseed rape to *L. maculans* infection.

Development of this method will also improve the process of breeding cultivars for control of crop diseases and is also possible to test effectiveness of resistance under a range of different controlled conditions.

*L. biglobosa* causes superficial stem lesions and considered as weakly virulent or avirulent (Shoemaker & Brun, 2001) while *L. maculans* causes more serious stem cankers and is considered as a highly virulent species (Howlett *et al.*, 2001). The defence capacity of plants can be enhanced by induced resistance, activated by biotic or abiotic agents. Previously weakly virulent *L. biglobosa* has been shown to induce resistance in host plants against *L. maculans* without the knowledge of presence or absence of mycoviruses in *L. biglobosa* (Liu *et al.*, 2006; 2007; Chen and Fernando, 2006). However, this study indicated that reduced disease was only observed on plants pre-inoculated with virus-infected *L. biglobosa* compared with virus-free *L. biglobosa* pre-inoculated plants.

*L. biglobosa* typically appears later in the growing season than *L. maculans* (West *et al.*, 2001) and causes disease at higher temperatures (Badawy *et al.*, 1992). This might be the reason why *L. biglobosa*-induced resistance has not been observed in crops. The application of virus-infected *L. biglobosa* prior to the natural infection by *L. maculans* might effectively induce resistance and significantly decrease infection and disease.

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## Appendices

### Appendix 1: List of materials

#### 1. Antibiotics

Antibiotics were dissolved in sterilised distilled water and filter-sterilised. Aliquots were stored at -20°C. Sterilised media were cooled to below 50°C prior to antibiotic addition to prevent heat inactivation.

**Table 1.1 List of antibiotics and their concentrations.**

| Antibiotics  | Stock concentration | Working concentration |
|--------------|---------------------|-----------------------|
| Penicillin   | 100 mg/ml           | 100 µg/ml             |
| Streptomycin | 100 mg/ml           | 100 µg/ml             |
| Ampicillin   | 100 mg/ml           | 100 µg/ml             |

#### 2. Media, solutions and buffers

##### 2.1 Malt extract agar (MEA)

|                             |         |
|-----------------------------|---------|
| Malt extract (Oxoid)        | 20 g    |
| Technical agar no.3 (Oxoid) | 15 g    |
| Sterilised distilled water  | 1000 ml |

##### 2.2 V8 agar

|  |        |
|--|--------|
| V8 (Vegetable juice)                   | 200 ml |
| Calcium carbonate (CaCO <sub>3</sub> ) | 2 g    |
| Technical agar no.3 (Oxoid)            | 20 g   |
| Sterilised distilled water             | 800 ml |

##### 2.3 Potato dextrose agar (PDA)

|                            |         |
|----------------------------|---------|
| Potato dextrose agar       | 39 g    |
| Sterilised distilled water | 1000 ml |

PDA and V8A media were prepared to culture *Leptosphaeria* species

## 2.4 LBA medium

|                             |         |
|-----------------------------|---------|
| Tryptone (Oxoid) or peptone | 10 g    |
| NaCl                        | 10 g    |
| Yeast extract               | 5 g     |
| Technical agar no.3 (Oxoid) | 12 g    |
| Sterilised distilled water  | 1000 ml |

## 2.5 1x LB medium

|                             |         |
|-----------------------------|---------|
| Tryptone (Oxoid) or Peptone | 10 g    |
| NaCl                        | 10 g    |
| Yeast extract               | 5 g     |
| Sterilised distilled water  | 1000 ml |

## 2.6 2x LB medium

|                             |         |
|-----------------------------|---------|
| Tryptone (Oxoid) or peptone | 20 g    |
| NaCl                        | 20 g    |
| Yeast extract               | 10 g    |
| Sterilised distilled water  | 1000 ml |

Different kinds of LB media were prepared to culture *E. coli* cells.

## 2.7 Transformation storage solution (TSS)

|                            |        |
|----------------------------|--------|
| 2x LB                      | 5 ml   |
| PEG 8000                   | 1 g    |
| Dimethyl sulfoxide (DMSO)  | 0.5 ml |
| MgCl <sub>2</sub> (1 M)    | 0.5 ml |
| Sterilised distilled water | 3 ml   |

## 2.8 X-gal solution

|                            |       |
|----------------------------|-------|
| IPTG                       | 10 µl |
| X-gal                      | 20 µl |
| Sterilised distilled water | 90 µl |

120 µl of X-gal solution is required for each LBA plate.

X-gal solution is freshly prepared every time.



### **2.9 Super optimal broth with catabolite repression (SOC - 10 ml)**

|                         |        |
|-------------------------|--------|
| Glucose (2 M)           | 200 µl |
| MgCl <sub>2</sub> (2 M) | 50 µl  |
| 1x LB                   | 10 ml  |

SOC solution is freshly prepared every time.

### **2.10 Extraction buffer for small-scale dsRNA extraction**

EDTA (20 mM) (pH 8.0)  
Tris-HCl (20 mM) (pH 7.5)  
1% SDS  
1% NaCl

### **2.11 TE buffer**

|                            |        |
|----------------------------|--------|
| Tris-HCl (1 M)             | 50ml   |
| EDTA (0.5 M)               | 2 ml   |
| Sterilised distilled water | 948 ml |

### **2.12 50x TAE buffer**

|                            |          |
|----------------------------|----------|
| Tris base                  | 242 g    |
| Glacial acetic acid        | 57.1 ml  |
| EDTA (0.5 M)               | 100 ml   |
| Sterilised distilled water | 842.9 ml |

### **2.13 Maleic acid buffer for determination of labelling efficiency and Immunological detection**

0.1 M Maleic acid, 0.15 M NaCl; adjust with NaOH (solid) to pH 7.5

### **2.14 Blocking solution for chemiluminescent detection procedure**

Dilute 10x blocking solution (vial 6) 1:10 with Maleic acid buffer

**2.15 Washing buffer for determination of labelling efficiency and Immunological detection**

0.1 M Maleic acid, 0.15 M NaCl; pH 7.5; 0.3% (v/v) Tween 20

**2.16 Detection buffer for determination of labelling efficiency and Immunological detection**

0.1 M Tris-HCl, 0.1 M NaCl, pH 9.5 (20°C)

**Appendix 2: List of oligonucleotide primers used to complete the dsRNA sequences of DsCV-1 and LbMV-1.**

**Table 2.1 Sequence of primers designed on the basis of multiple alignment of RdRPs of different members of *Chrysoviridae* family to sequence DsCV-1 dsRNAs.**

| <b>Name of primers</b> | <b>Sequences (5'-3' orientation)</b> |
|------------------------|--------------------------------------|
| DF 1                   | 5'-GTTCAACGTGGCTAACTAAGGG-3'         |
| DF 2                   | 5'-GGGACTGTACTCAGGATGG-3'            |
| DR 1                   | 5'-GCCATCCTGAGTACAGTCC-3'            |
| DF 3                   | 5'-TATAGACCATGGCGGTGATGA-3'          |
| DR 2                   | 5'-ACATCATCACCGCCATGGTC-3'           |
| DF 4                   | 5'-TTCGTGGCAGGCGATTGG-3'             |
| DR 3                   | 5'-CCAATCGCCTGCCACGAA-3'             |

**Table 2.2 Sequence of primers used for extending known sequence of DsCV-1 dsRNAs1 and 2 in both the 5'- and 3'- directions.**

| <b>Name of primers</b> | <b>Sequences (5'-3' orientation)</b> |
|------------------------|--------------------------------------|
| <b>RNA-2 primers</b>   |                                      |
| DR 463                 | 5'-GTG CTGATATGATCCCGTTC-3'          |
| DF 3.24                | 5'-GTGTCGCCAATTACTTTGATAAC-3'        |
| DR 3.24                | 5'-CACAGGGGTTTCGATTCTCC-3'           |
| DR 2434.1              | 5'-GCATAAGGAACCCCTTGAC-3'            |
| DR 2434.2              | 5'-CCTATCAGCACTTTGAACATGC-3'         |
| DR 2434.3              | 5'-CTTTGACTGACCAACCATCACC-3'         |
|                        |                                      |
| <b>RNA-3 primers</b>   |                                      |
| DR 13.2                | 5'-CTTCCATGAGGGCTACTTCAGC-3'         |
| DR 13.3                | 5'-GTGGCTGCGAAATAGGTATGC-3'          |

|           |                               |
|-----------|-------------------------------|
| DR 2701.1 | 5'-CCACGAACCCATGATATAATT G-3' |
| DR 2701.2 | 5'-ATCGATCCAGCCTTCCTCCC-3'    |
| DR 2701.3 | 5'-GATATCAATGAGTCGAAATCG-3'   |
| DR 2701.3 | 5'-CCACGAACCCATGATATAATT G 3' |
| DR 2701.4 | 5'-CTTACTCACATCTAGGTTCTT C-3' |
| DR 2844.1 | 5'-CTATCTTTCCCAGATCGACCTC-3'  |
| DR 2844.2 | 5'-CTTACTCACATCTAGGTTCTTC-3'  |
| DR 3298   | 5'-CATGCCCTCACTGTAGGTTG-3'    |
| DF UTR    | 5'-CAAAAATCCTAGCGGCCTGA-3'    |
| DF IJ1    | 5'-GTCGCATCTGTCTTATGAAG-3'    |
| DF IJ2    | 5'-GCGAGATAGATGTAAACGGA-3'    |
| DF IJ3    | 5'-GAGTTGGACGGCCTGAGTA-3'     |

**Table 2.3 Sequence of primers used for extending known sequence of LbMV-1 dsRNA(s) in both the 5'- and 3'- directions.**

| <b>Name of primers</b> | <b>Sequences (5'-3' orientation)</b> |
|------------------------|--------------------------------------|
| <b>RNA-1 primers</b>   |                                      |
| LBF 1                  | 5'-TTGAGCTGAGACCGACACCGC-3'          |
| LBR 1                  | 5'-AGAGTG TTAACAGCCCTGCGC-3'         |
| LBR 2                  | 5'-CATGACTACGCCTTTTGGGGC-3'          |
| LBR 3                  | 5'-GCTGCACGCCTCGCGATGG-3'            |
| LBR 4                  | 5'-CCTTGTCAGCTTCGTGTAACCAGG-3'       |
| LBR 5                  | 5'- GCTCTCACCTTAGACCACTCG-3'         |
| LBR 6                  | 5'-CTGGGATTCATCTCTCCTGTC-3'          |
| LBR 6A                 | 5'-CTCCTGTCTAATATCTCATCATCC-3'       |
| LBR 7                  | 5'-CTGTGGTGCTTCTGCGTGC-3'            |
| LBR 8                  | 5'-CGAAATCGGTACGCTGTGTGC-3'          |
| LBR 9                  | 5'-GCAGAAATCGTGCATGTCATC-3'          |
| LBR 9A                 | 5'-GCGCATCTATTATGTTCTTATTGGC-3'      |
| LBR 9B                 | 5'-CACGGATGCTTGTTGTTCTTTCAA-3'       |

| <b>RNA-2 primers</b> |                               |
|----------------------|-------------------------------|
| LBF 1                | 5'-GCTTGACGCATGCAATCTGAT C-3' |
| LBR 1                | 5'-GTC TCA CCG AAAGTGCCATC-3' |
| LB1F 1247.1          | 5'-CAATTCATCTCACAGGGCAGG-3'   |
| LB1R 1247.1          | 5'-GAATCCTTGCGCTTCATCAAC-3'   |
| LB1F 1247.2          | 5'-GTGATATACGGCCAGGAAGG-3'    |
| LB1F 2351.2          | 5'-GAGCGACAAAGTTAGAGCAGC-3'   |
| LB1R 1247.2          | 5'-GAAACCACGTTGCGTATGAATCC-3' |
| LB1R 1247.3          | 5'-CCAGCCCAACTCGCAATTG-3'     |
| LB1R 2351.1          | 5'-CGATATCACCCCAACCTT G-3'    |
| LB1R 2351.2          | 5'-GTTCCCTCGCTCAGTAGATTTG-3'  |
| LB1F 1593.1          | 5'-GACACCTAGCAGCAA CATGC-3'   |
| LB1F 1593.2-         | 5'-GTCTCCGAAACATTGGCTAG-3'    |
| LB1R 1593            | 5'-CGTTCGTGAACGTAAGTGG-3'     |
| LB1R 2518            | 5'-CTGCACTGTTCCCTCGCTCAG-3'   |

### **Appendix 3: Problems encountered while obtaining the clones of viral nucleic acid.**

Few problems which occurred during different phases of cloning and sequencing are listed below:

#### Phase 1: RT-PCR.

The different range of size of the PCR products (~100 bp-1000 bp) were obtained after RT-PCR. Sometimes, RT-PCR did not work and did not obtain any amplicons. After performing the RT-PCR and cloning, the clones were sent for sequencing. Normally, BLASTX was used to recognise the sequence similarity of the clones with the other known sequences in the database. However, sometimes more sophisticated programs were also used such as PSI BLAST which simply compares the protein query to a protein database. Sequence analysis showed that it was not possible to get the viral clones all the time when the RT-PCR was performed. We obtained combination of clones including fungal, bacterial and sometimes the clones with no known sequence similarity in the database.

Sequences of LbMV-1 dsRNAs 1 and 2 were confirmed after cloning and sequencing the individual clone three to four times. Approximately 10-12 different clones were used to assemble the final sequence.

#### Phase 2: Ligation of PCR products.

Sometimes freezing and thawing decreased the activity of enzyme and ligation did not work. Therefore, the reagents were aliquoted before use.

#### Phase 3: Blue-white screening to identify recombinant clones.

Sometimes there were few or no transformants (when competent cells did not work) or many satellite colonies (when ampicillin did not work) on the plate after transformation.

#### Phase 4: Overnight growth of individual colonies, plasmid extraction, restriction digestion and agarose gel electrophoresis.

Sometimes the selected white colonies did not show any growth next day in the LB media or if they grew, insert and plasmid were not observed on the gel after plasmid extraction thus they were confirmed as the satellite colonies.

**Appendix 4: Alignment of amino acid sequences of *Dothistroma septosorum* chrysovirus-1(DsCV-1) dsRNAs 2 and 3 ORFs with the comparable ORFs of *Isaria javanica* chrysovirus-1 (IjCV-1) dsRNAs 2 and 3.**

|                      |   |        |
|----------------------|---|--------|
| DsCV-1 dsRNA2 (160)  | FSNERYDEKTAAFNALRSGATALRRSKALSVKLTSDWPATRAGASNFREKQNMMLGRDIGS | (339)  |
| IjCV-1 dsRNA2 (6)    | + E K AAF ALRSGA AL + + L W P T GA + EKQ++ GRDIG              | (65)   |
| DsCV-1 dsRNA2 (340)  | VANYFDNKRSSALEVICKDEFTVNYQIYGDIREAVFGQNTLSIFFPIKWSQCEVNVSLY   | (519)  |
| IjCV-1 dsRNA2 (66)   | VA+YFDNK SSAL++IC+DEF ++YQI+GDI REA+FG N + +F +KW ++V LY      | (125)  |
| DsCV-1 dsRNA2 (520)  | PDVLDKPIPREKFTTAAREGIPNRDDIAKVTGWNRNVVRDIQDTDISMFKVLIGQVMVGQ  | (699)  |
| IjCV-1 dsRNA2 (126)  | P LD+ + R+K T+AAAREGIP+RD +AK TGWNRN VR +QD D+S FK L+ QV VGO  | (185)  |
| DsCV-1 dsRNA2 (700)  | SKLTRLVKGFMLMLECMERHDHIDVVLVQNTVLYNPTQVLNSFRANGRAYVYNSKPSSSV  | (879)  |
| IjCV-1 dsRNA2 (186)  | SKLTRLVKGFMLMLECMERHIDVVL VQ T++Y P V+ SF A+GRAYVYNSKPS SV    | (245)  |
| DsCV-1 dsRNA2 (880)  | HTAVLWRMCGAYPPPEIRGSHIQIPADGANVFMVLEGAVPAQQQRVRLTQGLIYASIMAY  | (1059) |
| IjCV-1 dsRNA2 (246)  | ++AVLWRMC AYPPE+ GSHI IP+DGA+V MV EG + G VRLT LIYAS+M Y       | (305)  |
| DsCV-1 dsRNA2 (1060) | AMDVSTQHLLQALIIACSLQQNRYFSRVQLPAVVSVMMLMIPAFMTTSSRLDKPILSIE   | (1239) |
| IjCV-1 dsRNA2 (306)  | AMD CT HLQAL+IACSLQQNRYFS+V+LP VVSV DLM+PAF +S+LDKPILS+       | (365)  |
| DsCV-1 dsRNA2 (1240) | MATSVGRRLRQMLFMNVKDVLTSAELSTSRGFDPAQSMRSYMSSQAALITQMSSEISALC  | (1419) |
| IjCV-1 dsRNA2 (366)  | MA SVGRL QML F ++D LT+AELSTS GFDP SMR+Y+ SQ ++++MSS IS L      | (425)  |
| DsCV-1 dsRNA2 (1420) | LVEAAIKMKVHEAMREDDFSDILNLSAFEGWLWCQEGTKSVKNGIISALVNGVSDLSGDM  | (1599) |
| IjCV-1 dsRNA2 (426)  | L+EA MK+H+ + DF D+L++S EGLWLCQE K+V NG+I +LV GVSD+S +         | (485)  |
| DsCV-1 dsRNA2 (1600) | CSYDIVRREMDLGRIVYDPAAMPKGAFTVGVWCVSPSIKKAVPAPRKRLSRQVTLVHPCE  | (1779) |
| IjCV-1 dsRNA2 (486)  | +YD+++RE+ L + + +P+G FTVGWV V+ PRKR + + LV C+                 | (545)  |
| DsCV-1 dsRNA2 (1780) | TKPGSTLRVSRKKRFMRNDHSGENETSRSPPTPETIPV                        | (1893) |
| IjCV-1 dsRNA2 (546)  | P R++RF R + +P T IP+  | (577)  |
|                      | FNPRLREIGRRRERFSR-----GKAPVTPPRGTQIPI                         |        |

**Figure 4.1 Alignment of the amino acid sequence of DsCV-1 dsRNA 2 ORF with the comparable ORF of IjCV-1 dsRNA 2. Consensus amino acids are highlighted in yellow.**

|                      |   |        |
|----------------------|---|--------|
| DsCV-1 dsRNA3 (154)  | KPKFEGGKTRRKETKENASVRRFTMGSSSEM----LPAPTTD--LRNFIGVVVPQGHGKTM   | (315)  |
| IjCV-1 dsRNA3 (159)  | KEDFWADQERARAKVADAI SRMKRLHGAEMPKHTIPVPSSEDEVRSDVFGVMPMGHGKTT   | (218)  |
| DsCV-1 dsRNA3 (316)  | LAREEGWidfdslissrslsdlrETAYDEIKAGRSIEDASTNFAAEARETLKLLNPDYPT    | (495)  |
| IjCV-1 dsRNA3 (219)  | LAREEGWVDFDSLISPKKRNELLDEVYERIAGGASIGCALSVLLPEAKKTLALLRPKERW    | (278)  |
| DsCV-1 dsRNA3 (496)  | ILMCQTFSLLEAIGVDCIGAVAVRPDVVLKHNKRRQLHELLMIEKNIEEVVNYDRWDSAE    | (675)  |
| IjCV-1 dsRNA3 (279)  | VLVAQDACLLAGLNVCEAGGIVMDERVVLEACQGRQEFEEILIRKNMAEVAE-QQAGAGT    | (337)  |
| DsCV-1 dsRNA3 (676)  | VALMEGMDDVRWYIYNICQHLGIPISOPHLYSMVDENIRSCYKTKGYPPLTEVVS DY YA   | (855)  |
| IjCV-1 dsRNA3 (338)  | LLTSESYEETRMLAYRICIAFGITVSRPSDFGLTDPLMSQGPGLTSTKMDLEDMVTYYDK    | (397)  |
| DsCV-1 dsRNA3 (856)  | GLIPREVV DHHVQAAGLRSYQGFQFTMNDWAKVVGHANSTRGSTTFSDIDWNAPMSLKG    | (1035) |
| IjCV-1 dsRNA3 (398)  | GKVPRESVDYQVHLSGLQSYKGFQFTSNKWAKFLSKVTETIGDVSFVDADWNPRVMSLDT    | (457)  |
| DsCV-1 dsRNA3 (1036) | LSESLDLAQHDDIRFIEAHKGEHERFILGLILHWKmlgltsglkskllplYAVKRVHWV     | (1215) |
| IjCV-1 dsRNA3 (458)  | FGSHSDLSGHDDVQYILSAQKGEHERFVLGLLHWKIGMHCGLGNRLLPFYGVRRCHWK      | (517)  |
| DsCV-1 dsRNA3 (1216) | AVFSKVRDGLASNGLFGQPLTVEERELVISMRLLAAGEFSQLQRLTSEQGS CPRRSPT     | (1395) |
| IjCV-1 dsRNA3 (518)  | SVMANLRECVASSGTFMGLNLTMEERKHMSLGLLVGGSLNELKGMFVKVSYPR IAPG      | (577)  |
| DsCV-1 dsRNA3 (1396) | RLIQDSASRGLDSVVFCA DSHQERELAFSEMLKQSRMSSLNEVDW-----DGKLRQLQSI   | (1560) |
| IjCV-1 dsRNA3 (578)  | LAVENKVIKQLDQVGFKEVGRACLVEKLLGQSSLS TISHLVWAVGDQPSAMPLNEAI      | (637)  |
| DsCV-1 dsRNA3 (1561) | AKSIGKDLRLRWRDEQRGLERSRILAGLLKRWHKACLIRDEWSDMTNALLEECSEHEAL     | (1740) |
| IjCV-1 dsRNA3 (638)  | AISLGLELCRRWGDND S ALERIPGITHKLI TKWYKVCVLRDEWSDFINRIMAAEVRNFGL | (697)  |
| DsCV-1 dsRNA3 (1741) | GDAIAAMLSCSIQEGSEGH DWSVRVMEALKGFVVCGLVCEGK GK---VVMQQTHKLLRPC  | (1911) |
| IjCV-1 dsRNA3 (698)  | AQATAKICSCDPQAGTSGLEWGVVCEALKGFIVCSIVTL-PGKLIGVVEDRATGQCRPC     | (756)  |
| DsCV-1 dsRNA3 (1912) | VLGMNEAEIWSKAISLNI PRGALGCFSSGISHLQLLNELCGWSTNKTVMVMEMINSSSWM   | (2091) |
| IjCV-1 dsRNA3 (757)  | VGGIEEGDLWKAI IQSNI PRHALGVFGGTVSALS LANELCEWQDSPTVAILEMVNCHSWE | (816)  |
| DsCV-1 dsRNA3 (2092) | PNMSKRATLAGLCRWRNF FTDVQERYIFEKIADSYTIRSMGRSYLKVEHRLMELISVKTS   | (2271) |
| IjCV-1 dsRNA3 (817)  | PRIGPGRMATCIYRWGRVCSGKVS DAVSSFMLNSHSYSRFGHSYARIASRLEFYARLSSR   | (876)  |
| DsCV-1 dsRNA3 (2272) | SGGLGCGPTLYDA-KV NK-GTDGFWNGHGKIGFGRKSRMSGDPGSIADMIELFERDDGRE   | (2445) |
| IjCV-1 dsRNA3 (877)  | CGGLGCHQTHIDSGSVEKIGPRGEWIGTGKI-HTLKEEKRADTTT LAEKISQFQ-DQGLQ   | (934)  |
| DsCV-1 dsRNA3 (2446) | QKTIAPK---SIYEVGLASLQVIRADNKSHLSR HARLLVRLA (2562)              |        |
| IjCV-1 dsRNA3 (935)  | YGT--PKDIRLAINLCGMVVCSLLQDQGKSALNINATLLTALA (975)               |        |

**Figure 4.2 Alignment of the amino acid sequence of DsCV-1 dsRNA 3 ORF with the comparable ORF of IjCV-1 dsRNA 3. Consensus amino acids are highlighted in yellow.**



## Appendix 5: Nucleotide sequences of LbMV-1 dsRNAs 1 and 2.

### 1. LbMV-1 dsRNA 1 sequence

TACGAAAAACACAATATCACCTACAGTACCACGCCGACCAGAGACAGCATGATCATCGGCAATATCGACTCCACA  
TACGAGGGGCTGGTTTCTGCCCTTGCATGAAACTCGCCACAGTGGGGCAGCGGTACGGCCAAGTTCGTTAGT  
AGCACCGTTGTGCCGGGCGATGACAGGGCGCGAAATGTCATTTCTACTATTTCCACGCTGACTAGCAAGTGGGCA  
GAGATAACTCACCTGAGCTCAAGGCATGACACGCTATGGACCGCAGTACCAAATGATTCACTTATGGATCCTGTG  
ACTAACGCCCAACCAGCGGTGTTATTTTCATGAAACAGAGGAAGTTGGAAGGTTACGCCGCTTAACCTCCCACCA  
ATAGCATTACAGAGGTGAAGTCATTGATGGCATGGTTACGTTGGCAAGTGAGAGCGTTGAATGCCACCAAATTATT  
ACGCCGAGCCCCGAAAAACCAAGTACTCAGACACTACGCCACGATCGCGTGCTTGATACCTAGAAGCAATCCA  
CTTACGTTACGAACGGCATAACCGTTGATGAAGCGCAAGGATTCATACGCAACGTGGTTTTCAATGATCACAATT  
GCGAGTTGGGCTGAAACCTATCACGAAACGCGGGCGTGGTTCTACGTACGCGCTTAACACAGAGGCACCTGCT  
ATCGCAACTAGCCATCTGGTGCAAACGAGTGTGAGCGGCAAGGTGGACAAGCGCAACTACGATTACATGAAACAA  
GAGATGACAACCTCTATCAATGACTACAAGCGTCAAGGCAAGCAAACAATGACGACAGATTACAGATCTGATAGAT  
GAAGCGCTCTTCGCAGCTGCCCTCATTACCCAGATTACTGGCAGGTTCTTTTACCAGACACGACCCACCAGTT  
GCGCGGGGTTGTGGTGGTCAAGTCAATTAATATTTTCGCAGGGCACTGGCTGCAATACCGTACAATCTTAAAGAA  
AAGTGTCAACTGCTAGCAGACATATCAGCGTGCGTCGCCATGGTGAAGGTACTTCCGATGAAGCCATGATCGAA  
CACCACGTTGGTGTAGTAGCAACGCATGCTCTATACGAAACCAAGGACTCTTATCGAGGCTAATGACTTCACAGAA  
AGCGATGTGCTGAGCGCACAAAGCTGCAGGCGTCTTTGCCCTTCGAAATGGCTTACAGCAGAATGCTTAACCTAGCT  
GCCACACAAACTAGTCACGTGCACTGGCAGAGGAAGCAGCACCTTAGGCAGTTGTGCGATTTTCGCTGGGCAGCTC  
ACTACCATTGCTGACGACGGGGTTTTGCTGAGCATTCACTCATTCAAAAACCCATGCCATGCGCAGCGGCAGT  
TCACAACACGGGTATGATGAGGGAACGTTCTTCTTTAGTTTACAGCACCAAGTGCAGATGTATCTTGACGAC  
AAGCCTGTTTTAGCCAAAGGTATACTGCAGACTAAAGATGCTATGTCATTCGATCAGCCTCAGTCAGCCAGGCTG  
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## 2. LbMV-1 dsRNA 2 sequence

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## Publications

**Type of presentation- Poster (second year PhD student)**

**MBPP Conference 2015, University of the West of England**

### **ISOLATION AND MOLECULAR CHARACTERISATION OF MYCOVIRUSES FROM THE PHYTOPATHOGENIC FUNGUS *LEPTOSPHAERIA BIGLOBOSA***

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*Leptosphaeria maculans* and *L. biglobosa* are closely related plant pathogens responsible for economically important diseases of oilseed rape (*Brassica napus*) worldwide. Mycoviruses are often associated with hypovirulence and can be used as biocontrol agents against plant diseases of fungal origin. The aim of the project is to assess the incidence of double-stranded (ds) RNA mycoviruses in *L. maculans* and *L. biglobosa* and to investigate effects of any dsRNAs on the growth and pathogenicity of the fungus. Forty-eight *Leptosphaeria* isolates were screened in total and five *L. biglobosa* isolates were found to possess dsRNA elements. Three different mycoviral dsRNAs investigated from *L. biglobosa* isolate C-Rox 12.8.1 were approximately 5000, 4500 and 4000 bp in length, respectively. Cloning, sequencing and sequence analysis of dsRNA 2 revealed that it is most closely related to the RNA-dependent RNA polymerase region of an Amasya cherry disease-associated mycovirus and has greatest similarity with members of the family *Totiviridae*. This constitutes the first report of dsRNA mycoviruses in *L. biglobosa*. The complete molecular characterisation of these dsRNAs, together with pathogenicity assays in order to detect possible hypovirulence, is in progress.

# ISOLATION AND MOLECULAR CHARACTERISATION OF MYCOVIRUSES FROM THE PHYTOPATHOGENIC FUNGUS *LEPTOSPHERA BIGLOBOSA*

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## Introduction

Mycoviruses are viruses that infect fungi and have the potential to control fungal diseases of plants when associated with hypovirulence. Typically, mycoviruses have double-stranded (ds) or single-stranded (ss) RNA genomes. Double-stranded (ds) RNA mycoviruses have been described in filamentous fungi, mushrooms, yeast and oomycetes. Mycoviruses are responsible for hypovirulence and hypervirulence effects on host fungi. Most of the mycoviral infections are cryptic. *Leptosphaeria maculans* and *L. biglobosa* are closely related plant pathogens which are responsible for economically important disease of oilseed rape (*Brassica napus*) worldwide. They were assessed for the incidence of dsRNA mycoviruses where dsRNAs viruses had not been reported previously and to investigate the effects of any dsRNAs on the growth and pathogenicity of the fungus.

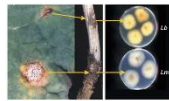


Fig. 1: Symptoms caused by *Leptosphaeria maculans* and *Leptosphaeria biglobosa*. *L. maculans* causes large pale lesions while *L. biglobosa* causes smaller and darker lesions on leaves. *L. maculans* presents basal stem canker while *L. biglobosa* presents upper stem lesions. Cultures of *L. maculans* do not exhibit pigmentation while *L. biglobosa* exhibit yellow pigmentation on potato dextrose agar (Fitt *et al.*, 2006).

## Aim

- Identification and characterisation of mycoviruses from fungal plant pathogens and investigations into their potential as agents of hypovirulence to protect plants against natural infection with highly pathogenic strains.

## Objectives

- To screen the large number of fungal isolates (*Hymenoscyphus pseudoalbivivus*, *Leptosphaeria maculans*, *Leptosphaeria biglobosa*, *Dothistroma septosporum* & *Pyrenopeziza brassicae*) using small scale extraction procedure.
- To perform large scale isolation of dsRNA elements found and identify putative viruses from *L. biglobosa*.
- To sequence the mycovirus genome and assess the sequence similarity between dsRNA elements from different isolates.
- To detect the effects of mycoviruses on fungal pathogenicity.

## Materials and Methods

- Fungal isolates were screened for the presence of dsRNA using massive screening procedures. More specifically, rapid small-scale isolation of nucleic acids from each strain was followed by large scale virus purification. After separation by gel electrophoresis, dsRNA elements were used either collectively or individually for reverse transcription, PCR amplification, cloning and sequencing using technology developed at the beginning of the project (Coutts *et al.*, 2004; Shah *et al.*, 2015).
- Leaves with phoma spots collected from different sites of UK (Fig. 3) were sampled and isolates of *Leptosphaeria* were obtained by single pycnidial isolation (Fig. 2). Pathogen species were identified on the basis of morphological characteristics of the colony and pigment production (Fig. 1).

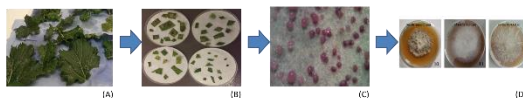


Fig. 2: Isolation of *Leptosphaeria* species from oilseed rape cultivars. (A) Leaves collected from field for the pathogen isolation. (B) Leaves with phoma spots were cut into small pieces and placed in Petri dishes on Whatman No. 1 filter paper that had been sprayed with distilled water. (C) Mature pycnidia observed under a microscope were sampled on PDA plate. (D) Cultures of *Leptosphaeria* were grown on V8 medium at 20°C in darkness for 20 days.

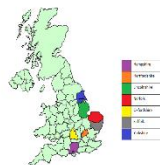


Fig. 3: Map showing the different locations (indicated with different colours) of UK from where *Leptosphaeria* isolates were obtained.

## Results

### Screening of *Leptosphaeria* isolates for dsRNA mycoviruses

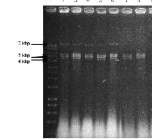


Fig. 4: Agarose gel electrophoresis (1% in TAE) of nucleic acids extracted from selected *L. biglobosa* isolates (lanes 2 to 8) and negative control (lane 9) obtained under the same condition with treatment of DNase and S1 nuclease. Hyperladder 1 (lane 1) was used as marker. The three/four dsRNAs derived from seven isolates can be seen on gel.

### Northern blot hybridization

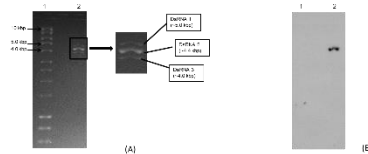


Fig. 5: (A) Agarose gel electrophoresis of *L. biglobosa* virus (LbV) dsRNA components with their approximate sizes (lane 2). Lane 1 contains Hyperladder 1. (B) Northern blot hybridization of LbV dsRNA2 using a DIG-labelled probe derived from its cloned sequence.

### *Leptosphaeria biglobosa* virus (LbV) dsRNA 2

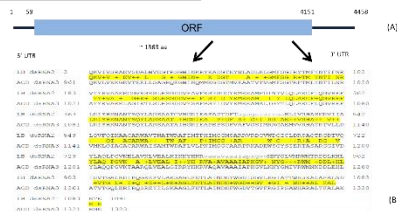


Fig. 6: Predicted genome organisation of LbV dsRNA 2 (A) and alignment of amino acid sequence of partial LbV dsRNA 2 ORF with the comparable ORF predicted from the sequence of ACD (Amasya cherry disease) associated mycovirus dsRNA 3. Consensus amino acids are highlighted in yellow (B).

## Discussion

- In total, sixty-nine *Leptosphaeria* isolates were screened (57- *L. maculans* and 12- *L. biglobosa*) and 7 *L. biglobosa* isolates were found to possess dsRNA elements which presents **10.1% incidence** of mycoviruses in *Leptosphaeria* isolates (Fig. 4).
- Northern blot hybridization result confirmed that the sequence obtained correspond to only LbV dsRNA2 (Fig. 5).
- Sequence analysis revealed that dsRNA 2 is most closely related to the RdRp gene of an Amasya cherry disease (ACD)- associated mycovirus which codes for RNA-dependent RNA polymerase and shows greatest similarity with members of the family *Totiviridae* (Fig. 6).

## Future work

- Continuation of cloning of dsRNA elements and obtaining new clones of other dsRNAs isolated from *L. biglobosa*.
- Northern blotting analysis and comparison of dsRNAs from all the isolates of *L. biglobosa* that contain dsRNA.
- Generation of isogenic lines of virus-free and virus-infected isolates and their growth comparisons-searching for hypovirulence.
- Transfection of virus-free isolates of *L. biglobosa* with purified virus obtained from a representative isolate.
- Development of infectivity assays on rape plants- screening for hypovirulence.

## References

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