Investigations on mycoviruses found in *Dothistroma septosporum* and *Beauveria bassiana* and their effects on fungal pathogenicity

By

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Statement of Originality

The material in this thesis has not been previously submitted for a degree in any university, and to the best of my knowledge contains no material previously published or written by another person except where due acknowledgement is made in the thesis itself.

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Abstract

Mycoviruses specifically infect and replicate in fungi, and they are frequently cryptic in nature and usually associated with hypovirulence (reduced virulence) or, more unusually, hypervirulence. The research aim of the project is to gain further understanding of the molecular biology of viruses infecting the entomopathogenic fungus *Beauveria bassiana* and the phytopathogenic fungus *Dothistroma septosporum*, together with any effects on pathogenicity.

D. septosporum is a foliar pathogen of pine that causes Dothistroma needle blight (DNB), the incidence of which has rapidly increased over the last few decades, particularly in the northern hemisphere. Screening of over 50 *D. septosporum* isolates for virus infection revealed one isolate D752.1 harboring a tetrapartite mycovirus with a double stranded (ds) RNA genome. The sequence of these dsRNA elements was determined using random cDNA PCR amplification combined with a genome walking strategy and RNA ligase-mediated rapid amplification of cDNA ends (RLM RACE). Following sequence analysis, the mycovirus was designated as *Dothistroma septosporum* chrysovirus (DsCV-1) and was found to belong to the family *Chrysoviridae*. Subsequently, attempts were made to construct virus-free and virus-infected isogenic lines, using matric stress, the protein synthesis inhibitor cycloheximide and the potent antiviral drug ribavirin; the latter was proven successful in eradicating the virus. Subsequently, small-scale field trial testing was developed in collaboration with Forest Research UK to investigate the virulence of *D. septosporum* isolates on *Pinus* saplings. The trials included virus-infected and virus-free isogenic lines of D752.1 and other isolates D622, D584, D233, D1216, D700 whose infection status was determined using RT-PCR. The DsCV-1-free D752.1 isolate was revealed to be more infectious, cause significantly more extended needle damage in the form of red bands and have significantly higher fungal burden as compared to the DsCV-1-infected D752.1 isolate, four other DsCV-1-infected *D. septosporum* isolates from Scotland and one DsCV-1-free *D. septosporum* isolate from Southern England, as shown by image analysis and qPCR.

B. bassiana is an entomopathogenic fungus with a worldwide distribution and a wide arthropod host range and is a popular biocontrol agent against insect pests. The effects of virus infection on the growth and pathogenicity of *B. bassiana* were investigated through a comparative study of virus-infected and virus-free isogenic lines of the fungus. Radial expansion experiments conducted on Czapek-Dox complete and minimal media revealed that the virus-infected (VI) *B. bassiana* isolate EABb 92/11-Dm grows faster as compared to the virus-free (VF) isogenic EABb 92/11-Dm, virus-free non-isogenic KVL-03-144 and KVL-03-122, and virus-free Botanigard and Naturalis which are commercially used as biocontrol agents. Subsequently, the pathogenicity of the

isogenic lines EABb 92/11-Dm VI and VF was studied in a controlled environment using the greater wax moth *Galleria mellonella* infection model. Larvae infected with EABb 92/11-Dm VF had a significantly higher survival rate as compared to EABb 92/11-Dm VI.

In summary, these experiments yielded promising results, as virus infection was shown to promote hypovirulence and hypervirulence respectively in *D. septosporum* and *B. bassiana*. Virus infection could therefore be utilised in the context of biological control in the future.

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Abbreviations

BbPmV-1	Beauveria bassiana polymycovirus-1
BbPmV-3	Beauveria bassiana polymycovirus-3
cDNA	complementary deoxyribonucleic acid
CDA	Czapek-Dox agar
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide phosphate
dsRNA	double stranded ribonucleic acid
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
g	gram
GFP	green fluorescent protein
Kb	kilobase
LB	Luria Bertani
DsCV-1	Dothistroma septosporum chrysovirus -1
DsCV-1. F	Dothistroma septosporum chrysovirus -1. Free
DsCV-1. I	Dothistroma septosporum chrysovirus -1. Infected
IPTG	isopropyl-beta-D-1-thiogalactopyranoside
mg	milligram
mL	millilitre
mRNA	messenger ribonucleic acid
NGS	next generation sequencing
ORF	open reading frame
PCR	polymerase chain reaction
PDA	potato dextrose agar
PEG	polyethylene glycol
PMMG	pine needle minimal medium + glucose
PmV-1. F	Polymycovirus-1 Free
PmV-1. I	Polymycovirus-1 Infected
PmV-3. F	Polymycovirus-3 Free
PmV-3. I	Polymycovirus-3 Infected
QC	quality control
RT-qPCR	reverse transcription quantitative polymerase chain reaction
RdRP	RNA dependent RNA polymerase
RLM-RACE	RNA ligase-mediated rapid amplification of cDNA ends
RNA	ribonucleic acid
rPCR	random polymerase chain reaction
RPM	revolutions per minute
RT-PCR	reverse transcription polymerase chain reaction
µL	microlitre
X-gal	5- bromo-4-chloro-3-indolyl-β-D-galactopyranoside
3’- UTR	3 prime untranslated region
5’- UTR	5 prime untranslated region

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Chapter 1.

Chapter 1. General Introduction.

1.1 Discovery of mycoviruses.

Mycoviruses are viruses that infect and replicate in fungal cells and are widespread in filamentous fungi, yeasts, mushrooms, and oomycetes (Wu *et al.,* 2012). In 1936, the presence of a virus in yeast was suspected due to a lytic phenomenon presumably caused by viral infection (Hirano *et al*., 1962). Sinden & Hauser, (1957) were the first to propose that virus infection might be associated with a disease in the mushroom *Agaricus bisporus*, causing a decrease in yield, premature degradation and alterations in mushroom tissue and morphology. Mycoviruses were first discovered by Gandy & Hollings (1962) in diseased mushroom *A. bisporus* (Gandy & Hollings 1962) and visualised by transmission electron microscopy (TEM). During an investigation of *P. stoloniferum* and *P. funiculosum*, the extract from several isolates was found to exhibit antiviral activity (Powell, 1952; Shope, 1953). TEM visualised polyhedral viral particles that were isolated from the antiviral fraction of *Penicillium stoloniferum* (Ellis & Kleinschmidt, 1967). Concurrently, it was discovered that the virus particles associated with *Penicillium spp.* contained dsRNA, which were responsible for the antiviral activity of the two moulds (Banks *et al*., 1968). Less than 10 years following these initial discoveries, mycoviruses had been described in over 60 species from approximately 50 genera of fungi (Lemke & Nash, 1974).

Mycoviruses are now believed to commonly exist in fungi and have since been isolated from various fungi, including mushrooms, medically, ecologically, and economically significant fungi, plant pathogenic and insect pathogenic species. To date, over 250 mycoviruses have been described and sequenced and this data has been recorded in the National Centre for Biotechnology Information (NCBI) database (Liu & Jiang, 2014). Mycoviruses have different genomic types and the majority possess single-stranded (ss) or double-stranded (ds) RNA as their genetic material. Those containing RNA have been assigned to several different viral families: *viz*. *Chrysoviridae*, *Megabirnaviridae, Quadriviridae, Totiviridae, Partitiviridae* and *Reoviridae,* which have dsRNA genomes, while *Endornaviridae*, *Hypoviridae*, *Alphaflexiviridae*, *Gammaflexiviridae, Barnaviride* and *Narnaviridae,* have ssRNA genomes (Ghabrial *et al*., 2015). Notably, *Endornaviridae* and *Hypoviridae* were originally thought to have dsRNA genomes but were recently reclassified as ssRNA viruses. Furthermore, *Metaviridae* and *Pseudoviridae* do not encode an RNA-dependent RNA polymerase (RdRP) but use reverse transcriptase to replicate (Wu *et al*.,

2012). Selected families accommodating dsRNA and ssRNA viruses are described in detail in the next sections.

1.1.1 *Chrysoviridae.*

This family accommodates two genera, *Alphachrysovirus* and *Betachrysovirus* (Kotta-Loizou *et al.*, 2020), and a list of the officially recognised chrysoviruses can be found in Table 1.

Table 1. Members of the *Chrysoviridae* family.

Genus	virus name & abbreviation	dsRNA & protein	5'-UTR	3'-UTR	accession number
Alphachrysovirus	Amasya cherry disease associated chrysovirus (ACDACV)	dsRNA 1 (3399 bp); RdRP (124 kDa)	86 nt	49 nt	AJ781166
		dsRNA 2 (3128 bp); CP (112 kDa)	95 nt	48 nt	AJ781165
		dsRNA 3 (2833 bp); alphachryso-P4 (98 kDa)	94 nt	84 nt	AJ781164
		dsRNA 4 (2498 bp); alphachryso-P3 (77 kDa)	105 nt	359 nt	AJ781163
	Anthurium mosaic-associated virus (AMAV)	dsRNA 1 (3550 bp); RdRP (126 kDa)	179 nt	71 nt	FJ899675
		dsRNA 2 (3448 bp); CP (113 kDa)	293 nt	161 nt	FJ899676
		dsRNA 3 (3244 bp); alphachryso-P4 (97 kDa)	393 nt	259 nt	FJ899677
	Aspergillus fumigatus chrysovirus (AfCV)	dsRNA 1 (3560 bp); RdRP (129 kDa)	128 nt	87 nt	FN178512
		dsRNA 2 (3159 bp); CP (109 kDa)	167 nt	130 nt	FN178513
		dsRNA 3 (3006 bp); alphachryso-P3 (100 kDa)	169 nt	161 nt	FN178514
		dsRNA 4 (2863 bp); alphachryso-P4 (95 kDa)	154 nt	165 nt	FN178515
	Brassica campestris chrysovirus 1 (BcCV1)	dsRNA 1 (3639 bp); RdRP (131 kDa)	101 nt	97 nt	KP782031
		dsRNA 2 (3567 bp); CP (126 kDa)	135 nt	81 nt	KP782030
		dsRNA 3 (3337 bp); alphachryso-P4 (113 kDa)	132 nt	139 nt	KP782029
	Colletotrichum gloeosporioides chrysovirus 1 (CgCV1)	dsRNA 1 (3397 bp); RdRP (126 kDa)	71 nt	59 nt	KT581957
		dsRNA 2 (2869 bp); CP (102 kDa)	71 nt	95 nt	KT581958
		dsRNA 3 (2630 bp); alphachryso-P4 (92 kDa)	66 nt	92 nt	KT581959
	Cryphonectria nitschkei chrysovirus 1 (CnCV1)	dsRNA 1 (partial; 2978 bp*); RdRP (110 kDa)	82 nt	7?	GQ290649
		dsRNA 2 (partial; 2980 bp*); CP (99 kDa)	242 nt	17?	GQ290645
		dsRNA 3 (partial; 2552 bp*); alphachryso-P4 (91 kDa)	?	77	HM013825
		dsRNA 4 (partial; 2960 bp*); alphachryso-P3 (81 kDa)	698 nt	30?	HM013826
	Dothistroma septosporum chrysovirus 1 (DsCV1)	dsRNA 1 (partial; 3354bp*); RdRP	152nt	110nt	NC033277
		dsRNA 2 (partial; 2800bp*); CP	145nt	108nt	NC_033317.1
		dsRNA 3 (partial; 2700 bp*); alphachryso-P4	?	102nt	NC_033318.1
		dsRNA 4 (partial; 2500 bp*); alphachryso-P3	160nt	117nt	NC_033278.1
	Fusarium oxysporum chrysovirus 1 (FoCV1)	dsRNA 1 (partial; 2574 bp*); RdRP	?	?	EF152346
		dsRNA 2 (partial; 648 bp*); CP	?	?	EF152347
		dsRNA 3 (partial, non-functional; 994 bp*); alphachryso-P4	?	?	EF152348

	Helminthosporium victoriae virus 145S (HvV145S)	dsRNA 1 (3612 bp); RdRP (125 kDa)	207 nt	144 nt	AF297176
		dsRNA 2 (3134 bp); CP (100 kDa)	293 nt	153 nt	AF297177
		dsRNA 3 (2972 bp); alphachryso-P4 (93 kDa)	302 nt	150 nt	AF297178
		dsRNA 4 (2763 bp); alphachryso-P3 (81 kDa)	412 nt	209 nt	AF297179
	Isaria javanica chrysovirus 1 (IjCV1)	dsRNA 1 (3593 bp); RdRP (129 kDa)	174 nt	65 nt	KX898416
		dsRNA 2 (3175 bp); CP (109 kDa)	176 nt	74 nt	KX898417
		dsRNA 3 (3165 bp); alphachryso-P3 (108 kDa)	147 nt	84 nt	KX898418
		dsRNA 4 (2874 bp); alphachryso-P4 (92 kDa)	212 nt	130 nt	KX898419
	Macrophomina phaseolina chrysovirus 1 (MpCV1)	dsRNA 1 (3712 bp); RdRP (129 kDa)	256 nt	138 nt	KP900886
		dsRNA 2 (3462 bp); CP (111 kDa)	374 nt	112 nt	KP900887
		dsRNA 3 (2985 bp); alphachryso-P4 (94 kDa)	278 nt	175 nt	KP900889
		dsRNA 4 (2927 bp); alphachryso-P3 (100 kDa)	127 nt	139 nt	KP900888
	Penicillium chrysogenum virus (PcV)	dsRNA 1 (3562 bp); RdRP (129 kDa)	144 nt	64 nt	AF296439
		dsRNA 2 (3200 bp); CP (109 kDa)	157 nt	94 nt	AF296440
		dsRNA 3 (2976 bp); alphachryso-P3 (101 kDa)	161 nt	83 nt	AF296441
		dsRNA 4 (2902 bp); alphachryso-P4 (95 kDa)	162 nt	196 nt	AF296442
	Persea americana chrysovirus (PaCV)	dsRNA 1 (3421 bp); RdRP (126 kDa)	97 nt	42 nt	KJ418374
		dsRNA 2 (3335 bp); CP (122 kDa)	95 nt	99 nt	KJ418375
		dsRNA 3 (2857 bp); alphachryso-P4 (92 kDa)	108 nt	135 nt	KJ418376
	Raphanus sativus chrysovirus 1 (RsCV1)	dsRNA 1 (3638 bp); RdRP (131 kDa)	106 nt	115 nt	JQ045335
		dsRNA 2 (3517 bp); CP (124 kDa)	128 nt	83 nt	JQ045336
		dsRNA 3 (3299 bp); alphachryso-P4 (110 kDa)	134 nt	177 nt	JQ045337
	Shuangao chryso-like virus (SCLV)	dsRNA 1 (3461 bp); RdRP (127 kDa)	45 nt	59 nt	MF176340
		dsRNA 2 (3140 bp); (108 kDa)	200 nt	57 nt	MF176342
		dsRNA 3 (3080 bp); (98 kDa)	153 nt	218 nt	MF176341
		dsRNA 4 (3059 bp); (106 kDa)	150 nt	74 nt	MF176343
	Verticillium dahliae chrysovirus 1 (VdCV1)	dsRNA 1 (3594 bp); RdRP (127 kDa)	91 nt	176 nt	HM004067
		dsRNA 2 (3313 bp); CP (113 kDa)	204 nt	58 nt	HM004068
		dsRNA 3 (2983 bp); alphachryso-P3 (84 kDa)	635 nt	53 nt	HM004069
		dsRNA 4 (2932 bp); alphachryso-P4 (90 kDa)	264 nt	196 nt	HM004070
Betachrysovirus	Alternaria alternata chrysovirus 1 (AaCV1)	dsRNA 1 (3647 bp); RdRP (124 kDa)	199 nt	94 nt	LC350277
		dsRNA 2 (2857 bp); CP (82 kDa)	389 nt	152 nt	LC350278
		dsRNA 3 (2785 bp); betachryso-P4 (83 kDa)	366 nt	94 nt	LC350279
		dsRNA 4 (2772 bp); betachryso-P3 (84 kDa)	334 nt	107 nt	LC350280
		dsRNA 5 (836 bp); 13 kDa	102 nt	386 nt	LC350281
	Botryosphaeria dothidea chrysovirus 1 (BdCV1)	dsRNA 1 (3654 bp); RdRP (126 kDa)	230 nt	73 nt	KF688736
		dsRNA 2 (2773 bp); CP (80 kDa)	272 nt	254 nt	KF688737
		dsRNA 3 (2597 bp); betachryso-P3 (82 kDa)	293 nt	63 nt	KF688738
		dsRNA 4 (2574 bp); betachryso-P4 (77 kDa)	292 nt	128 nt	KF688739

	Colletotrichum fructicola chrysovirus 1 (CfCV1)	dsRNA 1 (3620 bp); RdRP (126 kDa)	154 nt	67 nt	MG425969
		dsRNA 2 (2801 bp); CP (89 kDa)	233 nt	90 nt	MG425970
		dsRNA 3 (2687 bp); betachryso-P3 (76 kDa)	273 nt	290 nt	MG425971
		dsRNA 4 (2437 bp); betachryso-P4 (72 kDa)	340 nt	109 nt	MG425972
		dsRNA 5 (1750 bp); 54 kDa	61 nt	168 nt	MG425973
		dsRNA 6 (1536 bp); 33 kDa	67 nt	560 nt	MG425974
		dsRNA 7 (1211 bp); 10 kDa	252 nt	689 nt	MG425975
	Fusarium graminearum dsRNA mycovirus 2 (FgV2)	dsRNA 1 (3580 bp); RdRP (128 kDa)	82 nt	84 nt	HQ343295
		dsRNA 2 (3000 bp); betachryso-P4 (95 kDa)	93 nt	279 nt	HQ343296
		dsRNA 3 (2982 bp); CP (94 kDa)	105 nt	306 nt	HQ343297
		dsRNA 4 (2748 bp); betachryso-P3 (91 kDa)	78 nt	162 nt	HQ343298
		dsRNA 5 (2414 bp); 80 kDa	97 nt	184 nt	HQ343299
	Fusarium oxysporum f. sp. dianthi mycovirus (FodV)	dsRNA 1 (3555 bp); RdRP (129 kDa)	82 nt	53 nt	KP876629
		dsRNA 2 (2809 bp); betachryso-P4 (95 kDa)	84 nt	88 nt	KP876630
		dsRNA 3 (2794 bp); CP (92 kDa)	97 nt	138 nt	KP876631
		dsRNA 4 (2646 bp); betachryso-P3 (92 kDa)	97 nt	56 nt	KP876632
	Magnaporthe oryzae chrysovirus 1-A (MoCV1-A)	dsRNA 1 (3554 bp); RdRP (125 kDa)	118 nt	52 nt	AB560761
		dsRNA 2 (3250 bp); betachryso-P3 (99 kDa)	343 nt	102 nt	AB560762
		dsRNA 3 (3074 bp); betachryso-P4 (84 kDa)	144 nt	530 nt	AB560763
		dsRNA 4 (3043 bp); CP (85 kDa)	161 nt	443 nt	AB560764
		dsRNA 5 (2879 bp); 63 kDa	178 nt	865 nt	AB700631
	Penicillium janczewskii chrysovirus 1 (PjCV1)	dsRNA 1 (3698 bp); RdRP (127 kDa)	168 nt	149 nt	KT601115
		dsRNA 2 (2899 bp); CP (84 kDa)	186 nt	400 nt	KT601116
		dsRNA 3 (2942 bp); betachryso-P3 (90 kDa)	223 nt	253 nt	KT601117
		dsRNA 4 (2506 bp); betachryso-P4 (74 kDa)	179 nt	263 nt	KT601118
	Penicillium janczewskii chrysovirus 2 (PjCV2)	dsRNA 1 (3540 bp); RdRP (124 kDa)	94 nt	86 nt	KT950836
		dsRNA 2 (2699 bp); CP (84 kDa)	194 nt	153 nt	KT950837
		dsRNA 3 (2535 bp); betachryso-P3 (80 kDa)	164 nt	100 nt	KT950838
		dsRNA 4 (partial; 2155 bp*); betachryso-P4 (70 kDa)	190 nt	?	KT950839

(Table courtesy of Dr Ioly Kotta-Loizou)

Penicillium chrysogenum virus (PcV) is the exemplar of the prototype species for genus *Alphachrysovirus* (Ghabrial & Caston, 2011). PcV contains four monocistronic dsRNA segments, 2.4-3.6 kbp in size which are separately encapsidated in virions. DsRNA1 and dsRNA2 encode RdRP and capsid protein (CP), respectively. DsRNA3 and dsRNA4 encode P3 and P4 proteins of unknown function. The 5'- and 3'- untranslated regions (UTRs) of the four dsRNAs are highly conserved in sequence (Jiang & Ghabrial, 2004).

PcV has been studied comprehensively at the biochemical, biophysical, and ultrastructural levels which have revealed that the virions are 35-40 nm in diameter, have an icosahedral shape and are non-enveloped. Two related viruses, *P. brevicompactum* virus (PbV) *and P.*

cyaneo-fulvum virus (Pc-fV) have similar characteristics to PcV and initial confusion as to whether their genomes contained three or four dsRNA elements has now been resolved (see below) and both are considered *bona fide* members of the family *Chrysoviridae*.

The four dsRNA segments have sizes ranging from 2902-3562 bp. PcV dsRNA-1 is 3562 bp in length and encodes a protein of 128,548 Da which has motifs characteristic of eukaryotic RdRPs. PcV dsRNA-2 is 3200 bp in size and encodes the 108,806 Da CP. PcV dsRNA-3 is 2976 bp in length and encodes a putative protein of 101,458 Da and PcV dsRNA-4 is 2902 bp in length and encodes a putative protein of 94,900 Da.

The functions of the putative products of dsRNA-3 and dsRNA-4 are unknown. Sequences in the 5'- and 3'-UTRs are highly conserved among the four dsRNA segments and except for cherry chlorotic rusty spot associated chrysovirus (CCRSACV) and Amasya cherry disease associated chrysovirus (ACDACV), all viruses have long 5'-UTRs ranging between 140-400 nucleotides in length. Little is known about the replication mechanism of chrysoviruses.

A new member of the *Chrysoviridae* family was discovered which infects isolate NB IFR-19 of the entomopathogenic fungus *Isaria javanica*. This has been nominated Isaria javanica chrysovirus-1 (Fig. 1; IjCV-1; Herrero, 2017) and has direct relevance to the results presented in this thesis.

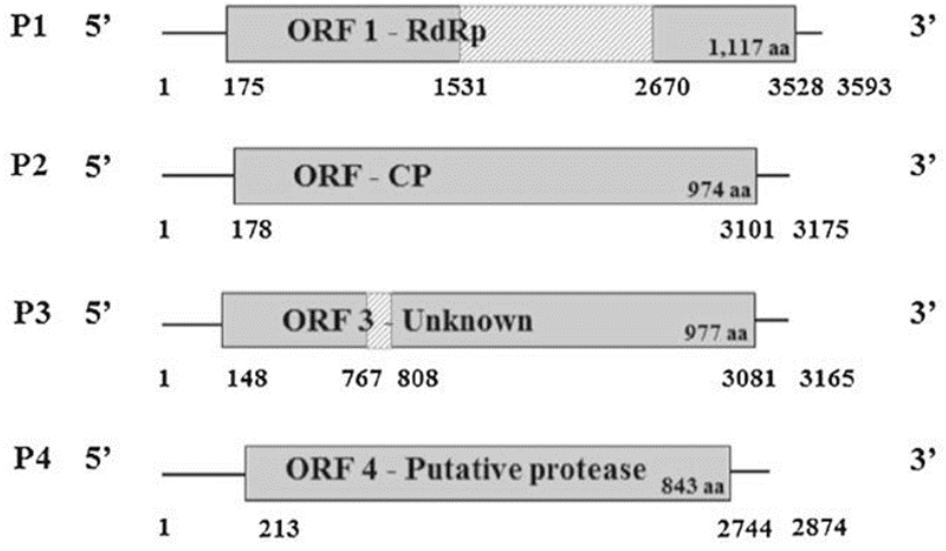


Figure 1. Isaria javanica chrysovirus-1.

Genome organisation of Isaria javanica chrysovirus-1 (IjCV-1) showing four dsRNA segments, their sizes and 5'- and 3'-UTRs (adapted from Herrero, 2017).

1.1.2 *Megabirnaviridae.*

The only genus currently in this family is known as *Megabirnavirus*, and Rosellinia necatrix megabirnavirus 1 (RnMBV1) is the prototype. RnMBV1 infects the plant pathogenic fungus *Rosellinia necatrix* and has two genome segments which are encapsidated separately in isometric particles *ca*. 50 nm in diameter. DsRNA1 encodes two overlapping open reading frames (ORFs) for CP and RdRP genes which are expressed by ribosomal frameshifting as a fusion protein (Salaipeth *et al*., 2014). DsRNA2 encompasses two non-overlapping ORFs of unknown function.

1.1.3 *Quadriviridae*.

The family *Quadriviridae* includes to date one genus, *Quadrivirus*, and one species, Rosellinia necatrix quadrivirus 1. Two well-characterised strains of this species were isolated in Japan, also from *R. necatrix* (Lin *et al*., 2012). Members of this family have four monocistronic genome segments ranging in size from 3.7-4.9 kbp in length which are encapsidated in isometric virus particles *ca*. 45 nm in diameter. DsRNA1 encodes a product of unknown function, while dsRNA2 and dsRNA4 encode CPs, and dsRNA3 encodes the RdRP. Each quadrivirus genome segment contains “CAA” repeats in the 5’-UTRs (Ghabrial *et al*., 2015).

1.1.4 *Totiviridae.*

Viruses infecting yeasts and filamentous fungi have been placed in two genera, *Totivirus* and *Victorivirus*, respectively (King *et al*., 2011, Ghabrial, 2008). Members belonging to this family contain mono segmented dsRNA genomes, 4.6-7.0 kbp in size, and usually, encompass two ORFs on one strand. These two ORFs encode the CP and the RdRP. Gene expression is achieved by 1 of 3 methods: (1) fusion protein (CP/RdRP) consequent to ribosomal frameshifting, for example in parasitic protozoan 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 frameshifting, 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; Li *et al*., 2011; Huang & Ghabrial, 1996; Soldevila & Ghabrial, 2000).

1.1.5 *Partitiviridae.*

There are four different genera of mycoviruses in this family: *Alpha-, Beta-, Gamma- and Deltapartitivirus* (Ghabrial *et al*., 2015). Gammapartitiviruses only infect filamentous fungi, whereas alpha- and betapartitiviruses infect filamentous fungi and plants. Members of this family have bisegmented genomes 1.4-2.4 kbp in length. Both segments encompass a single ORF. The larger ORF on RNA1 encodes an RdRP and the smaller ORF on RNA2 encodes CP. Two separate virus particles are produced at the end of the packaging process of two genome segments.

1.1.6 *Reoviridae.*

Reoviridae is a family of viruses with a wide host range, including vertebrates, invertebrates, plants, protists, and fungi. When virus particles were first observed in a fungal "reo-like" infection (Enebak *et al*., 1994), these viruses were placed in the genus, *Mycoreovirus*. Mycoreoviruses 1-3 (MyRV1-3) are accommodated in this genus. MyRV1 and -2 were discovered in the chestnut blight fungus *Cryphonectria* (formerly *Endothia*) *parasitica*, and MyRV3 was found in *R. necatrix (*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 segments and each virus genome ranges in size from 0.7-4.1 kbp (Kanematsu *et al*., 2004).

1.1.7 *Endornaviridae.*

It was reported by the International Committee for the Taxonomy of Viruses (ICTV) that, some mycoviruses were assigned to family *Endornaviridae* as being dsRNA viruses even though they are phylogenetically more closely related to alpha-like ssRNA viruses (Hacker *et al*., 2005; Ghabrial & Suzuki, 2009). It is now suspected that the dsRNA associated with endornavirus infections represents the replicative form of the virion ssRNA. Endornaviruses contain linear dsRNA genomes *ca.* 14-17 kb in length and encode long polypeptides encoding RNA helicases, RdRPs and UDP-glucosyltransferases. Endornaviruses do not produce virions, and they are typically found in cytoplasmic vesicles of infected fungi, plants, and oomycetes.

1.1.8 *Hypoviridae.*

Hypovirus is the sole genus of the *Hypoviridae* family initially classified as a dsRNA virus, with the replication characteristics of a positive sense ssRNA. The dsRNA found in mycelia appears to be replicative intermediate or replicative forms of genomic RNA. Cryphonectria parasitica hypovirus 1 (CHV-1) and other hypoviruses infect *C. parasitica,* which results in hypovirulence on chestnut trees and altered fungal morphology in culture (9th report ICTV, 2012).

1.2 Transmission of mycoviruses.

Mycoviruses can travel within the fungal hyphae, moving from one cellular compartment to the next through the pores perforating the septa (Fig. 2b). Mycoviruses are transmitted naturally in fungi *via* intracellular transmission (Buck, 1998). Horizontal transmission of mycoviruses is achieved by heterokaryosis (Lhoas, 1971) and vertical transmission by sporulation. Further investigations on genetically marked strains of *P. stoloniferum* and *Aspergillus niger* illustrated virus transmission from a virus-infected strain to an uninfected strain (Lhoas, 1971). Horizontal transmission through protoplasmic fusion is a successful route of transmission of mycoviruses between various fungal strains (Xie *et al*., 2006) as illustrated in Fig. 2b. Anastomosis depends on vegetative compatibility between fungi and concerns hyphal fusion between different fungal strains. This process is controlled by vegetative incompatibility and heterokaryon incompatibility genes in fungi ascomycetes (Shape 2000; Glass & Dementhon, 2006). Hence heterokaryosis is restricted by incompatibility between fungi and affects a range of mycoviruses. However, Pearson *et al*. (2009) reported transmission of mycoviruses between various species, despite the absence of a vector and mechanical transmission. Finally, mycoviruses can be transmitted vertically, from parent to offspring, *via* the production of asexual and sexual fungal spores.

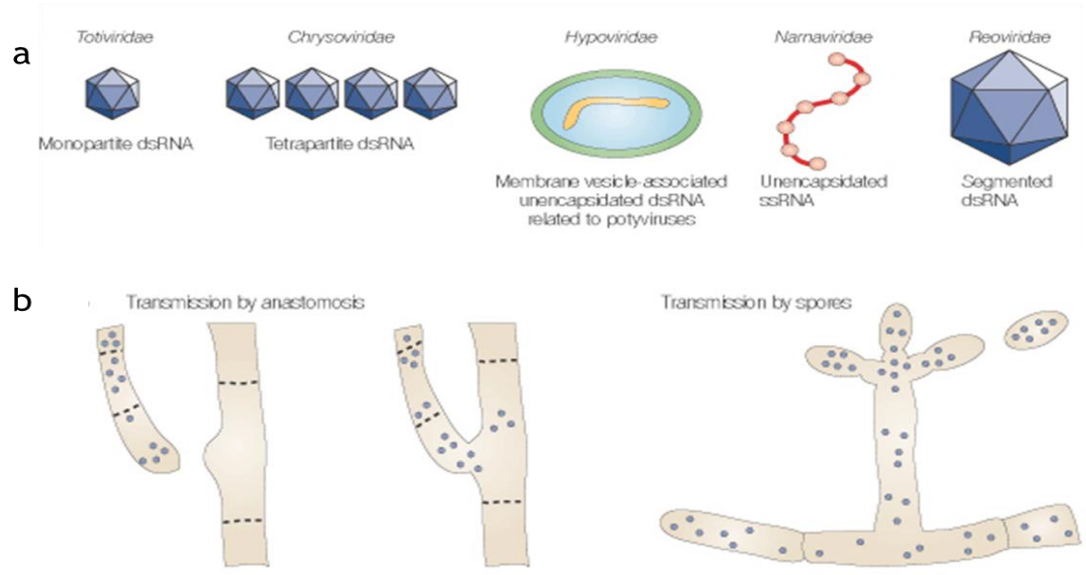


Figure 2. Mycovirus families and transmission.

(A) Important families of dsRNA viruses along with their structures and **(B)** mode of transmission used by mycoviruses (adapted from Nuss, 2005).

1.3 Interactions between mycoviruses and host fungi.

It is accepted that most mycovirus infections are cryptic with no overt symptomology; however, under certain environmental conditions symptoms can be induced (Pearson *et al*., 2009). Small but significant effects on growth and physiology have been observed when isogenic lines of virus-infected and virus-free fungi were compared (Van Diepeningen *et al*., 2006). Interactions between viruses and their hosts show virus-encoded determinants that are responsible for altering fungal host phenotypes (Liu & Jiang, 2014). However increasingly, genomic sequences of pathogenic fungi facilitate deciphering pathogen-virus interactions at the molecular level. There have been recorded data on two well characterized host-virus interactions systems: CHV-1 in *C. parasitica* and HvV190S in *H. victoriae*. Researchers have thoroughly studied biological control, virus replication and diversity (Nuss, 1992; 1998; Ghabrial & Suzuki, 2009; Hillman & Suzuki, 2004; Dawe & Nuss, 2001). For instance, using the *H. victoriae*-HvV190S system, Ghabrial *et al.,* (1992; 2002; 2013) and Li *et al*., (2012) determined the virion structure of HvV190S and the molecular mechanism of HvV190S translation and characterized interactions between HvV190S and its host.

1.3.1 Hypovirulence (reduced fungal pathogenicity).

Viruses belonging to the genus *Hypovirus* cause hypovirulence which is phenotypically expressed as a reduction in the growth rate of the host (Fauquet *et al.*, 2005). *C. parasitica* is a typical example of a fungus whose infection with CHV-1 results in hypovirulence (Choi & Nuss, 1992; Nuss, 2005).

Similarly, Doherty *et al.* (2006) reported that another group of mycoviruses, the mitoviruses which belong to the family *Narnaviridae*, confer hypovirulent traits to their hosts such as *Ophiostoma novo-ulmi*, the fungus responsible for Dutch elm disease. Additionally, *Botrytis cinerea* contains mitoviruses that reduce sporulation and laccase activity (Castro *et al.,* 2003).

Research carried on *Sclerotinia sclerotiorum* identified different mycoviruses (ssDNA, dsRNA, and ssDNA viruses), and provides an opportunity to explore *S. sclerotiorum*-mycovirus interactions. Sclerotinia sclerotiorum debilitation-associated virus (SsDRV) confers hypovirulence in the strain Ep-1PN. Thereafter extensive research on the *S.*

sclerotiorum-SsDRV system by Li *et al.* (2008) identified 150 genes down regulated in strain Ep-1PN, representing a broad spectrum of biological functions. For example, SsDRV suppressed the *S. sclerotiorum* integrin-like gene (SSITL). Further studies *via* forward and reverse genetics approaches by Zhu *et al*. (2013) suggested that targeted silencing of SSITL led to significant reduction in virulence and growth rate.

Likewise, studies conducted by Kwon *et al. (*2009) identified a mycovirus in *Fusarium graminearum* strain DK21 which triggered hypovirulence. Extensive studies using proteomic and transcriptomic approaches suggested FgV1 induced up regulation of a range of genes including a protein synthesis gene responsible for virus replication, cAMP signalling, and signal transduction resulting in significant suppression of *F. graminearum* infection (Cho *et al*., 2012).

1.3.2 Hypervirulence (increased fungal pathogenicity).

Beneficial effects of mycovirus infection in contrast to hypovirulence have been reported for the root pathogen *Nectria radicicola* in isolates containing dsRNA. Here inhibition of a signal transduction pathway increased fungal virulence (Ahn & Lee, 2001). Marquez *et al.* (2007) demonstrated that mycovirus dsRNA infection induced thermal tolerance and facilitated survival at a high temperature of both the pathogen and its host plant. Also, McCabe *et al.* (1999) mentioned that proteinaceous toxins encoded by some yeast dsRNA elements were fatal to other strains of the same fungus that did not produce the toxins, while the strains responsible for their production were immune. Thus, these toxins assist host strains to eradicate rival strains competing in the same niche. More recently several mycoviruses in the newly established family *Polymycoviridae*, which infect *Aspergillus fumigatus* and *Beauveria bassiana*, have been shown to cause hypervirulence (Kanhayuwa *et al*., 2015; Kotta-Loizou & Coutts, 2017).

1.4 Mycoviruses as biocontrol agents against plant diseases.

One of the most successful biocontrol agents known utilises mycovirus infected isolates of C. *parasitica* the causal agent of chestnut blight in chestnut trees (*Castanea spp*.). Heiniger *et al*. (1994) commented that a group of French scientists in the early 1960s identified some unusual *C. parasitica* isolates which were responsible for a reduction of pigmentation on canker as compared to wild-type strains. Subsequently, Macdonald & Fulbright (1991) extended these observations to illustrate that the effects were caused by mycovirus infection. Both *in vivo* and *in vitro* studies revealed the factors responsible for the hypovirulent phenotype were transmissible cytoplasmic genetic factors identified earlier as 8-12 kbp dsRNA elements (Dodds, 1980). Although hypoviruses have been used to control chestnut blight for over four decades, it should be noted that agroecosystems are primarily different from orchards and perennially planted forest trees. However, if a mycovirus-infected strain of a fungal pathogen is applied equally in fields at the appropriate time there is efficient establishment of mycoviruses within the crop (Liu & Jiang, 2014).

There are several potential advantages of using hypovirulence-associated mycoviruses to control fungal disease in crops. For instance, once hypovirulence-associated mycoviruses are transmitted to a virulent fungal strain they can inhibit lesion extension rapidly because viruses tend to invade actively growing regions of the fungal colonies and replicate (Boine *et al*., 2012). This property is essential because crop fungal diseases often damage or accelerate plant mortality during the growing season; therefore, a prompt response is required to suppress crop disease crucial for their successful biological control. Secondly the fitness of a hypovirulent strain on crop plants is not likely to be a problem. For instance, if plants are densely covered with the hypovirulent strain following spray inoculation whether the strain produces spores or other propagation bodies on plants is of no consequence because crops are harvested at the end of the growing season. Also, although hypovirulent strains cannot grow on their hosts to the same extent as virulent strains they do share a similar niche and can potentially compete with and reduce virulent strain growth (Anagnostakis, 1982). Furthermore, Xie & Jiang (2014) discovered that hypovirulent strains could be frequently isolated from sclerotia that were collected from diseased rapeseed, *Brassica napus* plants, suggesting that mycovirus-mediated hypovirulent strains are growing on the diseased plants. Lastly when hypovirulent strains grow on their hosts,

they are likely to produce pathogen-associated molecular patterns (PAMPs) and effectors that are recognised by their hosts which elicit a defence response that specifically targets infection of the virulent strain. Conversely, in cases of hypervirulence, the mycovirus may act as an enhancer of a biocontrol agent; such is the case in the entomopathogenic fungi.

1.5 *Dothistroma septosporum* and Dothistroma needle blight (DNB).

Dothistroma septosporum was first described by Doroguine (1911) in Saint Petersburg, Russia, and named *Cytosporina septospora*. Morelet 1968 is the anamorph of *Mycosphaerella pini* Rostr. 1957, a filamentous fungus belonging to family Mycosphaerellaceae, class Dothideomycetes, phylum Ascomycota. Its origin is unclear, but it has been suggested that it originated from South America and Nepal, with early reports in Eastern Europe in 1911, Western Europe 1920s and North America in 1941 (Ioos *et al*, 2010).

D. septosporum is a foliar pathogen of pine and together with the closely related species *D. pini* causes Dothistroma needle blight (DNB) or red band needle blight (Fig. 3), a disease which has rapidly increased in incidence over the last few decades particularly in the northern hemisphere (Bulman *et al.*, 2013; Watt *et al.*, 2009). Symptoms of DNB have been described and recorded in 1880 in Denmark (Munk, 1957) and 1914 in Ukraine (Barnes *et al.*, 2014). DNB was noted in Baltic and Nordic countries (Drenkhan & Hanso, 2009; Hanso & Drenkhan, 2008; Jovaišienė & Pavilionis, 2005; Markovskaja & Treigienė, 2009; Müller *et al*., 2009; Solheim & Vuorinen, 2011; Millberg *et al.*, 2016). Due to the low occurrence of DNB on native pines in indigenous areas, it has been speculated that *Dothistroma* spp. may originate from the Himalayas in Asia (Ivory, 1994). DNB was first observed in Japan in 1952 (Ito *et al*., 1975). Later, the disease was found in India (Bakshi & Singh, 1968) and Nepal (Ivory, 1990). Currently, there are reports of DNB from 12 Asian countries but the identity of the pathogen, as *D. septosporum*, has only been confirmed in one country (Drenkhan *et al*., 2016). Nevertheless, several studies (Barnes *et al*., 2014; Drenkhan *et al*., 2013; Mullett *et al*., 2017; Piotrowska *et al*., 2017) provide data supporting Europe as the origin of *D. septosporum.* However, the recent increase in recordings of the pathogen in Europe could be due to climate change, where models predict a further increase in the area suitable for DNB in northern Europe under climate change projections (Watt *et al*., 2011b). A recent study carried out by Adamson *et al*. (2018) suggested that the most diverse populations of *D. septosporum* are present in northern Europe, including the Baltic countries, Finland, and European Russia.

Figure 3. Dothistroma needle blight on Corsican pine. (Image courtesy of EPPO. N.d)

DNB is currently the most important foliar disease of pine worldwide (Barnes *et al*., 2004), reported more than 63 countries have been affected (Fig. 4), with over 82 different species of host pines world-wide (Drenkhan *et al*., 2016). In recent years, awareness of DNB and its causal agent have been raised, especially in Europe, through the Determining Invasiveness and Risk of Dothistroma (DIAROD) COST Action, which has led to a greater understanding of host range and geographical incidence of pathogens, and increased research efforts (Drenkhan *et al*., 2016). *D septosporum* is believed to be endemic on indigenous pine populations in the northern hemisphere (Drenkhan *et al*., 2013), DNB causes severe damage to exotic pine plantations in the southern hemisphere (Barnes *et al*., 2014). Comparative genomic studies have been carried out but there is a poor understanding of *D. septosporum* at the molecular level (Ohm *et al.*, 2012; de Wit *et al.,* 2012).

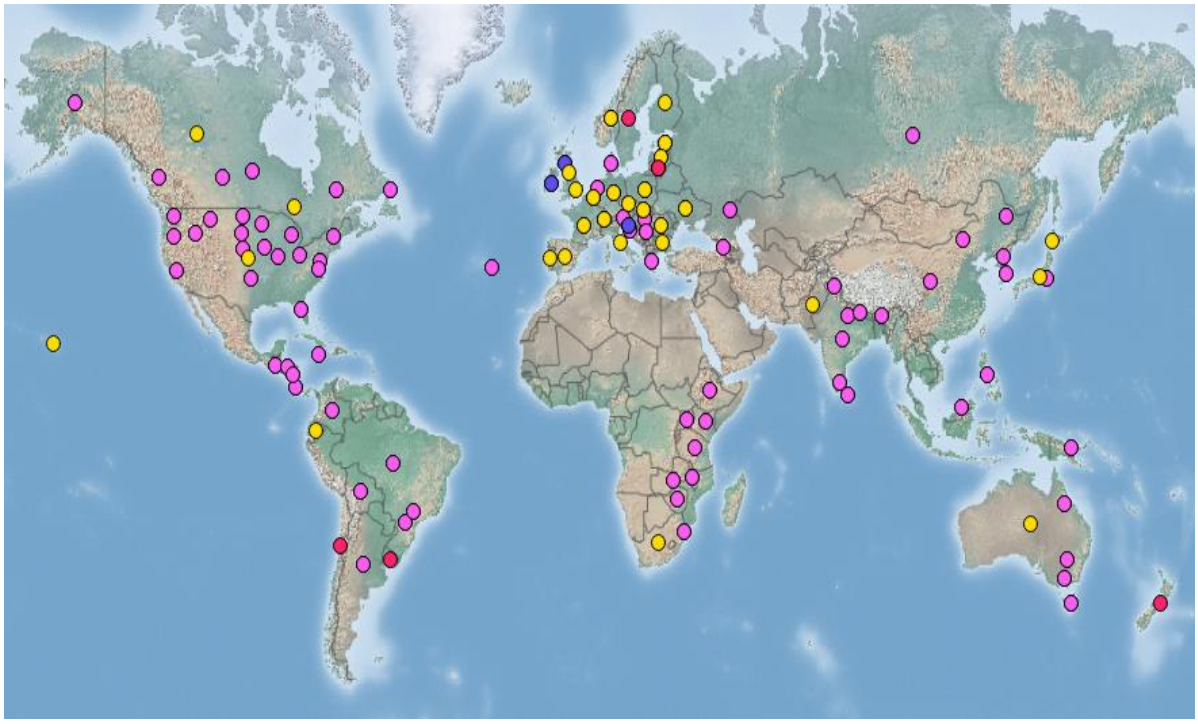


Figure 4. Distribution map of Dothistroma needle blight.

The coloured circles indicate the extent of DNB; purple: few occurrences; yellow: localised; pink: not recorded; red: widespread. (image courtesy of Centre for Agriculture and Bioscience International – CABI; https://www.cabi.org/isc/datasheet/49059#toDistributionMaps)

D. septosporum is a significant tree disease within the UK where commercial pine plantations and forest tree nurseries are infected causing premature defoliation and in some cases tree mortality (Forestry Commission, 2010). DNB was found in all Forestry Commission districts in England and Scotland and with an estimated 70% incidence in Wales which is increasing rapidly (Forestry Commission, 2010).

1.5.1 *D. septosporum* life cycle.

The critical period for infection with DNB in the UK is in spring and early summer when needles and fruiting bodies are formed (Archibald & Brown, 2007). Rainfall appears to be associated with dispersal of *D. septosporum* spores, and wind-borne ascospores (Funk & Parker, 1966) or rain-splashed conidia contribute to needle infection (Gibson, 1972). Spores land on the host, germinate on the needle surface and the hyphae penetrate stomata (Fig. 5).

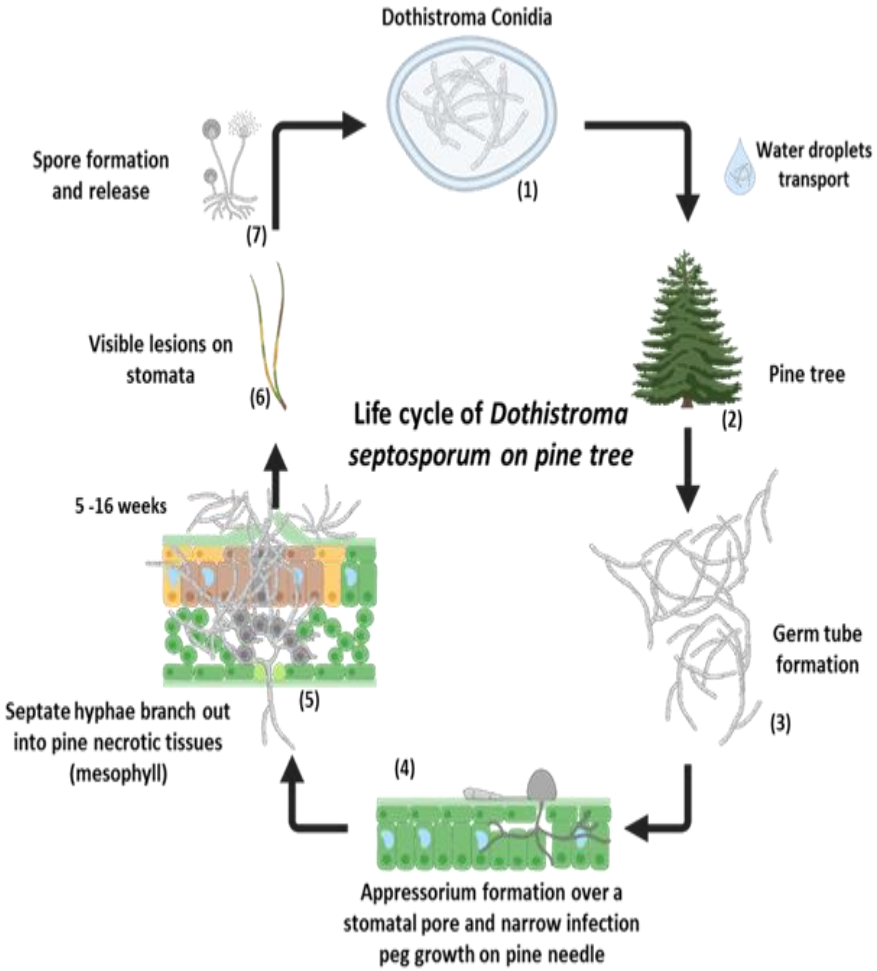


Figure 5. *D. septosporum* life cycle on pine trees.

(1) Water borne *D. septosporum*. (2) Conidia transported *via* water droplets settle on pine tree needles. (3) Germ tube formation and production of hyphae in response to favourable conditions. (4) Appressorium formation over a stomatal pore and narrow infection peg growth on pine needle. (5) Septate hyphae branch out into pine necrotic tissues. (6) Visible lesions on stomata orange-red brown distal ends, green bases, and visible fruiting bodies (approximately <0.5mm). (7) Spore formation and release (image constructed using Bio Render).

The optimum conditions favourable for *D. septosporum* spore germination are between 12°C-18°C under high humidity conditions. After infection in the second year, needles remain attached to the tree as an inoculum source and spores are released in the following summer. Early symptoms of DNB are visible by late autumn with yellow banding on the needles (Fig. 6). Murray & Batko (1962) reported that the initial symptoms of DNB infection are short-lived and progress to a reddish-brown coloration (Fig. 6). Dothistromin is a red coloured mycotoxin known to be associated with DNB and genes involved in its synthesis are expressed at an early stage of growth, suggesting a role in the initial stages of plant

invasion by *D. septosporum* (Schwelm *et al*., 2008). Confusingly for mycologists, symptoms of DNB are generic and can be mistaken with symptoms caused by other pathogens. However, these symptoms are unique in needles older than one year between spring and early summer (needle flush period) and include browning at their distal ends that remain green at the base (Fig. 6). In plantations where DNB thrives, defoliation usually starts at the lower end of the trees (Fig. 6), severely affecting foliage of all ages with total loss of needles after autumn, while the remaining needles on the branch tips become chlorotic. Symptoms of DNB are more pronounced on trees growing on calcareous soils.

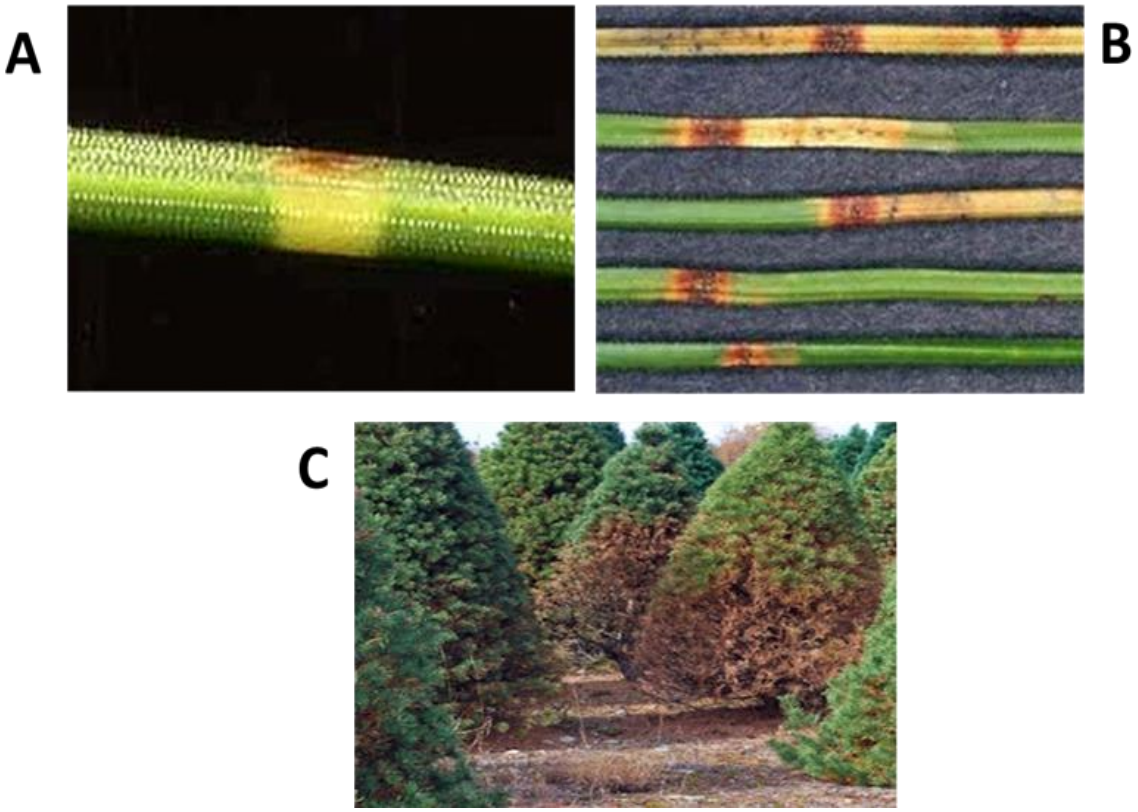


Figure 6. Progression of Dothistroma needle blight on pine trees.

(A) Initial DNB symptoms with yellow bands and tan spots; **(B)** progressive DNB symptoms with reddish-brown colouration; **(C)** DNB infection in the lower crown of Scots pine trees (image courtesy of Forestry Commission, 2010).

1.5.2 Dothistroma Needle Blight impact.

DNB mainly affects members of the genus *Pinus*, and in the UK, it has been found on Corsican or 'black' pine (*P. nigra*), lodgepole pine (*P. contorta*), Scot's pine (*P. sylvestris*), Ponderosa pine (*P. ponderosa*) and bishop pine (*P. muricata*). Different pine trees vary in their susceptibility to infection. For instance, *P. radiata* is the most economically important and is highly susceptible, together with *P. nigra*, *P. ponderosa* and *P. muricata*. In contrast, *P. contorta* and *P. sylvestris* are considered slightly susceptible to DNB. Some species, such as *P. radiata* and *P. muricata*, develop resistance with age and maturity whilst others, such as *P. nigra* and *P. ponderosa*, remain susceptible throughout their lifespan (Gibson, 1972; Kershaw *et al.*, 1988).

Studies made by Gibson (1972) reported that DNB infection spreads to younger pines, with more impact on photosynthetically active needles confirming a sigmoidal relationship between infection and impact on growth. It was consistently demonstrated that DNB led to a significant reduction of tree growth, affecting more its yearly diameter increment than its height, with over 25% of needles being infected (Hocking & Etheridge, 1967; Gibson, 1972). Additionally, Van der Pas (1981) illustrated that disease levels of 50% result in a 50% volume loss of pine wood after three years. Old & Dudzinski (1999) also discovered that, in cases of needle loss over 25%, every 1% additional needle loss up to 75% results in 1% volume loss of pine wood. In severe cases of DNB high levels of defoliation and the accompanying reduction in photosynthesis results in the death of the host.

In the UK, Mullett (2014) reported mortality rates of up to 6% each year in *P. nigra* subsp. *laricio* stands and 20% in unthinned, mature *P. contorta* var. *latifolia* (inland provenance) stands. The economic effects of DNB are challenging to evaluate, but several attempts have been made to detail an accurate value of yearly losses due to DNB. In the UK, an estimated £8.6 million is lost per annum mainly because of DNB impact on Corsican pine. The resulting decline in timber with an estimated loss of *ca.* £5 million per year and an expansion loss from growing Corsican pine of *ca*. £3.5 million per annum is devastating (Price, 2010). These estimated losses are like those reported by Sturrock *et al.,* (2011), who approximated the revenue loss of timber caused by DNB infection at £8 million per year.

Progeny-population trials using pine trees artificially inoculated with *D. septosporum* and carried out under controlled environmental conditions have been restricted to

investigating populations from native Caledonian pinewoods (Perry *et al*., 2016). Here susceptibility to *D. septosporum* and DNB severity was assessed under conditions ideal for fungal development (high temperature, humidity, and leaf wetness) using both visual, non-destructive, and detailed, destructive procedures. These studies demonstrated that there were differences in susceptibility to *D. septosporum* among pine populations indicating that variation in this trait is heritable and evolvable and concluded that native Scots pine populations contain sufficient genetic diversity to evolve lower susceptibility to *D. septosporum* through natural selection in response to increased prevalence of the pathogen (Perry *et al*., 2016). Yet no studies under similar controlled conditions to those described above have been performed comparing a range of different geographical *D. septosporum* isolates and a single pine species. Additionally, the effect of mycovirus infection on *D. septosporum* pathogenicity through a comparison of isogenic virus-free and virus-infected lines is yet to be addressed.

1.6 Entomopathogenic fungi.

Fungi and insects are known to establish mutualistic or commensal relationships that can progress to obligate pathogenicity. Fungi which cause lethal infections and regulate insect and mite populations in nature by causing epizootics are called entomopathogenic fungi (Carruthers & Soper, 1987). One of the earliest studies on entomopathogenic fungi was carried out in the 1800s, they were centred on the development of mechanism to avoid and prevent pests that devastated the silkworm industry (Steinhaus, 1975).

Entomopathogenic fungi include about 700 species belonging to approximately 100 orders, including Entomophthorales (phylum Entomophthoromycota; Hibbett *et al.*, 2007; Humber, 2012) and Hypocreales (phylum Ascomycota). Entomopathogenic fungi are ubiquitous and can be found in tropical rainforests, Antarctica, and the Arctic (Aung *et al.*, 2008; Augustyniuk-Kram & Kram, 2012).

The species diversity of entomopathogenic fungi in nature is influenced by several biotic and abiotic factors within the ecosystem. The geographical distribution of entomopathogenic fungi is governed primarily by biotic factors, i.e., the distribution of their target insects etc., and is secondarily affected by abiotic factors, including altitude, latitude, temperature, and humidity. Sun & Liu (2008) found that entomopathogenic fungi inhabit a wide range of altitudes (>5200m) and latitudes. However, it is essential to note that not all entomopathogenic fungi colonise the same range of altitudes and latitudes, or at least differences arise at the level of frequency of occurrence of individual species. For example, *Metarhizium anisopliae* occurs more frequently in northern latitudes than *Beauveria bassiana* (Quesada-Moraga *et al.*, 2007). In addition, other factors including temperature and humidity affect the incidence of entomopathogenic fungi; for example, the optimum growth temperature of hyphomycetes is generally between 20-30^{o}C, while Entomophthorales thrive at 15-25^{o}C. However, more tolerant entomopathogenic fungi are occurring at lower temperatures (*ca.* 8^{o}C) and high temperatures (*ca.* 37^{o}C) (Bidochka *et al.*, 2002; Sosnowska *et al.*, 2004; Gul *et al.*, 2014).

Entomopathogenic fungi have evolved to obtain all necessary nutrients for their growth and reproduction from their insect host; To this end, they produce various enzymes and secondary metabolites which help with the infection process and play an important insecticidal role on insects. The cuticle of insects constitutes 70% protein, and the

penetration process is facilitated by extracellular fungal proteases including chymotrypsins, subtilisins, trypsins and metalloproteases, usually with multiple isoforms of each (Charnley, 2003). Toxic metabolites with insecticidal effect include substances such as destruxins (produced by *Metarhizium* species), beauvericins, isarolides, bassianolides, beauverolides, oosporein (produced by *Beauveria* species or *Isaria fumosorosea*), efrapeptins (produced by *Tolypocladium* species) and hirsutellin (from *Hirsutella thompsoni*; Charnley, 2003; Gul *et al.,* 2014). Due to the action of destruxins many different species of insects and arthropods are killed by penetration through the cuticles and overcoming the insect immune system causing tetanic paralysis leading to insect death. Tetanic paralysis is attributed to muscle depolarization by direct opening of the Ca^{2+} channels in membranes (Gabarty *et al.,* 2014).

1.6.1 Entomopathogenic fungi as biocontrol agents.

Arthropod pests are a threat for human health as well as the global production of crops used as biofuels, fibres and most importantly food. Additionally, many insects act as vectors transmitting deadly diseases to humans, including malaria, yellow fever, and dengue. Likewise, insect pests cause damage to crop, and this threat is growing each year worldwide. Most of the crops produced worldwide suffer extensive losses caused by pests, in addition to plant pathogens and weeds (Oerke, 2006). It has been reported that a decade ago the reduction of the annual crop yield caused by insect pests was approximately 18%, not including losses of stored gains (Bergvinson & Garcıa-Lara, 2004). Additionally, it has been reported that an approximate reduction of 8% of the major crops in Brazil was observed due to pests despite control measures being taken (Oliveira *et al*. 2014).

In the last decade insects and pests have been controlled effectively through synthetic chemical insecticides (Meyling *et al.,* 2018). However, increased resistance development to chemical pesticides in pest populations was observed due to their extensive use in agriculture for many years. Moreover, the broad application of these pesticides has potential consequences of pesticide poisoning as well as environmental pollution, leading to regulatory bans of certain insecticides (Chandler *et al*., 2011). As existing agrochemicals are being withdrawn from the market due to environmental and health concerns, environmental and human friendly alternatives are urgently required. There has therefore

been increased interest in the development of biological control agents as substitutes for synthetic chemical insecticides (Aktar *et al*., 2009; Thomas & Read, 2007).

Biological control is important mainly because of protection of the environment. Unlike biopesticides, chemical pesticides release toxic contaminants to the environment. Although chemical pesticides are used mostly in a direct control of pests on above-ground plant parts, a large amount of these pesticides can reach the soil, groundwater, or the atmosphere. They can also cause long-term negative effects and movement over long distances (Van der Werf, 1996). Another major reason why chemical pesticides need to be replaced by biological pesticides or mycoinsecticides is the increasing amount of resistance that insects are developing against various chemical substances contained in plant protection products. Additionally, due to globalisation of the transportation industry invasive species spread to new regions. Since there may not be biological predators or natural pathogens to control the bio invasion, mycoinsecticides provide opportunity to solve this problem (Augustyniuk-Kram & Kram, 2012). For these reasons it is imperative to use or develop alternative pesticides and alternative sustainable control methods.

Reports made by Eilenberg *et al.* (2001) define biological control as the use of living organisms to suppress the population density or the impact of a specific pest organism, making it less abundant or less damaging. Microbial control agents represent sustainable and environmentally friendly alternatives to chemical insecticides (Lacey *et al.,* 2015). These biocontrol agents are based on viruses, bacteria, or fungi with abilities to infect and kill insects (Thomas & Read, 2007). Among these microbes are various entomopathogenic fungi, which effectively infect a wide range of insect and arthropod species through the cuticle (Vega, & Kaya, 2012), rendering them promising for usage as biological control agents (de Faria *et al*., 2007; Augustyniuk-Kram & Kram, 2012).

The use of entomopathogenic fungi as biological control agents constitutes an important component of what is known as integrated pest management (IPM). These fungi have great potential in the management of insect pests infecting various crop systems since they have a big impact on the population dynamics of many economically important species of insect, arthropods, spiders, and mites. Entomopathogenic fungi are very widely used in horticulture where crops are grown under protection rather than the open fields. Therefore, investing more in the research of entomopathogenic fungi will place them in a

better position for IPM than they are now. This way, new paradigms in the development, utilization and regulation of these insecticides will be established (Liu *et al.,* 2012).

Although mycoinsecticides constitute an environmentally friendly alternative to and are comparatively more cost effective than chemical insecticides, currently they are not widely used largely due to a failure of identifying strains consistently active at low doses that eliminate pests rapidly (Leger & Wang, 2010). Efficacy of entomopathogenic fungi can vary depending on abiotic conditions constraining the take-up of these products (Lacey *et al*., 2015). Entomopathogenic fungi cannot be mass produced as efficiently as chemical insecticides, and it is usually hard to meet optimal environmental and host conditions for their application in the field. Plans to alleviate these constraints will considerably improve the reliability and predictability of these biocontrol agents (Meyling *et al.*, 2018). Hence the development of biopesticides able to control different types of plant pathogens and insect pests under a range of environmental conditions can have enormous value for plant protection in the agricultural industry (Ownley *et al.,* 2004). Additionally, it is crucial to optimise their application to ensure maximum efficiency and reliability (Leger & Wang, 2010): establish the stages of the host life cycle that are susceptible to each biopesticide and ensure the timeliness of the treatment is appropriate for full protection. In this respect, entomopathogenic fungi such as *Beauveria bassiana* have the advantage of being able to adapt and grow in soil and in plants as an endophyte.

Large numbers of entomopathogenic fungi have been discovered and several are marketed as biocontrol agents. Among the fungal-based biopesticides, entomopathogenic fungi within the *Ascomycota* and order *Hypocreales* are represented in over 150 commercially available products (de Faria & Wraight, 2007). At least twelve fungal species or subspecies have been used as mycoinsecticides and mycoacaricides. Products based on *B. bassiana* (33.9%), *Metarhizium anisopliae* (33.9%), *Isaria fumosorosea* (5.8%), and *B. brongniartii* (4.1%) are the most common amongst >200 commercial products available (Kotta-Loizou *et al*., 2017).

In the EU, active substances present in the pesticides, both chemical and biological such as entomopathogenic fungi, need to be approved by the European Commission for safety before they reach the market as a commercially available product. More specifically, substances must be proven safe for human and animal health and the environment. All substances undergo intensive evaluation and peer-review by EU countries and the

European Food Safety Authority prior to approval. Currently, it takes 2.5 to 3.5 years in total for a new active substance to be approved by the European Commission and the full process, including field and environmental trials, may take up to 6 years. In the UK specifically, active substances need to be approved by Chemicals Regulation Division (CRD) of the Health and Safety Executive (HSE) prior to marketing, research and development work or import into the UK.

1.6.2 The entomopathogenic fungus and popular biocontrol agent *Beauveria bassiana.*

Beauveria bassiana (Balsamo) Vuillemin is a soil-borne entomopathogenic ascomycete belonging to the Phylum: *Ascomycota*, Subphylum: *Pezizomycotina*, Class: *Sordariomycetes*, Subclass: *Hypocreomycetidae*, Order: Hypocreales, Family: *Clavicipitaceae*, Genus: *Beauveria*, Species: *Beauveria bassiana*. Due to its significance in insect pathology, it affects a wide range of over 700 arthropod species with widespread geographical distribution. *B. bassiana* isolates have not just been recovered from soil but have also been discovered on several plant species as endophytes (Vega, 2008). ATCC 74040 and GHA, trade names Naturalis and Botanigard respectively used to control a variety of arthropod pests (de Faria & Wraight, 2007). Reports made by Chandler *et al*. (2005) are the first to present data on a broad range of entomopathogenic fungal formulations against red spider mite *Tetranychus urticae*. They found that *B. bassiana* Naturalis-L gave a 97% reduction of the *T. urticae* on tomato grown under commercial conditions.

1.6.3 *B. bassiana* life cycle.

Life cycles of entomopathogenic fungi such as *B. bassiana* are integrated with insect host stages and environmental conditions (Fig. 8**;** Shahid *et al.,* 2012). This way, the infection process of fungal entomopathogens of their hosts could be summarized in six main steps: adhesion, germination, differentiation, penetration, development, colonization, sporulation, and dispersion (Vaněček, 2015).

There are several factors that influence the degree of pathogenicity of entomopathogenic fungi. During the adhesion stage, spores attach or adhere to the insect cuticle, which is facilitated by a layer of mucus composed of proteins and glucans that cover the whole spore. After adhesion, the spores begin to germinate and then the fungus differentiates. During this period, the germinating spore produces specialized structures called

appressoria, which fix the spores to the epicuticular surface. The next stage of infection is penetration of the insect cuticle or entrance through natural openings of the insect host such as the mouth or anus. The effects of mechanical pressure and enzymatic activity of the germ tube help penetration.

The important point in this stage is the secretion of several proteases, lipases and chitinases. Immediately entomopathogenic fungi penetrate the insect body, they grow and spread through the hemocoel as yeast-like propagules (blastospores), hyphal bodies or protoplasts lacking a cell wall (Maciá-Vicente, 2011). Entomopathogenic fungi kill the insect by mechanical damage when mycelia grow inside the insect or by the production of toxins. After host death fungi colonize the cadaver, and for 2-3 days they form aerial hyphae and then sporulate (Augustyniuk-Kram & Kram, 2012). Sporulation takes place through a massive production of conidia that will be dispersed in different ways. Species like *Metarhizium* and *Beauveria spp*. produce hydrophobic conidia, which are passively spread, while fungi of the order Entomophthorales produce conidia actively discharged under hydrostatic pressure that are carried by the wind or by co-occurring insects (Roy *et al*., 2001).

Mycoviruses have been described in *B. bassiana* previously and in a descriptive investigation Dalzoto *et al*. (2006) reported the presence of dsRNA elements, virus-like particles and associated hypovirulence of the fungus. Subsequently two viruses belonging to the genus *Victorivirus*, family *Totiviridae*, isolated from a small collection of *B. bassiana* isolates were characterised and sequenced (Herrero *et al.,* 2012; Yie *et al.,* 2014). More recently in a survey of an extensive worldwide collection of *B. bassiana* isolates several mycoviruses were discovered which included several polymycoviruses (Kotta-Loizou & Coutts, 2017) which are novel members of the previously proposed tetramycovirus family (Kanhayuwa *et al*., 2016). Polymycoviruses contain at least four and up to seven genomic segments; the three smallest ones are individual and non-homologous among different members of the family, exhibiting an exceptional dynamic genomic organisation with respect to sequence and segment number. Polymycoviruses cause mild hypervirulence in *B. bassiana* (Kotta-Loizou & Coutts, 2017) which is a desirable trait for the improvement of insecticidal fungi and merits further investigation.

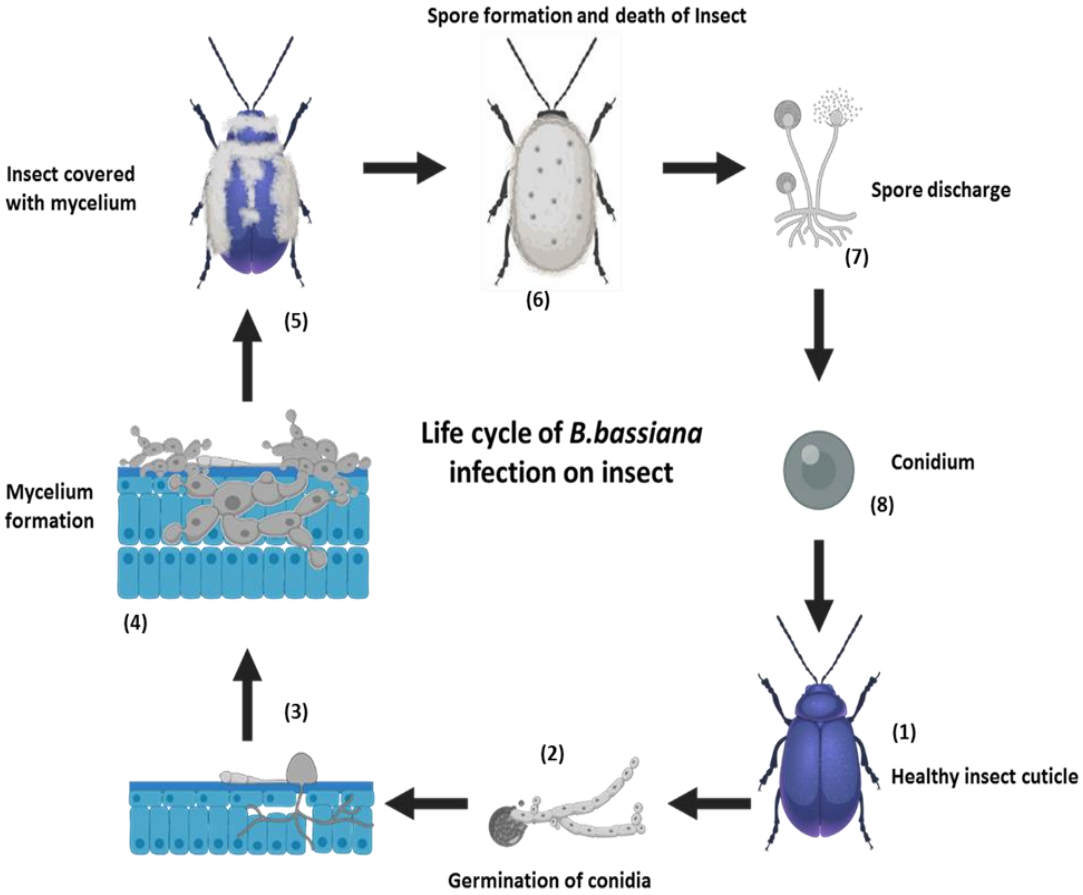


Figure 7. *B. bassiana* life cycle on arthropods.

(1) Metabolically dormant conidia fuse with healthy insect cuticle. (2) Germination and production of hyphae in response to favourable growth condition. (3) Appressorium formation and formation of infection in the arthropod host. (4) Mycelium formation. (5) Mycelium matures and covers the insects. (6) Spore formation occurs resulting in insect death. (7) Sporulation occurs yielding dormant spores. (8) Airborne *B. bassiana* conidia release (image constructed using Bio Render).

1.7 Aim and Objectives.

The overall aim of the PhD was to investigate a novel virus found in *D. septosporum*, designated as Dothistroma septosporum chrysovirus (DsCV)-1. It is hypothesised that DsCV-1 may have a hypovirulent effect on its fungal host and therefore could be a good candidate for the development of a biological control approach to protect pine trees from DNB and I sought to test this hypothesis. The individual objectives of the PhD are illustrated below:

- To clone and sequence the DsCV-1 dsRNA genome from *D. septosporum* isolate D752.1 using random cloning, genome walking and RLM-RACE.
- To perform detailed sequence analysis using bioinformatics tools and phylogenetic analysis to establish relationships between DsCV-1 and other known chrysoviruses.
- To investigate the prevalence of DsCV-1 and related viruses in selected *D. septosporum* isolates from Scotland and England.
- To cure *D. septosporum* isolate D752.1 of infection with DsCV-1 to generate virus-free and virus-infected isogenic lines for assessing the effects of DsCV-1 on its fungal host.
- To assess the putative effect of DsCV-1 on the production of the fungal toxin dothistromin *in vitro*.
- To assess the effect of DsCV-1 on symptomatology, growth, and pathogenicity of *D. septosporum* on pine trees under controlled conditions and with individual pine needles in a novel *in vivo* assay.

D. septosporum is a remarkably slow growing fungus and establishing experimental procedures for the first time with *D. septosporum* is very time consuming. Therefore, some initial work with *B. bassiana* served as developmental technology to be used in main project, including fungal cultures, spore isolation and quantification, growth and pathogenicity assays.

- To investigate the effects of virus infection on the growth and pathogenicity of *B. bassiana* through a comparison of virus-infected and virus-free isogenic lines of the fungus.
- To investigate the pathogenic effects of a virus-infected isogenic line on the greater wax moth *G. mellonella* in a controlled environment.
- To investigate the suitability of a plasmid expressing a hygromycin resistance gene as a transfection marker for *B. bassiana.*

Chapter 2.

Chapter 2. General materials and methods.

2.1 Source of fungal isolates.

All isolates of *Dothistroma septosporum*, the causative agent of red band, Dothistroma needle blight (DNB), were obtained from Dr Anna Brown (current Head of Tree Health at Forestry Commission Scotland, previously Forest Research, UK) on malt extract agar (MEA) plates (Table 2).

Table 2. Provenance of *D. septosporum* isolates.

***D. septosporum* isolate**	**Geographical origin, host, year of isolation (Mullett *et al*. 2017)**
D233	South England, LAT. 51.1525 LONG. -0.8364 Corsican pine - *P. nigra subsp nigra*, 2011
D554	Inverness, Ross and Skye, Scotland, LAT. 57.2830 LONG. -4.8057 Lodgepole pine - *P. contorta*, 2010
D577	Inverness, Ross and Skye, Scotland, LAT. 57.2618 LONG. -4.4340 Lodgepole pine - *P. contorta*, 2010
D584	Inverness, Ross & Skye, LAT. 57.1666 LONG. -4.5588 Lodgepole pine - *P. contorta*, 2010
D587	Inverness, Ross and Skye, Scotland, LAT. 57.1759 LONG. -4.5577 Lodgepole pine - *P. contorta*, 2010
D622.1	Moray and Aberdeenshire, Scotland, LAT. 57.5721 LONG. -3.0424 Lodgepole pine - *P. contorta*, 2010
D700	Inverness, Ross & Skye, LAT. 57.2434 LONG. -3.6951 European spruce - *Picea abies*, 2011
D752.1	West Argyll, Scotland, LAT. 55.3437 LONG.-5.5662 Corsican pine - *P. nigra subsp nigra*, 2012
D1216	Cowell and Trossachs, Scotland, LAT. 56.2330 LONG. -4.5517 Lodgepole pine - *P. contorta*, 2013

Developmental technology at the early stages of the project utilised isolates of *Beauveria bassiana* obtained from various sources through Dr Ioly Kotta-Loizou on Czapek-Dox agar (CDA). All isolates belong to a collection of *B. bassiana* maintained at Imperial College, London. In addition to virus-infected *B. bassiana* isolates (Table 3), the study included two *B. bassiana* virus-free isolates KVL-03-144 and KVL-03-122, supplied by Dr Nicolai Vitt-Meyling, University of Copenhagen (Filippou *et al*., 2018), two commercially available, virus-free isolates Botanigard and Naturalis and an isogenic, virus-free line of EABb 92/11-Dm were compared. Isolate EABb 92/11-Dm was naturally doubly infected with a unirnavirus and a polymycovirus and was freed of virus infection using a combination of cycloheximide treatment and single spore isolation (Kotta-Loizou & Coutts, 2017). Additionally, isolate EABb 92/11-Dm was transfected with plasmid pAN7-1 that confers resistance to hygromycin B.

Table 3. Provenence of *B. bassiana* isolates harbouring dsRNA elements.

***B. bassiana* isolate**	**Arthropod Host (if known)**	**Location**
IMI 392612	Adult spider, Araneidae, Araneoidea, Arachnida	Ecuador
IMI 331273 (ARSEF 757)	Terebrinoid beetle, Terebrionidae, Terebrionidea, Coleoptera	Brazil
IMI 391043	*Eurygaster* sp., Pentatomoidea, Hemiptera	Syria
EABb 92/11-Dm	*Dociostaurus maroccanus*, Acrididae, Acridoidea, Orthoptera	Spain
ATHUM 4946	Air-borne	Greece

2.2 Inoculation and growth of fungal isolates.

D. septosporum (Table 2) and *B. bassiana* isolates (Table 3) were respectively grown on MEA and CDA plates respectively containing streptomycin (100 mg/mL) before inoculation of Petri plates with agar plugs infected with fungal mycelia. Initially autoclaved cellulose discs were placed on the surface of the media to facilitate harvesting mycelia. Subsequently, isolates were subcultured (Fig. 8, 9). All inoculated plates were grown at 25°C to facilitate growth and proliferation of fungal mycelia.

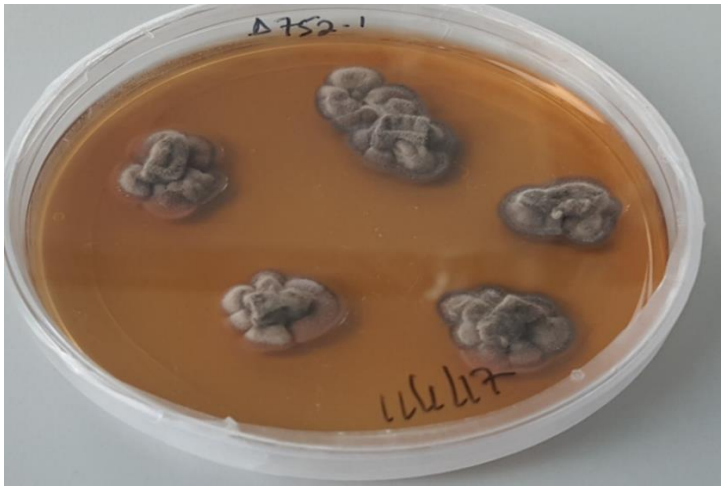


Figure 8. *D. septosporum* isolates grown on MEA.

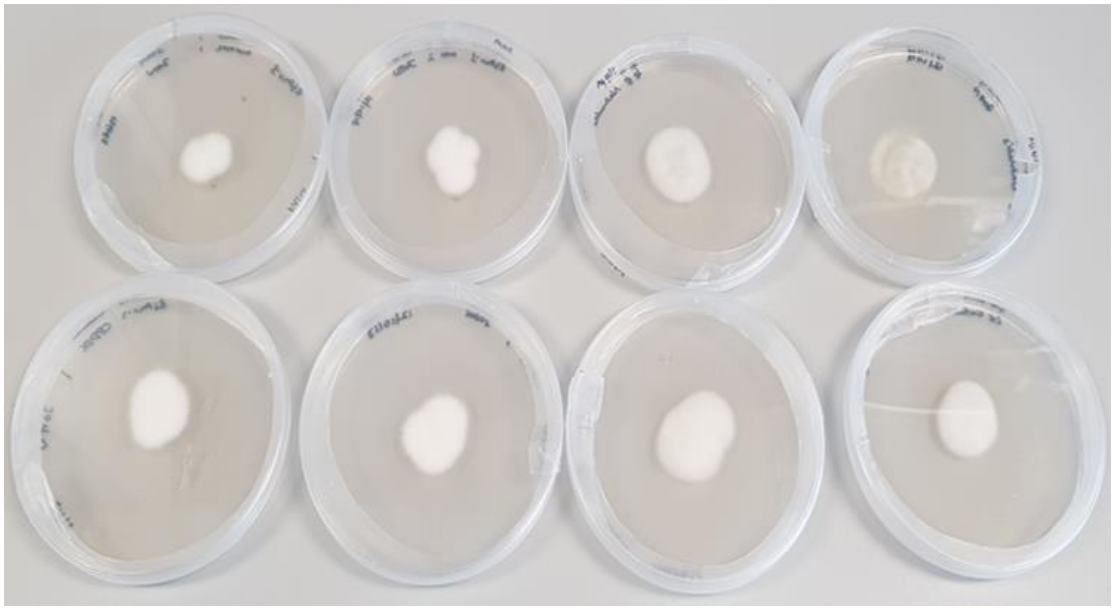


Figure 9. *B. bassiana* isolates grown on CDA.

Additionally, pine minimal medium (MM) with glucose (PMMG) as described by MacDougal *et al*. (2011) was used for *D. septosporum* sporulation and radial expansion assays. The media consists of; magnesium sulphate ($MgSO_4.7H_2O$), 0.2 g/L; dipotassium hydrogen orthophosphate (K_2HPO_4), 0.9 g/L; potassium chloride (KCl), 0.2 g/L; ammonium nitrate (NH_4NO_3), 1.0 g/L; iron sulphate ($FeSO_4.7H_2O$), 0.002g/L; zinc sulphate ($ZnSO_4.7H_2O$), 0.002 g/L; manganese chloride ($MnCl_2.4H_2O$), 0.002 g/L; asparagine, 2.0 g/L; glucose, 3 g/L and agar, 20 g/L added to Milli Q water in which pine needles had been freshly soaked (10% $^w/_v$) for 24-48 h at room temperature and adjusted to pH 6.2 (Dr Rosie Bradshaw, Massey University, New Zealand personal communication). Pine needles from *Pinus radiata* were collected from trees grown in the pinetum at the Bayfordbury site of UH.

2.3 Small-scale double-stranded (ds) RNA extraction protocol.

Mycovirus dsRNA was isolated from fungi, *D. septosporum* and *B. bassiana*, using a small-scale extraction procedure.

2.3.1 Sample preparation for dsRNA extraction.

Fungal mycelium was harvested and crushed using a mortar and pestle in liquid nitrogen. 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 (Section 2.3.2).

2.3.2 Phenol and Sevag treatment.

Phenol was prepared by melting solid phenol (Sigma Aldrich Ltd.) at 68°C, adding 0.1% (w/v) para hydroxy-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. Chloroform (Sigma) was used for nucleic acids extraction and protein denaturation mixed with equal volumes of phenol. Alternatively, TRIZOL was used to isolate nucleic acids. To extract nucleic acids from aqueous solutions mixtures were vortexed and centrifuged at 13,000 rpm for 5 min, to separate the aqueous and organic phases. The upper aqueous phase was then transferred into sterile Eppendorf tubes mixed with Sevag (24:1 chloroform and isoamyl alcohol), vortexed and centrifuged as above to eliminate phenol. The aqueous supernatant containing nucleic acids was processed as described below.

2.3.3 Precipitation of nucleic acids.

Nucleic acids were precipitated by the addition of 1/10 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 to precipitate nucleic acids. 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 the further enzymatic digestions, electrophoresis, or storage at -80°C.

2.3.4 DNase 1 treatment.

Precipitated 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 1 buffer (10x) and 10 µl DNase 1 (1 U/µL; Promega). The mixture was incubated at 37°C >1 h to ensure complete removal of DNA. After incubation, water (400 µL) was added, and nucleic acids were extracted with an equal volume of phenol as above to remove DNase 1 (Section 2.3.1).

2.3.5 S1 nuclease treatment.

DsRNA solutions from above were treated with S1 nuclease to remove contaminating fungal ssRNA. Following DNase 1 treatment as above and overnight ethanol precipitation of dsRNA, mixtures were centrifuged, and the supernatant decanted before air drying the pellets. Pellets were resuspended in 88 µL of water followed by the addition of 10 µL S1 nuclease buffer (10x) and 2 µL S1 nuclease enzyme (1:10 enzyme dilution) (95 U/ µL: Promega). The mixture was incubated at 37°C for >1 h. Then, phenol and Sevag were used to remove the enzyme as above (Section 2.3.1).

2.4 Virus purification using polyethylene glycol (PEG).

Large-scale processing of fungal mycelia was performed to isolate purified virus for dsRNA extraction. To generate sufficient quantities of mycelia, fungi were grown in Spezieller Nahrstoffarmer liquid media (NSLM) or CD liquid media until growth ceased, then the tissue was harvested and frozen at -80°C in 50-60g portions until required. Frozen mycelia were thawed, weighed, and transferred into a blender with the addition of two volumes (w/v) of TE buffer. After homogenising the mycelia 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 x g for 20 min to pellet residual fungal debris. After centrifugation, the supernatant was transferred to another sterilised bottle, and 10 % PEG 6000 and 0.6 M NaCl were added to precipitate the virus by stirring overnight at 4°C. The solution was transferred to Nalgene tubes the next day and centrifuged at 10,000 x g for 20 min to collect the precipitate. The precipitate was resuspended in 20 mL of TE buffer and centrifuged again at 10,000 g for 20 min. The clarified supernatant was subjected to ultracentrifugation at 105,000 x g (30,000 rpm) for 90 min. The pellet was resuspended in 500 µL of TE buffer and transferred into a 1.5 mL microcentrifuge tube before centrifugation at 10,000 rpm for 20 min. To extract dsRNA the supernatant was further purified by phenol and Sevag extraction method which was followed by precipitation of dsRNA with ethanol and sodium acetate as described above (Section 2.3.2 and 2.3.3).

2.5 Total RNA extraction.

The Qiagen RNeasy mini kit was used to extract total RNA from mycelia. Fungal mycelia (100g) were 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 resultant supernatant was carefully transferred to a microcentrifuge tube without disturbing the pellet. The volume of cleared lysate was estimated, and half volume of absolute ethanol was added to it. This mixture was applied to the RNeasy spin column and centrifuged for 15 sec at ≥ 10,000 rpm. The flow-through was discarded and 700 µL RW1 washing buffer was added to the RNeasy spin column followed by centrifugation for 15 sec at ≥ 10,000 rpm. The flow-through was discarded, and 500 µL RPE washing buffer was added for 15 sec at ≥ 10,000 rpm to wash the spin column membrane. The flow-through was discarded and the washing step of the spin column with 500 µl RPE buffer repeated but with centrifugation for 2 min at ≥ 10,000 rpm. Any residual RPE buffer was removed by centrifugation at 13,000 rpm for 1 min. Total RNA extracts were eluted in 30-50 µL water by centrifugation at 13,000 rpm for 1 min.

2.6 DNA extraction.

FastPrep-24 Lysing Matrix tubes were used to isolate pine needle DNA. The tubes, which contain lysing matrix A (garnet matrix and ¼ ceramic spheres), were numbered with a nursery letter identifier and a sample number e.g. “A1” for Alfa sample 1, irrespective of the actual sample name. Records of the respective labels and nursery samples (one microcentrifuge tube for each sample) were incorporated into a spreadsheet. Prior to DNA extraction individual needle samples were placed onto clean paper towels and visually inspected for DNB fruit bodies and colour coded regarding banding. Individual, symptomatic needles (50 mg fresh material or 10 mg dried material) were handled using scalpel and forceps and were sterilised using 0.4 M HCl, rinsed in sterile distilled water and industrial methylated spirit, flame dried and placed into microcentrifuge tubes prior to storage at -20°C and DNA extraction. The Kingfisher Plant DNA kit was used to extract DNA from pine needles. After the pine needles were homogenised using the Qiagen power lyzer 24 DNA was isolated according to the manufacturer’s instructions.

2.7 Quantitation of nucleic acids.

For the quantitation of nucleic acids, a traditional spectrophotometer and a nanodrop were used.

2.7.1 Traditional spectrophotometry.

A spectrophotometer was used for quantitation of nucleic acids, proteins, and the measurement of the OD of bacterial cultures. Samples were placed in plastic or quartz cuvettes with a 1 cm path length, and the absorbance of light at an appropriate wavelength was compared with that of appropriate blank samples and concentrations calculated according to the relationship:

1 A_{260} = 50 µg/mL dsRNA or DNA

1 A_{260} = 40 µg/mL ssRNA

The following equation was used to quantify the dsRNA concentration.

A_{260} × dilution factor × 50 = µg/mL dsRNA

2.7.2 Nanodrop spectrophotometry.

For the quantitation of small amounts of nucleic acid, the Nanodrop ND-1000 (Thermo Fischer) was used according to the manufacturer's instructions. After cleaning the lower and upper optical surfaces of the micro spectrophotometer and opening the nucleic acid module, 2 µL of deionised water was loaded on the lower optical surface, and a blank measurement was taken. The nucleic acid sample was measured by loading 2 µL of sample. The software automatically calculates the nucleic acid concentration if the appropriate constant has been chosen for the measurement (e.g., 50 for dsDNA or RNA and 40 for ssRNA).

2.8 Molecular technology.

Nucleic acids were manipulated and amplified using variations of the polymerase chain reaction (PCR), such as random PCR, reverse transcription (RT) followed by PCR, genome walking, and RNA mediated ligation followed by rapid amplification of cDNA ends (RLM-RACE). Real time quantitative (q) PCR was used to quantify nucleic acids.

2.8.1 Oligonucleotide primer design for 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 nucleotides 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 oligonucleotides 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. Additionally, the OligoCalc tool can also check the self-complementarity for potential hairpin formation.

2.8.2 Reverse transcription (RT) and PCR amplification.

RNA (8 µL) extracted from different sources including *D. septosporum*, *B. bassiana* and their associated mycovirus dsRNAs, together with pine needle DNA and RNA were denatured by heating at 90°C for 10 min, snap cooled on ice for 2 min and 1 µL of sequence specific oligonucleotide primer (100 µM) was added which annealed with the denatured template. This mixture was incubated for 20 min at room temperature and subsequently kept on ice for 1 min. Denatured RNAs were mixed with preheated (50-55°C for 1 min) reaction mixture to synthesise the first strand cDNA following incubation at for 1 min. Superscript™ III RNase H-Reverse Transcriptase (Invitrogen) was then added to the mixture prior to incubation for 1 h at 55°C to synthesise cDNA.

<u>Reaction mixture for first strand cDNA synthesis</u>:

Reverse transcription buffer (5x, Invitrogen) 10 µL

Dithiothreitol (DTT, 100mM, Invitrogen)	4 µL
dNTP mix (20mM, Promega)	1 µL
RNasin™ RNase inhibitor (40 U/µL, Promega)	1.25 µL
Sterilised distilled water	23.75 µL

Following 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 µl of sterilised distilled water. The solution containing cDNA was transferred into a sterile tube for further use PCR amplification.

Reaction mixture for PCR amplification

First strand cDNA	50 µL
PCR reaction buffer (5x, Bioline)	20 µL
dNTP mix (20mM, Promega)	1.2 µL
Sequence-specific primer (100 µM)	1 µL
$MgCl_2$ (25 mM)	8 µL
Random hexamer (100 µM)	1 µL
Sterilised distilled water	18.3 µL
GoTaq DNA polymerase (5 U/ µL, Promega)	0.5 µL

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 amplification (GoTaq DNA polymerase)

94°C for 2 min	1 cycle
94°C for 1 min	
60°C for 1 min	30 cycles
72°C for 3 min	
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% agarose gel electrophoresis (section 2.9).

2.8.3 Random PCR.

A random cloning procedure was used to generate amplicons from mycovirus dsRNAs. Approximately 0.5 µg of purified dsRNA was used as a template for the synthesis of a random cDNA library following protocols described previously by Froussard (1992) and PCR amplification was performed as described (Section 2.7.3).

This PCR amplification programme was used mainly for amplification of dsRNA cDNAs from *D. septosporum*.

Thermocycling conditions for PCR amplification (FROUSSARD)

95°C for 10 min	1 cycle
94°C for 30 sec	
60°C for 30 sec	40 cycles
72°C for 90 sec	
72°C for 5 min	1 cycle
Store at 4°C	

This PCR amplification programme was used mainly for amplification of cDNAs from *B. bassiana* and *D. septosporum* dsRNA. Alterations to the protocols above were employed to amplify fragments of the 18S ribosomal RNA genes from pine needles, *D. septosporum* and *B. bassiana* DNA and for real time quantitative (q) PCR amplification of fragments of the constitutive β-tubulin gene and genes involved with dothistromin toxin production during *D. septosporum* growth, see later. Changes to the protocols above including thermocycling conditions for PCR amplification are described below.

Reaction mixture for real time qPCR amplification

Template DNA (0.8-20 ng)	2 µL
PCR reaction buffer (1x, Hotgoldstar, Eurogentec)	10 µL

dNTP mix (0.2 mM, Promega)	0.8 µL
Forward and Reverse primer (0.3 µM)	0.3 µL
$MgCl_2$ (5 mM)	5 µL
Molecular grade water	4.3 µL
Taq polymerase (Hotgoldstar, Eurogentec)	0.5 µL

Each reaction contains equal concentrations of forward primer, reverse primer, and probe for each of the three pathogens and the positive control (18S rDNA target).

Thermocycling conditions for real time qPCR amplification

95°C for 10 min	1 cycle
95°C for 15 sec 60°C for 55 sec	40 cycles

2.8.4 Genome walking.

A single primer genome walking procedure was used to generate amplicons (Kotta-Loizou & Coutts, 2017) which could be sequenced to characterise mycovirus dsRNAs. Here oligonucleotide primers were designed from known sequences of dsRNAs obtained using the Froussard procedure described earlier, cDNA synthesis and PCR amplification were performed. Normally, the specificity of PCR amplification is determined by two oligonucleotide primers of known nucleotide sequence, one at each end of the target sequence. 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). Therefore, 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.

2.8.5 RNA ligation-mediated rapid amplification of cDNA ends (RLM-RACE).

This procedure involves the modification of the 3'-termini of dsRNA, by ligating a LIG-Rev oligonucleotide primer (5'-kinated-PO4; 3'-OH-blocked; 5'-GATCCAACTAGTTCTAGAGCGG-3'; Coutts & Livieratos, 2003). The RLM-RACE procedure is divided into three phases.

Step 1:

Firstly, 7 µL of dsRNA was mixed with LIG-Rev primer (1 µL), heated at 90°C for 2 min followed by snap cooling on ice. Secondly, a 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 held at room temperature for 2 min prior to adding it to dsRNA plus dimethyl sulphoxide (DMSO; 10 µL), RNase inhibitor (2.5 µL), T4 DNA ligase (1 µL) and T4 RNA ligase (5 µL). The whole reaction mixture was incubated at 17°C overnight.

Step 2:

Following overnight incubation, the volume of the reaction mixture (100 µL) was increased by the addition of 400 µL sterilised water and concentrated using a Nanosep 30K column (VWR) by centrifugation (5,000 X g: 5-10 min), resulting in the recovery of *ca*. 100 µL. The ligated dsRNA (100 µL) was then added to a 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 reaction mixture final volume of (200 µL) was incubated at 68°C for 3 h and the nucleic acids precipitated.

Step 3:

Following pelleting of nucleic acids and washing with 70% ethanol and further pelleting, pellets were resuspended in 8 µL of sterile water and kept on ice for 30 min. Then the ligated nucleic acid was mixed with 2 µL of (LIG-For primer; 5'-CCGCTCTAGAACTAGTTGGATC-3') denatured by heating at 90°C and snap cooled on ice for 2 min. The mixture was incubated at room temperature for 20 min and kept on ice for 2 min. To synthesise first strand cDNA, denatured dsRNA was added to the first initial cDNA synthesis reaction mixture which was pre-heated at 50°C for 1 min, followed by addition of denatured dsRNA and 1 µL of SuperscriptTM III RNase H-Reverse Transcriptase (200 U/ µL; Invitrogen). The entire mixture was incubated at 50°C for 1 h and 70°C for 15 min. this mixture could be stored at -20°C or used on the same day for PCR amplification.

<u>First strand cDNA synthesis reaction mix:</u>

Reverse transcription buffer (5x, Invitrogen)	10 µL
DTT (100 mM, Invitrogen)	4 µL

RNasinTM RNase inhibitor (40 U/ µL, Promega)	1.25 µL
dNTP mix (10 mM, Promega)	2.4 µL
SuperscriptTM III RT (200 U/ µL; Invitrogen)	1 µL
Sterilised distilled water	19.35 µL

Before PCR amplification, the mixture was size-fractioned and concentrated using a Nanosep 30K column as previously. The volume of the first strand cDNA solution was increased to 500 µL with H_2O and centrifuged in the column (5000 x g for 5-10 min), resulting in the recovery of *ca*. 100 µL of solution. Then PCR amplification was then set up as described below.

PCR Mixture:

First strand cDNA	2.5 µL
PCR reaction buffer (5 X, Promega)	10 µL
LIG-FOR primer (100 mM)	0.5 µL
Sequence-specific primer (100 mM)	0.5 µL
dNTP mix (10 mM, Promega)	1 µL
Sterile distilled water	35 µL
GoTaq polymerase (5 U/ µL, Promega)	0.5 µL
Total volume	50 µL

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

2.9 Purification of PCR amplicons.

To purify amplicons generated by polymerase chain reaction (PCR) amplification the QIAquick PCR Purification kit (Qiagen) was used, following the manufacturer's protocol. Amplicons were mixed with five volumes of PB binding buffer and mixed by inversion. Sodium acetate was added to optimise the pH to 5.5 and the mixture was placed onto the QIAquick column and centrifuged at 13,000 rpm for 1 min followed by washing with 700 µL of PE washing buffer. 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).

2.10 Gel electrophoresis.

Nucleic acids were separated by agarose gel electrophoresis. Here 1% agarose gel was prepared by dissolving agarose powder by boiling in 1x TAE buffer. When the temperature was *ca*. 50-60°C, the gel was poured into a casting tray and left to solidify for at least 45 min before loading the samples which contained 5x loading buffer. The gel was electrophoresed in 1x TAE buffer for 2 h at 30-50 V. Then, gels were stained with Gel Red as described by the manufacturer for 15 min and destained for 10 min to visualise dsRNA under UV light**.**

2.11 Gel extraction of nucleic acids.

Following gel electrophoresis, bands of interest were excised and extracted 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 QG bidning buffer were added to each gel volume and the mixture incubated at 55°C for 10 min until the gel slices melted. This process was facilitated by inverting tubes every 2 min during incubation. When the mixture turned to orange or violet in colour after the gel dissolved, 10 µL of sodium acetate (3 M) was added to adjust the pH shown as a yellow colour. One gel volume of isopropanol was then added to the sample and the whole mixed. The melted agarose solution was transferred to a silica membrane based MinElute spin columns 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 after addition of 750 µL of buffer PE. The column was centrifuged again for 1 min to remove residual buffer. After removal of impurities, the column was placed in a 1.5 mL Eppendorf 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 in a tube with a small amount of buffer or water which was stored at -20°C until use. All centrifugations were performed at 13.000 rpm.

2.12 Molecular cloning.

2.12.1 Bacterial strains

The following *Escherichia coli* strains were used for cloning purposes.

E. coli JM109 (Promega). The genotype of this strain is: traD36 proA$^+$ proB$^+$ *lacI*^{q} *lacZ*ΔM15/*rec*A1 *gyr*A96 (Na^r) *thi hsd*R17 *sup*E44 *rel*A1 Δ (*lac-pro*AB) *mcr*A.

E. coli XL 10 Gold Ultracompetent cells (Agilent). The genotype of this strain is: Tet^{r}Δ (*mcrA*) 183Δ (mcrCB-*hsd*SMR-*mrr*) 173 *endA1 supE44 thi-1 recA1 gyrA96 relA1 lac* H*te* [F′ proAB lacI^{q}ZDM15 Tn*10* (Tet^r) Amy Cam^r].

2.12.2 Preparation of *E. coli* competent cells.

Preparation of *E. coli* competent cells was carried out using the transformation storage solution (TSS) method adapted from Chung *et al*. (1989). Frozen *E. coli* JM109 cells were thawed, and 1 µL was inoculated into sterile 2x LB broth (5 mL). These cells were grown overnight at 37°C on a shaker (250 rpm). Then 500 µL of the overnight culture was diluted into 1X LB broth (50 mL). This suspension was incubated at 37°C until the optical density of the cells reached 0.40-0.60 at 600nm. The cells were then chilled on ice for 10 min in pre-cooled 50 mL centrifuge tubes and centrifuged (3000 x g; 10 min) at 4°C. The pelleted cells were resuspended in 5 mL chilled TSS (Appendix 1), aliquoted (100 µL) into Eppendorf tubes and stored at -80°C.

Alternatively, preparation of *E. coli* competent cells was carried out using the calcium chloride ($CaCl_2$) 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 X g for 10 min at 4°C. The supernatant was decanted, and pellet was resuspended in cold 25 mL $CaCl_2$ (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 CaCl2) and aliquots were stored at -80°C.

2.12.3 Cloning vectors.

The vectors used in *E. coli* cloning are listed below with their applications and antibiotic markers (Table 4).

Table 4. List of vectors used for cloning in *E. coli*.

Vector	Source of reference	Selection
pGEM-T Easy	Promega	Ampicillin
pUC18	Fermentas	Ampicillin
pUC19	Promega	Ampicillin

2.12.4 Ligation.

PCR amplicons (3 µL) were ligated with pGEM-T-Easy plasmid (Promega; 1 µL, Table 4) in the presence of 2x ligation buffer (5 µL) using T4 DNA ligase (1 µL). Ligation reactions were incubated at 4°C for a minimum of 16 h.

2.12.5 Transformation.

Transformation was used to transfer recombinant plasmids into *E. coli* cells. To perform transformation previously stored (-70°C) competent *E. coli* cells were thawed on ice. Ligation mixture (5 µL) was mixed with the 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 on ice for 20 min. The transformation tube was heat-shocked by placing it into a 42°C water bath for 45 sec. The tube was returned to ice for 2 min. Then, Super Optimal broth with Catabolite repression (SOC) solution (900 µL; Appendix 1) was added to the mixture and incubated at 37°C on 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 pellet was resuspended gently by pipetting in remaining solution. The transformation mixture was spread on LB agar antibiotic (ampicillin) plates containing X-Gal (5-bromo-4-chloro-indolyl-β-D-galactopyranoside; Appendix 1) and IPTG for selection of blue and white colonies. Plates were incubated overnight (16-20 h) at 37°C. After overnight incubation plates could be stored at 4°C. Recombinant clones were identified as white colonies which were distinguished from blue, wild-type colonies. White colonies were selected under sterile conditions and transferred into 5 ml LB medium

containing ampicillin using sterile toothpicks and incubated overnight at 37°C with shaking (200 rpm).

2.13 Plasmid extraction and restriction digestion.

The QIA Mini prep kit (Qiagen) was used to isolate plasmids from individual recombinants amplified in cultures grown overnight as described above. Bacteria were pelleted by centrifugation at >8,000 rpm for 5 min and cells were resuspended in P1 resuspension buffer (250 µL) and resuspended by vortexing and then transferred into a 1.5 mL microcentrifuge tube. Before the addition of 250 µL of P2 alkaline buffer (0.2 N NaOH and 1% SDS) and tube inversion 4 to 6 times to assist lysis of bacterial cells. After allowing the solution to clear for 3 min, 350 µL of N3 neutralisation buffer was added to neutralise the pH with tubes being inverted 4 to 6 times gently to mix. Following this lysed cell were removed by centrifugation (13,000 rpm for 10 min) and the supernatant carefully transferred onto QIA Miniprep columns. Columns were centrifuged (13,000 rpm for 1 min) to allow binding of plasmid DNA to the columns, and the flow-through was discarded. The columns were then washed using 500 µL PB buffer, centrifuged for 1 min (13,000 rpm) and the flow-through discarded. Columns were washed with 750 µL PE buffer followed by centrifugation (13,000 rpm for 1 min) removal of flow-through and re-centrifuged as above to remove residual washing buffer. Finally, columns were placed into 1.5 mL microcentrifuge tubes and 50 µL of EB buffer or nuclease-free water was added to elute plasmid DNA. The columns with the elution buffer were allowed to stand for 1 min and then they were centrifuged to collect the DNA. The restriction endonuclease digestion with *Eco*R1 prior to sequencing set up is described as follows.

<u>Restriction Digestion</u>

*Eco*R1 buffer (10x)	2 µL
Plasmid DNA	10 µL
*Eco*R1 enzyme	1 µL
Sterilised distilled water	7 µL

Tubes were incubated at 37°C overnight; the restriction digestion products were visualised following agarose gel electrophoretic separation.

Chapter 3.

Chapter 3. Characterisation and sequence determination of Dothistroma septosporum chrysovirus (DsCV-1).

3.1 Chapter 3 Introduction.

Recent developments in recombinant DNA technology and DNA sequencing, such as next-generation sequencing, have led to an increase in the number of mycoviruses whose genome has been fully or partially sequenced. Although obtaining the mycovirus genome sequence does not directly provide any evidence on mycovirus-mediated phenotypes and the potential of mycoviruses as biological control agents, this knowledge significantly contributes to our understanding of the diversity and evolution of mycoviruses together with their potential effects on their hosts.

The latter is achieved by investigating eukaryotic linear motifs (ELMs) in viral proteins. ELMs are compact protein interaction sites composed of short stretches of adjacent amino acids (Dinkel *et al.*, 2016). Six classes of ELMs are recognised: CLV (cleavage sites), DEG (degradation sites), DOC (docking sites), LIG (ligand binding sites), MOD (post-translational modification sites) and TRG (targeting sites). ELMs play crucial roles in cell regulation and ELM mimics are often used by pathogens to manipulate their hosts' cellular machinery. Mycovirus proteins are liable to interact with fungal proteins *via* ELMs, affecting the hosts' phenotype and potentially leading to hypovirulence (Dawe & Nuss, 2013).

In a previous investigation a panel of 44 *D. septosporum* isolates from the United Kingdom were screened for mycoviruses and one isolate D752.1 was discovered to contain dsRNAs (Shah, 2017). *D. septosporum* isolate D752.1 was originally discovered in 2012 at West Argyll, Scotland in Corsican pine and was identified morphologically and by molecular analysis as mating type 1. Resistance to DNase 1 and S1 nuclease treatment and sensitivity to RNase III of the four nucleic acid elements confirmed dsRNA provenance. Partial sequencing suggested the presence of a chrysovirus provisionally nominated Dothistroma septosporum chrysovirus (DsCV)-1.

3.1.1 Chapter 3 Objectives.

The main objectives for this chapter were:

- To clone and sequence the DsCV-1 dsRNA genome from *D. septosporum* isolate D752.1 using random cloning, genome walking and RLM-RACE.

- To perform detailed sequence analysis using bioinformatics tools and phylogenetic analysis to establish relationships between DsCV-1 and other known chrysoviruses.
- To investigate the prevalence of DsCV-1 and related viruses in selected *D. septosporum* isolates from Scotland and England.

3.2 Chapter 3 Materials and Methods.

The materials and methods for chapter 3 include cloning and sequencing of DsCV-1, bioinformatics analysis of DsCV-1 and development of a diagnostic assay for the presence of DsCV-1 in *D. septosporum*.

3.2.1 Cloning and sequencing of DsCV-1.

DsCV-1 dsRNA was extracted using a small-scale dsRNA extraction protocol (section 2.3). The purified DsCV-1 dsRNAs were separated by electrophoresis and visualized after staining with GelRed (section 2.9). The sizes of the dsRNA elements were estimated in relation to DNA size markers (Hyperladder 1; Bioline). Approximately 0.5 µg of purified dsRNA was used as a template for random RT-PCR and cloning. Over thirty different clones were sequenced and used to assemble 10 contigs (Fig. 12). Sequence-specific primers (Table 5) and genome walking were used to fill gaps in the assembled sequences not already covered by the clones derived from random RT-PCR and RLM-RACE (section 2.8) was performed to determine the terminal sequences of the four dsRNA molecules.

Table 5. List of primers used for cloning DsCV-1.

List of primers	
RNA-1 primers	**Sequences (5'-3' orientation)**
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'
RNA-2 primers	**Sequences (5'-3' orientation)**
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'
RNA-3 primers	**Sequences (5'-3' orientation)**
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’-CATGCCCCTCACTGTAGGTTG-3’
DF UTR	5’-CAAAAATCCTAGCGGCCTGA-3’
DF IJ1	5’-GTCGCATCTGTCTTATGAAG-3’
DF IJ2	5’-GTCGCATCTGTCTTATGAAG-3’
DF IJ3	5’-GAGTTGGACGGCCTGAGTA-3’
RNA-4 primers	**Sequences (5’-3’ orientation)**
DS1.34.F	5’- GAAGAGCCTACACTCATACACGC- 3’
DS1.34.R	5’- CTTGGGCACATGTCCCTTGTC- 3’
DS1.RDRPEX.R	5’- GAATCTAGAACGGATCACGGGTC- 3’
DS4.2.R	5’- CGCTGATATACGCTCATGAACACTC- 3’
DS4.2.R	5’- GGCACTGATATACGCTCATGAACA- 3’
DS4.2.F	5’- GACCGGTTAGCTGCAGTCACATTG- 3’
DS4.57.R	5’- GTTTCTACTCACAGCGCCACCG- 3’
DS4.57.F	5’- TCGCTCGGTGCGTGCGGC- 3’
DS4.45.F	5’- GGGCACTGTACTCCCATCCTG- 3’
DS4.15.R	5’- CGAACTATTGTTATTGTATGGGCGTC- 3’

The amplicons were then ligated into the cloning vector pGEM-T Easy and transformed into competent *E. coli* cells (section 2.12). Recombinant plasmids were extracted and verified using restriction digestion (section 2.13). Plasmids containing inserts of interest were sequenced at Genewiz UK using the universal M13 primers (M13 FORWARD 5’-GTA AAA CGA CGG CCA GT-3’ and M13 REVERSE 5’-AAC AGC TAT GAC CAT G-3’). Each nucleotide was sequenced in at least three independent experiments. The work flow in Fig. 10 summarises the methodology used to obtain the sequence of DsCV-1.

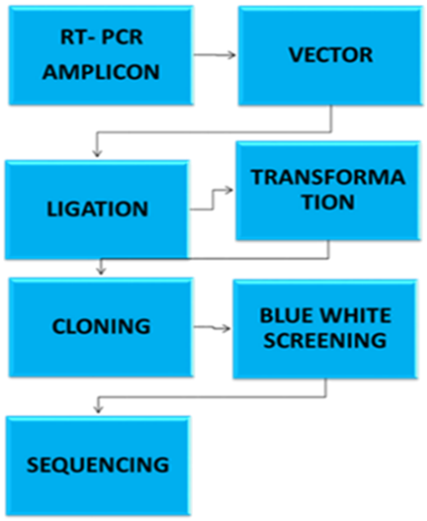


Figure 10. Workflow for DsCV-1 sequencing.

Flow diagram of procedural steps for the production and analysis of cDNA clones generated using DsCV-1 dsRNAs as a template.

3.2.2 Bioinformatics analysis of DsCV-1.

Full length clones of the DsCV-1 genome were assembled from the cloned and sequenced segments using the ClustalX (Thompson *et al*., 1992) and MAFFT (Katoh & Standley, 2013) programmes. The DsCV-1 genome sequence was compared for similarity with sequences available in public databases using the Basic Local Alignment Search Tool (BLAST) (https://blast.ncbi.nlm.nih.gov/Blast.cgi) on the National Centre for Biotechnology Information (NCBI) web server (Altschul *et al*., 1990). Multiple sequences were aligned using MULTALIN (http://multalin.toulouse.inra.fr/multalin/) and ClustalX (Thompson *et al*., 1997). The alignments were improved manually and highlighted using GeneDoc (Nicholas *et al*., 1997).

For phylogenetic analysis of DsCV-1 and related viral sequences, the RdRP sequences were aligned with MUSCLE as implemented by MEGA 7 (Kumar *et al*., 2016). All positions with less than 30% site coverage were eliminated and the LG+G+I+F substitution model was used. The maximum likelihood (ML) method was used to construct a phylogenetic tree, with bootstrap test values based on 1,000 replications.

Finally, the Eukaryotic Linear Motif (ELM) database (http://elm.eu.org/) was used to detect ELMs in DsCV-1 and related viral sequences. The cell compartment and taxonomic content were specified as 'cytosol' and 'fungi' respectively. Revigo (http://revigo.irb.hr/) was used for the visualisation of the Gene Ontology (GO) analysis of the ELMs.

3.2.3 DsCV-1 diagnostic assay.

In addition to D752.1, eight *D. septosporum* isolates (Table 2; Fig. 11) from the Forest Research UK fungal collection were chosen for investigation by RT-PCR amplification (section 2.8) to establish whether they were infected with DsCV-1 or not. The cohort of isolates investigated included the virus-infected (VI) and virus-free (VF) isogenic lines of isolate D752.1. The VF isolate was generated from a culture of *D. septosporum* isolate D752.1 which had been stored at -80^{o}C for over two years and had spontaneously lost the virus as established on a separate occasion by Dr Ioly Kotta-Loizou. Two oligonucleotide primer pairs were used to generate DsCV-1 amplicons of 465 bp employing forward primer 5'-ATT GGC TGA CAT GGT GGA GG-3' and reverse primer 5'-ATG CGC CTG TCA GGA TCA AA-3' plus a combination of oligonucleotide primers shown in Table 5 to generate 1001 bp

amplicons. All the larger amplicons were sequenced to determine potential heterogeneity of the DsCV-1 sequences in the *D. septosporum* isolates examined.

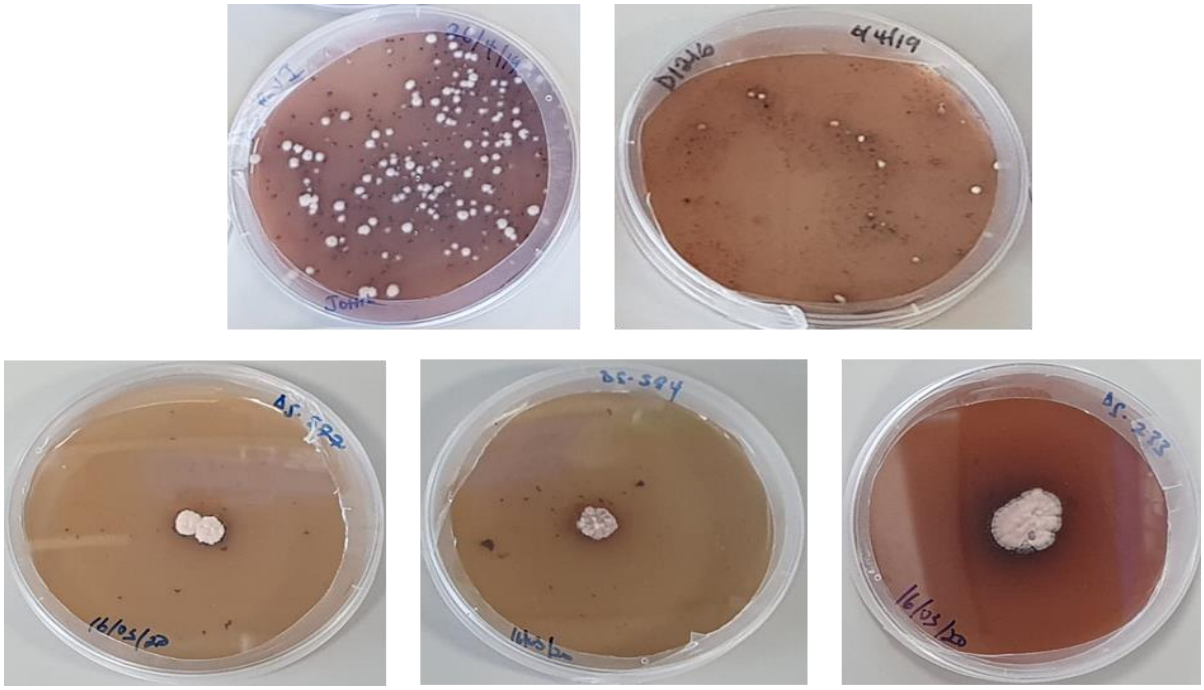


Figure 11. *D. septosporum* isolates grown on PMMG.

Representative photographs of *D. septosporum* isolates from top left to bottom right: D752.1, D1216, D577, D584 and D233.

3.3 Chapter 3 Results.

The results of chapter 3 include description of sequence properties of DsCV-1, phylogenetic analysis of DsCV-1 and related chrysoviruses, population studies of DsCV-1 in the UK and sequence-based prediction of putative virus-host interactions. The objectives were achieved with one exception: the 5' terminus of dsRNA 3 was not sequenced despite multiple attempts to do so employing different approaches such as RLM-RACE, genome walking and use of degenerate primers.

3.3.1 Sequence properties of DsCV-1.

Small scale dsRNA extraction and agarose gel electrophoresis indicated that *D. septosporum* isolate D752.1 harboured four dsRNA elements *ca*. 3.6, 3.0, 2.8 and 2.8 kbp in size (Fig. 12).

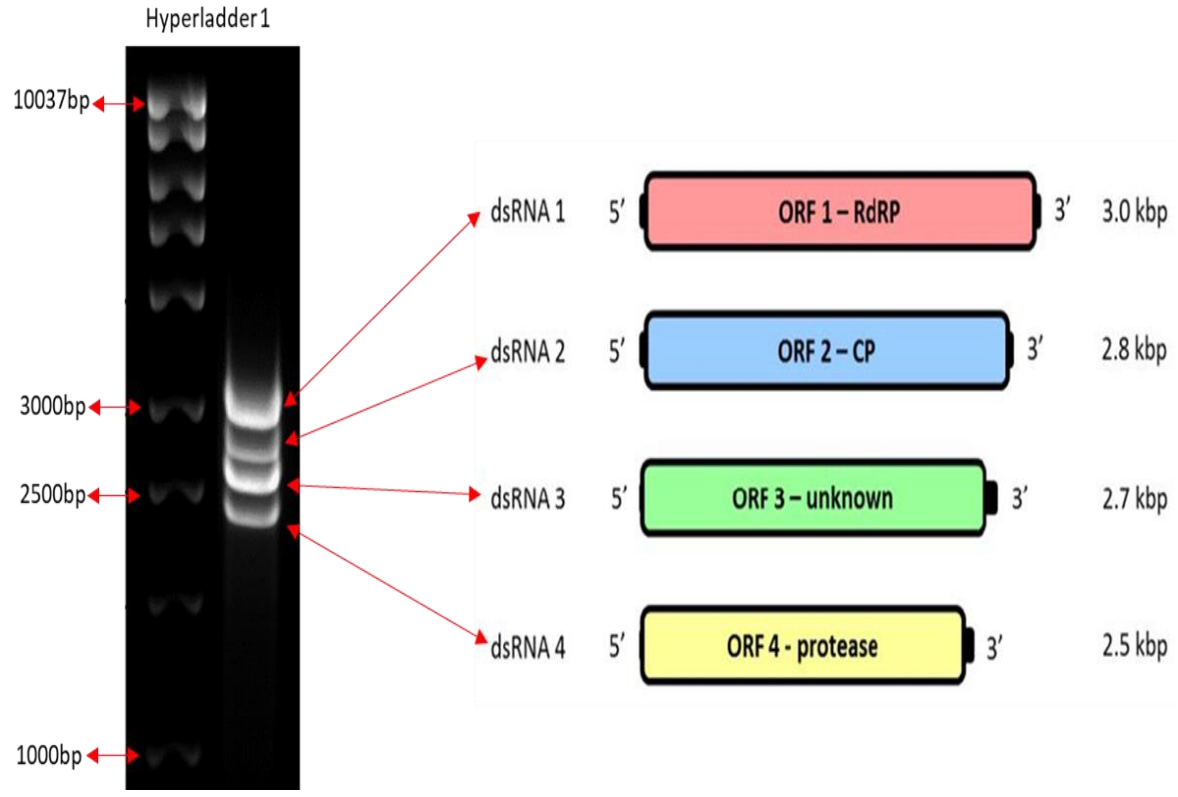


Figure 12. Electrophoretic profile of DsCV-1 dsRNAs.

Separation of DsCV-1 four dsRNAs, whose approximate sizes are shown to the right of the gel. The dsRNAs were displayed on a 1.5% ($^{w}/_{v}$) agarose gel stained with GelRed and electrophoresed in 1 X TAE. Hyper Ladder™ 1kb (Bioline) was used as molecular weight marker and sizes are shown to the left of the gel.

The genome organization of DsCV-1 is shown in Fig. 12. The contigs used to assemble the sequences of the four dsRNAs are shown underneath the assembled sequences. The dsRNA elements comprising the genome are numbered 1 to 4 according to decreasing size. Dr Unnati Shah completely sequenced DsCV-1 dsRNAs 2 and 4, while this thesis focused on determining the sequence of dsRNAs 1 and 3. I am indebted to Dr Shah for allowing me access to these sequences so that I could use them to perform a full sequence analysis of the viral genome.

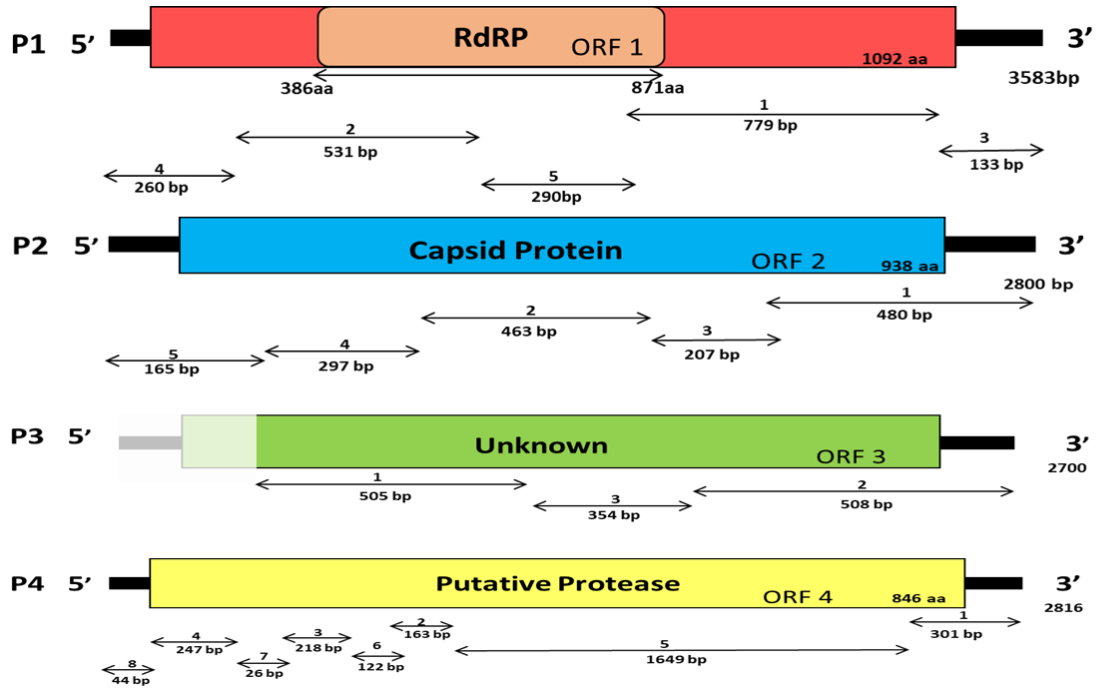


Figure 13. Genome organization of DsCV-1.

The genome consists of four dsRNA monocistronic segments. The double headed arrows represent the location of individual amplicons generated with random RT-PCR, genome walking or RLM-RACE. Rectangular boxes represent the open reading frames (ORFs); ORF 1 encodes an RNA-dependent RNA polymerase RdRP (P1), ORF 2 the capsid protein (CP; P2), and ORF 3 (P3-partial) and ORF 4 (P4) respectively encode an unknown protein and a putative protease. Stippled areas within the ORF boxes represent conserved domains in P1 (RdRP conserved motifs) and P3 (phytoreovirus S7 domain).

The complete sequence of dsRNA 1 is 3583 bp in length and contains a single ORF encoding a 1095 amino acid (aa) protein (126.45 kDa) designated P1. Analysis of the deduced aa sequence of dsRNA 1 revealed the presence of eight conserved motifs typically present in RdRPs of dsRNA viruses of simple eukaryotes (Figs. 13 and 14; Bruenn, 1993). Further

searches showed that P1 shares a high degree of identity to RdRPs of members of the *Chrysoviridae* family in the RdRP_4 family PF02123 with the highest identity to Isaria javanica chrysovirus-1 (IjCV-1) RdRP (Herrero, 2017) with 65 % identity. Sequence analysis of dsRNA 2 revealed that it is 2800 bp in size containing an ORF that encodes a 938 aa protein (104.21 kDa nominated P2. P2 showed significant identity to capsid protein (CP) genes of different members of the family *Chrysoviridae*, with IjCV-1 CP being the most closely related (44 % identical). DsCV-1 dsRNA 3 is approximately 2700bp in length, notwithstanding its 5' terminus (*ca.* 500 bp). DsCV-1 dsRNA 3 includes a single ORF that encodes a protein at least 859 aa in length nominated P3. As with the IjCV-1 dsRNA 3 the protein encoded by DsCV-1 dsRNA 3 is expected to be longer than that encoded by dsRNA 2, whereas most CPs from members of the *Chrysoviridae* family are normally longer than the proteins encoded by the shorter dsRNAs. BLAST searches of the aa sequence deduced from dsRNA 3 showed the highest identity with a hypothetical protein of unknown function encoded by IjCV-1 dsRNA 3 (36 % identity). As found in other members of family *Chrysoviridae* this hypothetical protein contains a ''phytoreovirus S7 domain'', in this case VVVPQGHGKTML found in viral core proteins with nucleic acid binding activity (Ghabrial *et al*., 2015; 2018). Additionally, as observed in similar P3 proteins from other members of the family *Chrysoviridae* the N-terminal region of the protein shares a degree of identity with comparable N-terminal regions of chrysovirus RdRPs. DsCV-1 dsRNA 4 is 2816 bp in size and contains an ORF that encodes an 846 aa protein (93.49 kDa) designated as P4. Analysis of P4 revealed the presence of motifs typical of cysteine proteases which contain the conserved motif PGDGXCXXHX (in this case PGDGRCGIHA) which forms the conserved core of the ovarian tumour gene-like superfamily of predicted proteases. BLAST searches showed a high degree of identity of P4 to putative proteases from other members of the *Chrysoviridae* family (the highest identity was to IjCV-1; 62 % identity).

The termini of the 5'- and 3'-untranslated regions (UTRs) of the four dsRNAs constituting the DsCV-1 genome were highly conserved (Fig. 15 A, B). The 5'-UTRs of DsCV-1 dsRNAs 1, 2 and 4 are respectively 189, 144 and 158 nt in length and considered to be relatively long for a mycovirus (Fig. 15 A). An analysis of the four 5'-UTRs showed the presence of a highly conserved region of *ca*. 65 nt, like the ''box 1'' region found in most chrysoviruses (Ghabrial *et al*., 2018). A second region of strong sequence similarity is found immediately downstream from ''box 1'' containing a few CAA repeats (Fig. 15 A). The CAA repeats are

similar to the enhancer elements found in the 5'-UTRs of tobamoviruses (Gallie & Walbot 1992; Ghabrial *et al*., 2018). The 3'-UTRs were 109, 107, 102 and 117 nt long for dsRNAs 1-4 respectively and contained a conserved 3'-terminus of 11 nt (Fig. 15 B).

Figure 14. Conserved motifs in chrysovirus RdRP sequences.

Comparison of the eight conserved motifs in the RdRP sequences of members of the genus (A) *Alphachrysovirus* and (B) *Betachrysovirus* (Table 1). The strictly conserved GDD triplet motifs are circled. Numbers in parentheses correspond to amino acid (aa) residues present separating the motifs. Asterisks and colons respectively signify identical and highly conserved aa residues at the positions indicated (figure courtesy of Dr Ioly Kotta-Loizou).

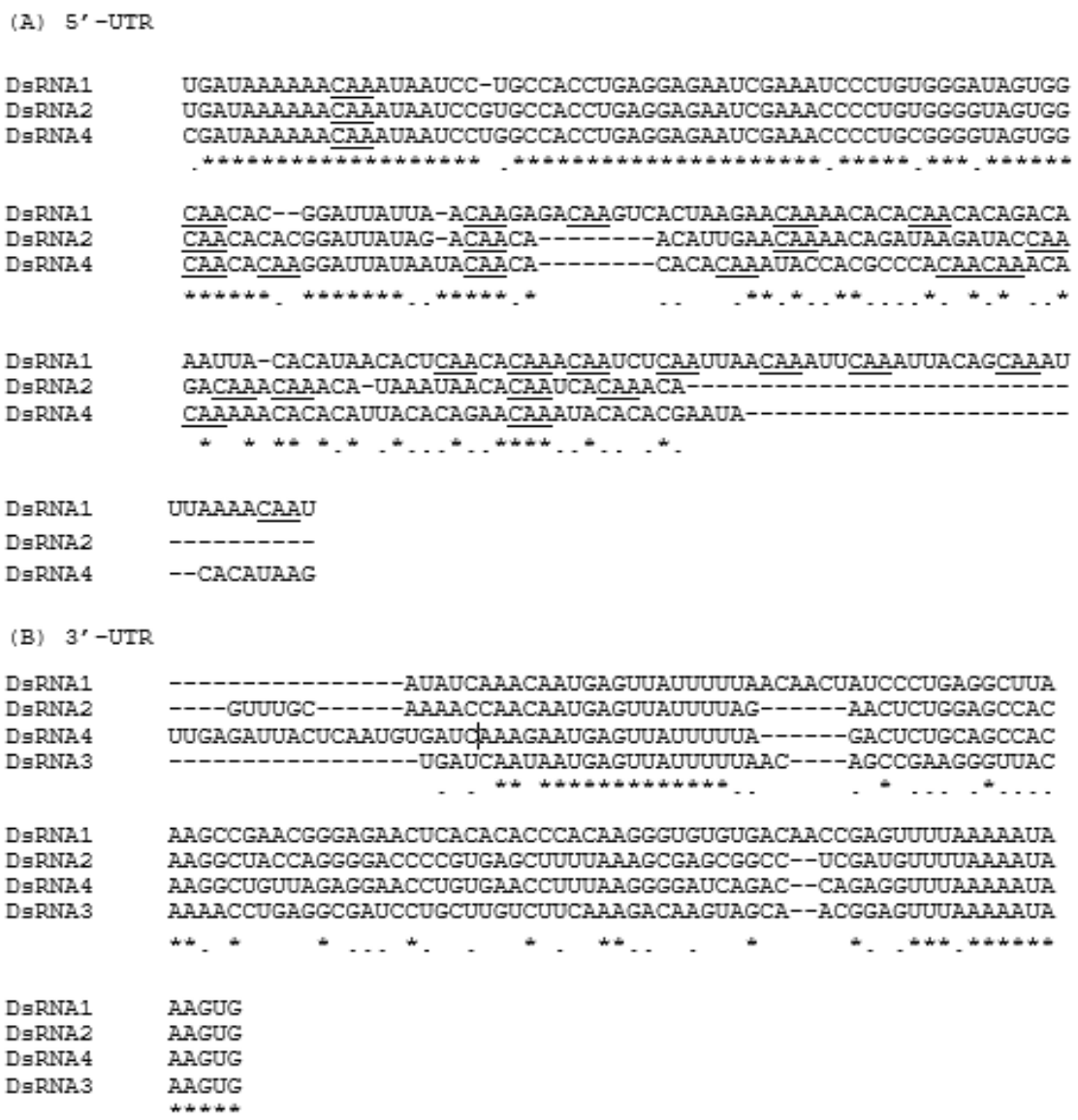


Figure 15. DsCV-1 5' and 3' UTRs.

Nucleotide sequence alignment of the **(A)** 5'- and **(B)** 3'-UTRs of the DsCV-1 dsRNA segments. The CAA repeats in the 5'-UTRs are underlined. Asterisks designate identical nucleotides at the indicated positions while dots specify that three out of four nucleotides are conserved at the respective position

3.3.2 Phylogenetic analysis of DsCV-1.

Phylogenetic analysis, based on the complete or almost complete aa sequence of the RdRP genes of all members of the *Chrysoviridae* family and that of DsCV-1, was performed (Fig. 16). The phylogenetic tree constructed revealed that the putative RdRP encoded by DsCV-1 most strongly resembled viruses within the genus *Alphachrysovirus*, family *Chrysoviridae*, which exclusively infect plants and fungi (Fig. 16). A complete list of known and sequenced alpha- and betachrysoviruses, their names, acronyms, and accession numbers of their genomic dsRNAs are shown in Table 1.

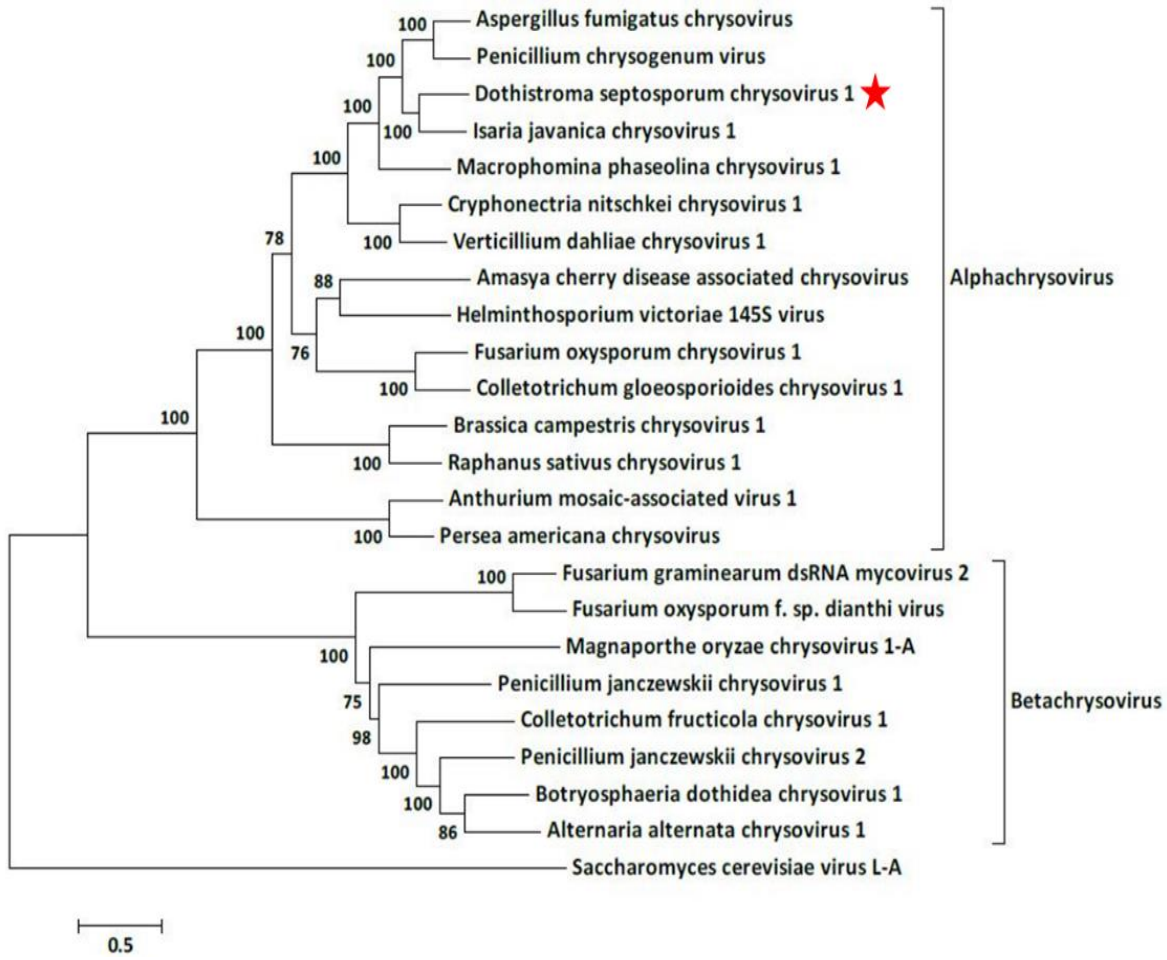


Figure 16. Phylogenetic tree of the chrysovirus RdRP sequences.

The phylogenetic tree was created based on the RdRP sequences of members of the genus *Alphachrysovirus* and *Betachrysovirus* (Table 1). The sequence of Saccharomyces cerevisiae virus L-A totivirus was incorporated as an outgroup in the tree. DsCV-1 is indicated by a red star.

3.3.3 Assessment of DsCV-1 infection of *D. septosporum* isolates.

Diagnostic RT-PCR amplification revealed that all eight *D. septosporum* isolates (D554, D577, D584, D587, D622.1, D700, D752.1 and D1216) apart from cured D752.1 VF (not shown) and one wild type isolate D223 were infected with DsCV-1 (Fig. 17). Notably, two independent cultures of D752.1 were tested (Fig. 17, lanes 4 and 6), confirming the presence of DsCV-1. Numerous screens for the presence of mycoviruses in fungi from diverse families reveals a much lower rate of infection of only 15-20% (Ghabrial & Suzuki 2009); however, there have been reports of similar high rates of infection when screening geographically related isolates (Shah, 2017). Following analysis of the proteins predicted from all the 1001 bp amplicons only limited sequence heterogeneity between the prototype D752.1 VI isolate was found as compared to all the other isolates (Fig. 18).

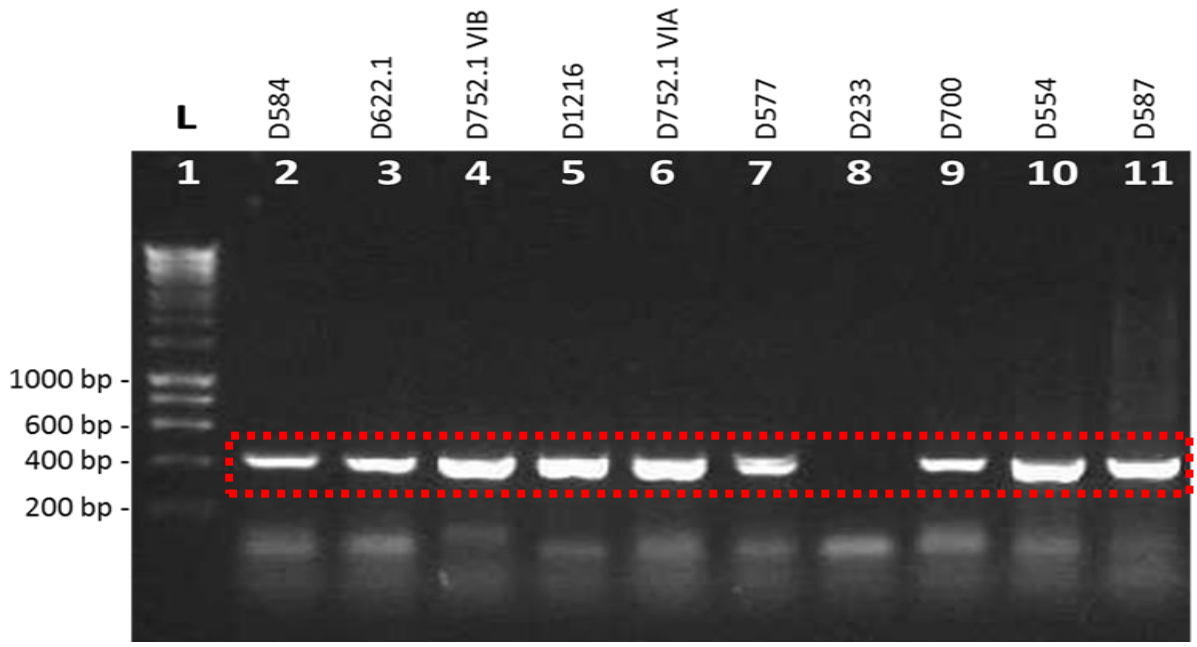


Figure 17. Electrophoresis of DsCV-1 specific PCR amplicons.

Screen of representative *D. septosporum* isolates (Table 2) for the presence of DsCV-1 using RT-PCR amplification of a 465 bp fragment of the DsCV-1 dsRNA 1 RdRp gene. Amplicons were displayed on a 1% ($^{w}/_{v}$) agarose gel stained with GelRed and electrophoresed in 1 X TAE. Hyper Ladder™ 1kb (Bioline) was used as molecular weight marker and sizes are shown to the left of the gel. The area of the gel where amplicons would be located is indicated with a red box.

Figure 18. Multiple sequence alignment of DsCV-1 specific PCR amplicons.

Amino acid sequence alignment of proteins predicted from the sequences of 1001 bp amplicons of fragments of the DsCV-1 dsRNA1 encoded RdRP gene for *D. septosporum* isolates (Table 2). Asterisks and colons respectively indicate identical and conserved amino acid residues.

3.4.4 Predicted interactions between DsCV-1 and the host fungus.

ELMs associated with fungal proteins localised in the cytosol were identified in the three conserved proteins of alphachrysoviruses, the RdRP, the CP and the putative protease (Figs. 19, (A), (B) and (C) respectively); in all cases, docking (DOC), ligand-binding (LIG) and modification (MOD) sites are abundant, while cleavage (CLV), degradation (DEG) and targeting (TRG) sites are rare. Based on what is known on the biology of members of *Chrysoviridae*, these viruses are localised in the cytoplasm throughout their replication cycle and do not encode polyproteins, so targeting (TRG) sites for specific membrane bound organelles within hyphae are unnecessary while cleavage (CLV) and degradation (DEG) sites would be harmful.

ELMs conserved across all alphachrysoviruses were selected (Fig. 20 A), since it is most likely that they have biological significance, and GO analysis was performed on docking (DOC) and ligand-binding (LIG) sites which signify an interaction of the viral proteins with host proteins and small molecules, respectively. Docking (DOC) sites were found to be associated with 'regulation of cell cycle', 'cell proliferation' and 'transcription from RNA polymerase II promoter' (Fig. 20 B). Ligand-binding (LIG) sites were found to be associated with 'intrinsic apoptotic signalling pathway in response to DNA damage', 'cell proliferation', 'gene expression', 'autophagy' and 'autophagosome assembly' (Fig. 20 B). Taken together, these results indicated that DsCV-1 proteins might affect fungal growth, an observation potentially linked to a mycovirus-mediated hypovirulent phenotype. The links between viral proteins and transcription of fungal messenger (m) RNAs suggested that DsCV-1 has wider effects on the host transcriptome, while it also influences homeostasis (apoptosis, autophagy).

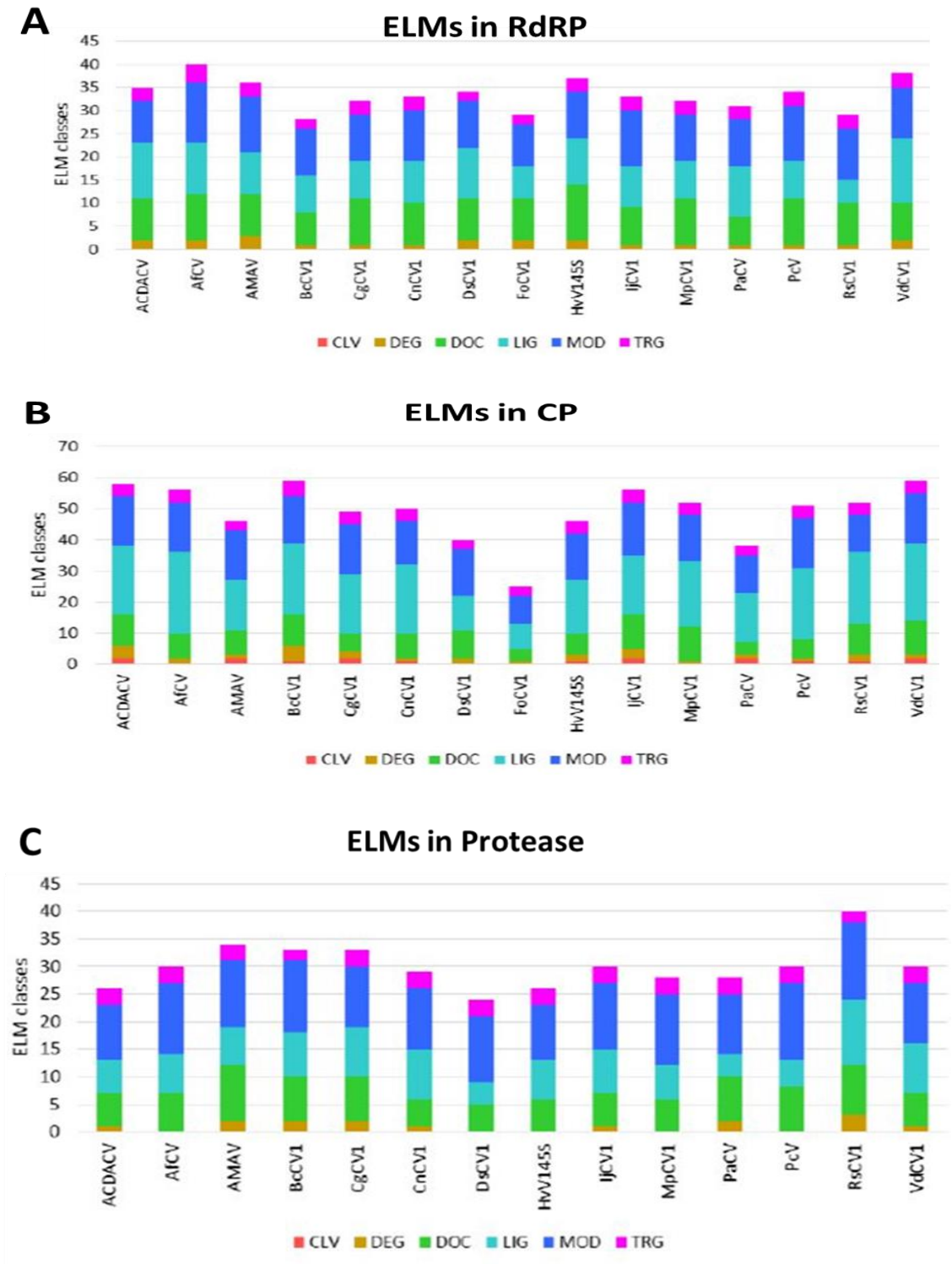


Figure 19. ELMs in alphachrysovirus proteins.

Putative cleavage (CLV), degradation (DEG), docking (DOC), ligand-binding (LIG), modification (MOD) and targeting (TRG) sites were predicted in the **(A)** RdRP, **(B)** CP and **(C)** putative protease of different members of the genus *Alphachrysovirus* within the family *Chrysoviridae*.

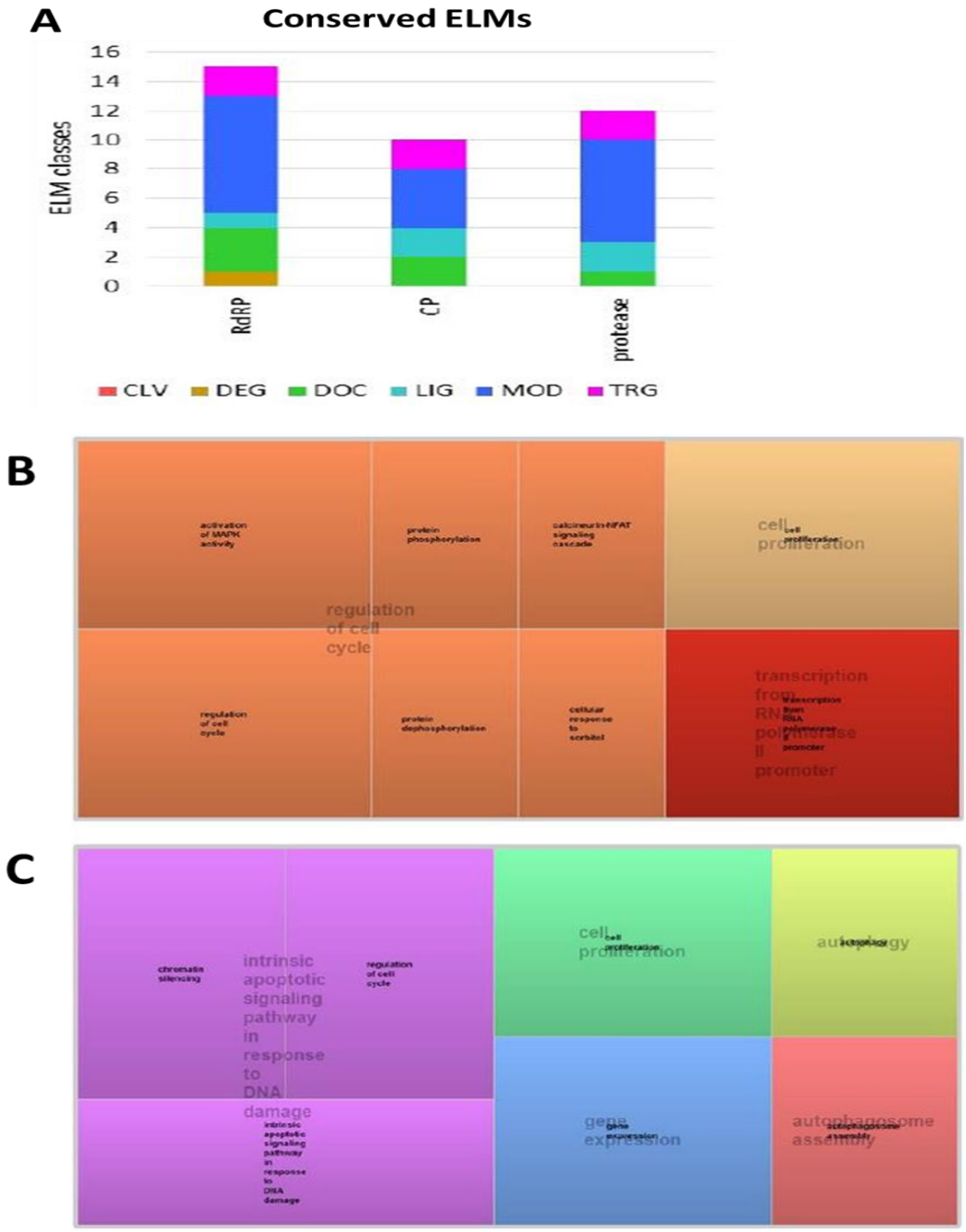


Figure 20. GO analysis of ELMs.

(A) Conserved ELMs in alphachrysovirus proteins. **(B)** GO analysis of docking (DOC) sites and visualisation using Revigo. GO terms include 'regulation of cell cycle', 'protein phosphorylation', 'protein dephosphorylation', 'activation of MAPK activity', 'calcineurin-NFAT signalling cascade' and 'cellular response to sorbitol' (orange squares); 'cell proliferation' (beige square); and 'transcription from RNA polymerase II promoter' (red square). **(C)** GO analysis of ligand-binding (LIG) sites and visualisation using Revigo. GO terms include 'intrinsic apoptotic signalling pathway in response to DNA damage', 'regulation of cell cycle' and 'chromatin silencing (purple squares); 'cell proliferation' (green square); 'gene expression' (blue square); 'autophagy' (lime square) and 'autophagosome assembly' (pink square).

In conclusion the banding pattern and the molecular features of the four dsRNAs found in *D. septosporum* isolate D752.1, the structural characteristics of their 5'-UTRs (like those from other chrysoviruses) and the phylogenetic analysis indicate that these four dsRNAs represent the complete genome of a new member of the *Alphachrysovirus* genus in the family *Chrysoviridae.* DsCV-1 is the first virus to be identified in *D. septosporum*.

Chapter 4.

Chapter 4. Eradication of DsCV-1 and pathogenic effects of DsCV-1 infection on pine trees.

4.1 Chapter 4 Introduction.

To understand more about the effect of mycovirus infection on the pathogenicity of *D. septosporum* it is essential to compare isogenic lines of virus-infected and virus-free isolates, to ensure that any observed effects are due to the presence of the virus and not due to differences in the genetic background of the host. Ghabrial & Suzuki (2009) reported the difficulties of constructing isogenic lines were majorly due to lack of simple methods for the artificial inoculation of mycoviruses, which has hampered progress in exploring mycovirus–host interactions. The alternative approach is to cure the fungus of virus infection. Mycoviral infections can often be persistent and difficult to eliminate from their hosts (Martins *et al.,* 1999; Romo *et al.,* 2007). Several different methods to cure fungi of virus infection have been reported, e.g., single conidium subculture, hyphal tip transfer, incubation at low or high temperatures, treatment with cycloheximide, a drug which specifically blocks translational initiation and the elongation process in protein synthesis (Carroll & Wickner, 1995; Romo *et al.,* 2007; Azevedo *et al.,* 2000), matric stress (Thapa *et al.,* 2016) and treatment with ribavirin, a potent synthetic guanosine nucleoside antiviral agent which interferes with viral RNA synthesis and viral mRNA capping. These methods were not always successful.

Once virus-infected and virus-free isogenic lines have been established, numerous potential effects of mycovirus infection can be investigated. Traditionally, *D. septosporum* pathogenicity trials are performed on pine trees following artificial inoculation (e.g. Perry *et al*., 2016). To this end, a collaboration with Forest Research UK was forged to take advantage of the in-house facilities, expertise and training opportunities. In parallel with the experiments on pine trees, a novel assay was set up to assess virulence of different *D. septosporum* isolates on single pine needles. This was designed to be a cost-effective alternative to the traditional trials as implemented at Forest Research UK (Table 6). To the best of our knowledge, such a single pine needle assay has not been reported in the literature previously.

Table 6. Advantages and disadvantages of pathogenicity assays.

single needle pathogenicity assay	traditional pathogenicity trials at FR
Requires a small number of fungal spores (Approximately 1000 spores/ml; 50 to 150μL)	Requires many fungal spores
Requires a small number of pine needles	Requires numerous potted nursey pine trees
Less contamination (Provided a stringent aseptic technique is used)	More contamination (Other fungi, insects, and nematodes)
Relatively easy to set up	Extremely cumbersome to set up
Cheap to set up and maintain	Very expensive to set up and maintain
Less material available for further analysis	More material available for further analysis

4.1.1 Chapter 4 Objectives.

The main objectives for this chapter were:

- To cure *D. septosporum* isolate D752.1 of infection with DsCV-1 to generate virus-free and virus-infected isogenic lines for assessing the effects of DsCV-1 on its fungal host.
- To assess the putative effect of DsCV-1 on the production of the fungal toxin dothistromin *in vitro*.
- To assess the effect of DsCV-1 on symptomatology, growth, and pathogenicity of *D. septosporum* on pine trees under controlled conditions and with individual pine needles in a novel *in vivo* assay.

4.2 Chapter 4 Materials and Methods.

4.2.1 Eradication of DsCV-1.

Several methods of eradicating DsCV-1 from *D. septosporum* isolate D752.1 were investigated, and these are described below. To assess the success of the treatments on the eradication of mycoviral dsRNAs, total RNA was extracted from the fungal mycelia (section 2.5). RT-PCR assays were then performed (section 2.8.2) using sequence-specific oligonucleotide primers, forward primer 5'-ATT GGC TGA CAT GGT GGA GG-3' and reverse primer 5'-ATG CGC CTG TCA GGA TCA AA-3', designed to generate a 465 bp amplicon representing a fragment of the coding region of the RdRP gene discovered on the largest dsRNA element found in *D. septosporum* isolate D.752.1.

4.2.1.1 Cycloheximide treatment on MEA.

Attempts to eradicate DsCV-1 infection from *D. septosporum* isolate D752.1 with cycloheximide were made in solid MEA media amended with a range of concentrations of the drug (Table 7). Cycloheximide 50 mM solutions were filter-sterilised and incorporated into media at ~50°C. Inoculated plates were incubated at 22°C.

Table 7. Cycloheximide treatment.

Dilution series and preparation of different cycloheximide concentrations.

Desired concentration (mM)	Amount of cycloheximide (mL) in 20 mL of MEA
0.0001	0.001
0.001	0.01
1	0.1
2	0.2
4	0.4
8	0.8
10	1.0
12	1.2
14	1.4
16	1.6
18	1.8
20	2.0

4.2.1.2 PEG induced matric stress treatment on SNLM.

Matric stress was induced using polyethylene glycol (PEG 8000) in a modified Spezieller Nahrstoffarmer liquid medium (SNLM) to cure DsCV-1 infection of *D. septosporum* isolate D752.1. SNLM contains 0.02 g/L sucrose, 0.02 g/L glucose, 0.08 g/L KNO_3, 0.08 g/L KH_2PO_4, 0.04 g/L $MgSO_4.7H_2O$ and 0.04 g/L NaCl, together with PEG to produce water potential gradients of -1 MPa, -2 MPa, -3MPa, -4 MPa, -5 MPa and -6 MPa (Table 8). The amounts of PEG 8000 added to the media were calculated as described previously (Michel, 1983; Thapa *et al.,* 2016). Because of agar solidification difficulties only low PEG concentrations were utilised in solid cultures prepared by adding 2.5 g MEA to 50 mL of SNLM + PEG. *D.septosporum* isolate D752.1 was then grown in SNLM amended with increasing concentrations of PEG until confluent mycelial growth was obtained before being used to inoculate solid SNLM/MEA media. After 14 days, individual colonies, presumably arising from single spores, developed at the periphery of the main colonies. These were subcultured onto solid SNLM/media for 14 days prior to harvesting mycelia.

Table 8. Matric stress treatment.

SNLM, PEG 8000 and MEA used to achieve desired water potential in liquid and solid media.

Water potential Ψ	PEG concentrations (mg/mL)	SNLM + PEG + MEA (g)
1 MPa	8.15	4.079
2 MPa	8.42	4.21
3 MPa	8.68	4.34
4 MPa	8.92	4.46
5 MPa	9.18	4.59
6 MPa	9.42	4.71

4.2.1.3 Ribavirin treatment on PMMG.

To assess the potential curing effect of the drug on DsCV-1 infection of *D. septosporum* isolate D752.1, ribavirin (Sigma) concentrations ranging from 10 µM to 200 µM on MEA plates were investigated. All inoculated plates were incubated at 22°C (Herrero & Zabalgogeazcoa, 2011) and after 28 days incubation mycelia were harvested.

4.2.2 *D. septosporum* pathogenicity assays.

All pine experimentation was undertaken at Forest Research, Alice Holt Lodge, Wrecclesham, Farnham GU10 4LH, under the guidance of Dr Katherine Tubby.

4.2.2.1 Preparation of *D. septosporum* inocula.

D. septosporum (VI and VF D752.1 plus several other isolates) was maintained and grown on MEA and PMMG plates. To inoculate the first-generation plates, segments (*ca*. 0.5 cm) were excised from the growing edges of the MEA cultures, placed onto PMMG plates (3 segments/plate) and incubated in darkness at 22^{o}C for 7-10 days. Spores of the various isolates were harvested by agitating the surface of individual plates with 5 mL sterile distilled water and re-inoculation of plates with 50 µL of the resulting spore suspensions. For both individual needle and tree inoculation 50 µL of a fungal spore suspension (1x10^{6} spores/mL in water) was spread on PMMG plates using a glass spreader. This volume and concentration are sufficient for a good spore distribution over the plate with optimum density and avoids overcrowding problems which can result in the production of shorter than average spores with poor germination (Dr Rosie Bradshaw, Massey University, New Zealand, personal communication). This protocol was repeated with repeat harvesting and new plate inoculation 3 to 4 times at 2-week intervals to promote high levels of sporulation for inoculation purposes.

4.2.2.2 Preparation of pine (*Pinus nigra* subsp. *laricio*) seedlings.

Corsican pine trees (*Pinus nigra* subsp. *laricio* – designated *P. nigra* var Corsicana) were grown in pots at Heathwood Nursery and kept under cover to prevent infection with DNB. Sapling trees were selected for inoculation at the age of 24 months. Potted trees were placed on capillary matting and were watered at regular intervals. Insecticide was applied to control aphids and other insect infestations when required. Prior to inoculation all dead needles were removed from the trees to prevent confusion with DNB symptoms. The pine host used in these investigations is known to be highly susceptible to DNB.

4.3.2.3 Artificial inoculation of pine saplings.

Inoculum concentrations were adjusted to 2x10^{6} spores/mL in water, which is optimal when inoculating trees using a hand or pressure sprayer. Since pine needles have a slender and twisted morphology they need to be rotated and sprayed from all sides to ensure the inoculum is distributed evenly over the needle surface. After spraying, seedlings were left to dry for 20 min before further spraying in the controlled environment chamber. Inoculation of the seedlings with individual isolates was staggered by at least 15 min

between each treatment to minimise the potential for aerial cross-contamination. Thereafter extreme high needle wetness was maintained for 4-7 days by enclosing individual seedlings in plastic sleeves within the assay chamber (see Fig. 22). After this time, the plastic sleeves were removed, and wetness conditions maintained by 'rain' (artificial chamber) or by spraying plants 2-3 times daily by hand.

4.3.2.4 Controlled environment chamber and microclimate.

All inoculated trees were incubated for the duration of the trials in a controlled environment chamber. A microclimate was maintained in the chamber with a 16 h day and a maximum light intensity of 120 microMol/m^2/s at tree top height. Chamber temperature was 20^{o}C day, 12^{o}C night with hourly 'rainfall', 6 min during daylight and 3 min during darkness on the hour. Relative humidity was maintained at 80-100%.

4.3.2.5 Experimental design.

For both individual pine needles and pine saplings eight *D. septosporum* isolates were tested in two trials including some duplicates (Fig. 21). The various *D. septosporum* inocula used in the trials were colour coded by tagging as shown in brackets (Table 9, column 1). Isolates investigated in both trials are shown with asterisks. The non-isogenic isolates were selected for investigation because of known variability in origin, pathogenicity, and growth morphology.

Table 9. *D. septosporum* isolates used in pathogenicity trials.

***D. septosporum* isolate**	**Designation (colour & number)**	**DsCV-1 infection**
Control – water only	Black – numbers 7 (trial 1) & 6 (trial 2)	N/A
D752.1 VIA *	Purple – number 1 (both trials)	+
D752.1 VIB *	Green – number 2 (both trials)	+
D752.1 VF *	Red – number 3 (both trials)	–
D700	White – number 4 (trial 1 only)	+
D622.1	Light blue - number 5 (trial 1 only)	+
D233	Yellow – number 6 (trial 1 only)	–
D584	Blue – number 4 (trial 2 only)	+
D1216	Orange – number 5 (trial 2 only)	+

Asterisks (*) refer to isolates examined in both trials 1 and 2.

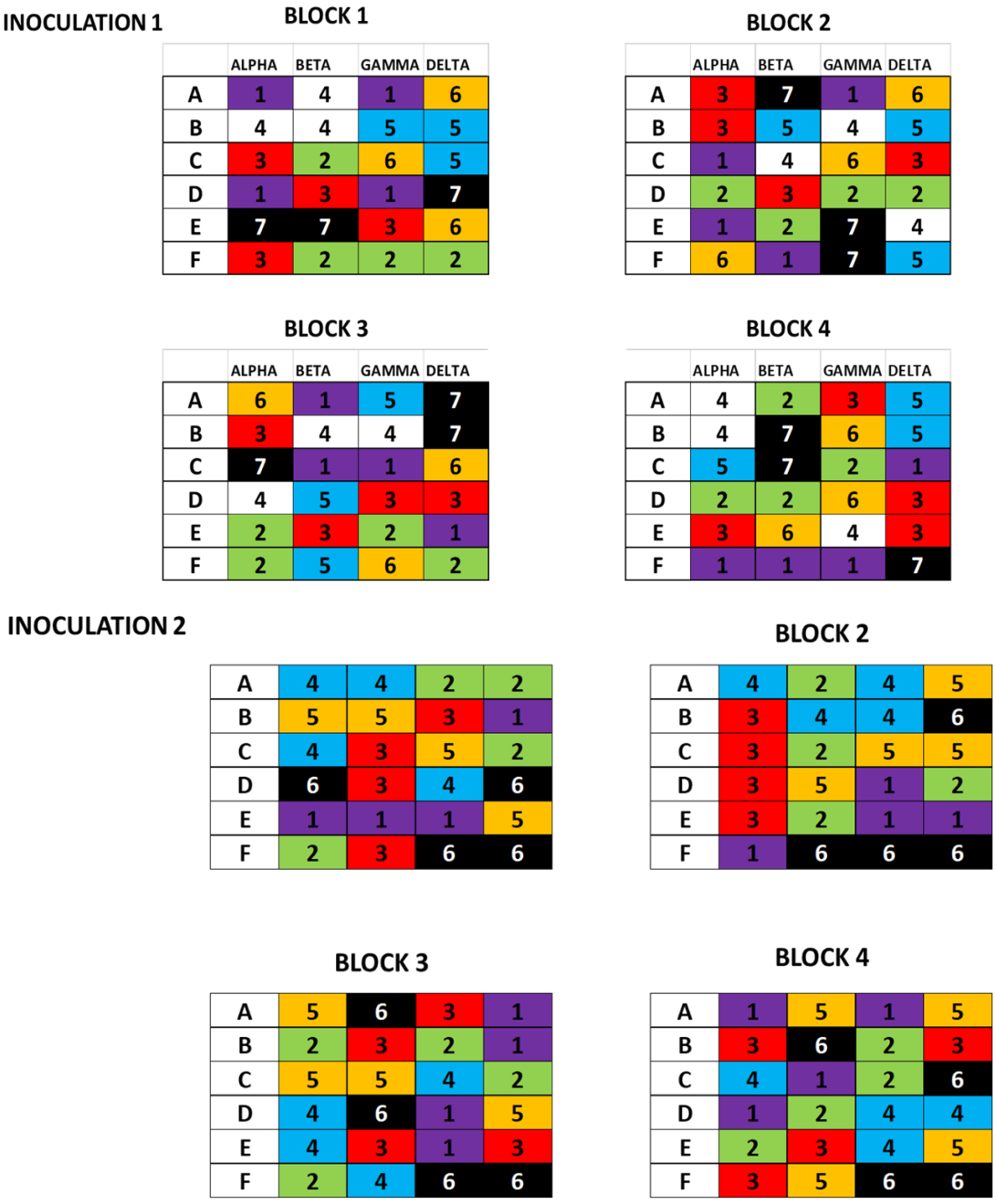


Figure 21. Experimental design for *D. septosporum* pathogenicity trials on pine.

Colour coded blocks of *D. septosporum* inoculated pine seedlings as described in Table 9 for inoculation trials 1 and 2. In trial 2, isolates D622 (5) and D233 (6) were replaced with isolates D584 (5) and D1216 (6) respectively.

'Rainfall' in the growth chamber was achieved through ceiling mounted nozzles. Prior tests illustrated water coverage of the chamber was uneven, so the chamber was split into 4 blocks, 1 on the upper left, and 1 on the lower left of a central aisle and the other 2 at the top right

and bottom right of the aisle. Each block contains nursery trees 20-40 cm tall in 9 x 9 cm square pots. Individual blocks containing the trees were inoculated with each *D. septosporum* isolate and several pots were inoculated with water as controls (Fig. 21). These treatments were fully randomised within each of the four blocks. Because of an initial shortfall of viable spore numbers for inoculation the experiment was split into two trials separated by a period of 4 weeks.

Figure 22. Pine trees inoculated with *D. septosporum*.

Corsican nursery pine trees in an artificial climate chamber inoculated with spores of *D. septosporum* isolates.

The image above shows nursery pine trees in an artificial climate chamber, infected with isolates of *D. septosporum* spores at the Forest Research institute in Farnham UK. All trees were bagged to prevent cross contaminations between the isolates. For the listed provenance of *D. septosporum* isolates see Table 9.

4.3.2.6 Infection assessment of pine seedlings.

Any needle that was not completely green was henceforth considered to exhibit DNB symptoms. DNB severity was defined as the numbers of needles with symptoms consistent with DNB (needles with lesions and necrotic needles i.e., those which were not entirely

green). To follow the time course of DNB infection, assessment and sampling was carried out at regular intervals during the experiments at 7, 14, 21, 28, 35, 42, 48- and 61-days post inoculation (dpi). Quantitative assays of growth and virulence of *D. septosporum* were assessed for all isolates by qPCR amplification. Target genes for growth and virulence of *D. septosporum* were respectively beta-tubulin (housekeeping gene) and dothistromin, a potential virulence factor for DNB (Kabir *et al*., 2018). Growth and development of the pine host throughout the time course of the investigation was assessed using qPCR of 18S ribosomal RNA as a reference. The oligonucleotide primers used in qPCR amplifications are shown in Table 10.

Table 10. List of primers used for qPCR amplification.

Primer / probe	Sequence	Target	Amplicon size (bp)	Slope	Y-intercept	Efficiency
D. septosporum						
DStub2-F1	5'-CGAACATGGACTGAGCAAAA-3'	β-tub	89	-3.348	32.375	99%
DStub2-R1	5'-TGCCTTCGTATCTGCATTTC-3'					
DStub2-P1	5'-ROX-TGGAATCCACAGACGCGTCA-BHQ2-3'					
dotA.F	5'-TCGAGACCATCATCGCCAAC-3'	*dotA*	136	-3.418	29.567	96%
dotA.R	5'-GCGGTCGAATTCATCACCAG-3'					
dotC.F	5'-CGGACCAGAGGAACATACTTG-3'	*dotC*	149	-3.680	33.933	87%
dotC.R	5'-CCAAAAGCGAGTCCATCAATC-3'					
pksA.F	5'-ACGAGGCTCTGAAGATCATTTC-3'	*pksA*	149	-3.509	32.758	93%
pksA.R	5'-AGCAAGATCGAGTTCAAGGTC-3'					
vbsA.F	5'-TGAAATGGATGCGGGTAACG-3'	*vbsA*	146	-3.322	33.513	100%
vbsA.R	5'-TGTAGAACATCTCACGGTTGTG-3'					
18SrRNA.F	5'-CGACCTCCAACCCTTTGTGA-3'	18S rRNA	183	-3.152	37.124	107%
18SrRNA.R	5'-GCATTTCGCTGCGTTCTTCA-3'					
DsCV-1						
DsCV-1. F	5'-ACGGGGAAGTGAGTTTAGCG-3'	RdRP	180	-3.568	19.927	91%
DsCV-1. R	5'-TCTTGCTCGCCATCCAACAT-3'					
Pinus spp.						
18S uni-F	5'-GCAAGGCTGAAACTTAAAGGAA-3'	18S rDNA	150	-3.929	27.912	80%
18S uni-R	5'-CCACCACCCATAGAATCAAGA-3'					
18S uni-P	5'-JOE-ACGGAAGGGCACCACCAGGAGT-BHQ1-3'					

Table 10 above shows a list of oligonucleotide primers used for qPCR amplification of selected genes in the *D. septosporum* genome (GenBank accession: AY808228) including the constitutive β-tubulin gene (Ioos *et al.,* 2010), a range of genes involved in dothistromin

synthesis and the 18S rRNA as a reference gene; the RdRP encoding sequence of DsCV-1; and the pine 18S rDNA sequence (Ioos *et al.,* 2010) as a positive control.

4.3.2.7 Infection assessment of single pine needles.

A novel method of assessing *D. septosporum* growth and virulence used individual pine needles of known species origin obtained from the University of Hertfordshire pinetum at Bayfordbury and from Forest Research at Alice Holt. Pine needles were sterilised in 2.5% sodium hypochlorite, washed extensively in sterile distilled water and finally absolute ethanol. Individual needles were then mounted and held in place with sterile 2% ($^{w}/_{v}$) tap water agar on glass slides in large Petri plates on Whatman filter paper stacks. The needles were then inoculated at their extremities or centrally with 100 µL *D. septosporum* spore suspension (10^4 spores/mL). The inside of the Petri dish tops was ringed with filter paper to absorb moisture and avoid condensation falling back onto the needles. The dishes were then sealed with Parafilm and maintained under conditions of extreme high humidity at 24^{o}C in the dark using constant hydration with sterile tap water. An exemplar single needle inoculation arrangement is shown in Fig. 23. The development of DNB infection on the needles was monitored weekly over an extended time course and samples taken for qPCR assessment (section 2.8.2). The isolates examined in these assays duplicated those investigated in the tree inoculations described earlier.

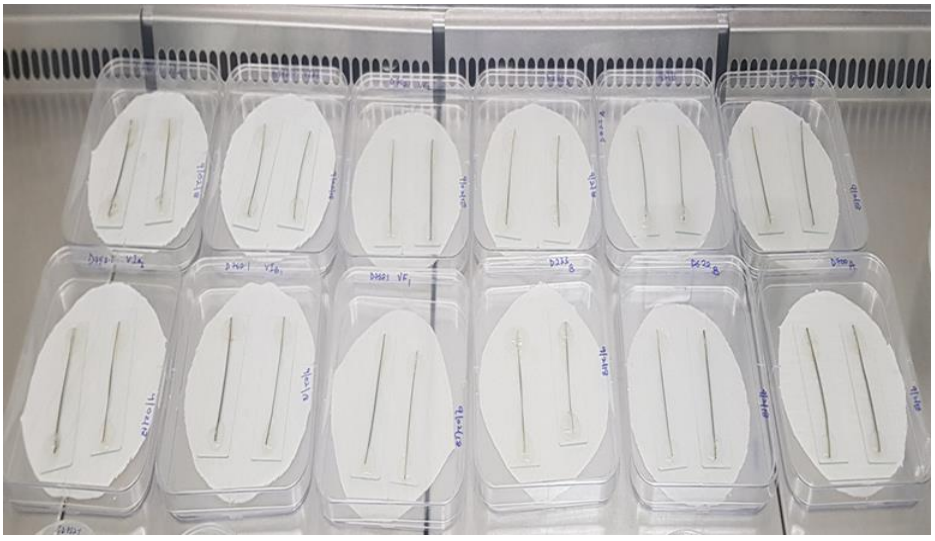


Figure 23. Single pine needles inoculated with *D. septosporum*.

Pairs of detached single pine needles in large Petri dishes inoculated with spores of *D. septosporum* isolates.

4.3 Chapter 4 Results.

The results of chapter 4 include eradication of DsCV-1 from *D. septosporum* using ribavirin treatment, investigation of potential links between DsCV-1 infection of *D. septosporum* and synthesis of the putative virulence factor dothistromin, pathogenicity assays of *D. septosporum* on pine trees and the set up of a novel single pine needle assay. All objectives were achieved.

4.3.1 Eradication of DsCV-1 from *D. septosporum* using cycloheximide, matric stress and ribavirin.

Neither matric stress nor cycloheximide were successful in eradicating DsCV-1 but the virus was successfully eradicated from cultures treated with ribavirin at concentrations of 80 µM and 100 µM. These cultures together with the D752.1 VF generated by Dr Ioly Kotta-Loizou (section 3.2.3).

4.3.1.1 Cycloheximide treatment.

Attempts were made to eradicate DsCV-1 dsRNA from *D. septosporum* isolate D752.1 using cycloheximide treatment (section 4.2.1.1). Unfortunately, higher concentrations of cycloheximide resulted in fungal death while lower concentrations of the drug severely impeded fungal growth (Fig. 24) resulting in insufficient mycelial material being available to analyse further.

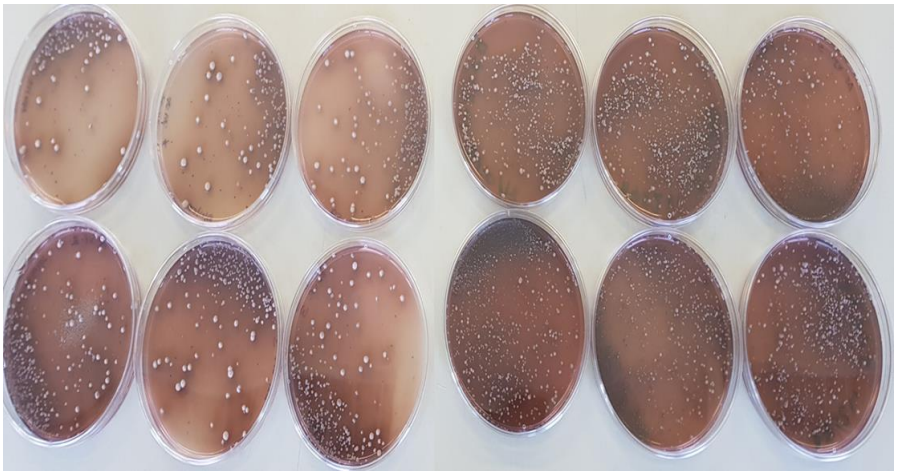


Figure 24. Growth of *D. septosporum* in the presence of cycloheximide.

D. septosporum isolate D752.1 did not grow sufficiently on cycloheximide, impeding further analysis.

4.3.1.2 PEG induced matric stress treatment.

Attempts to eradicate DsCV-1 infection from *D. septosporum* isolate D752.1 using PEG induced matric stress were made using liquid and solid media (section 4.2.1.2). Increasing matric stress appeared to cause significant increased colouration of the cultures. This colouration is suspected to be caused by the toxin dothistromin (Fig. 25). Following RNA extraction and RT-PCR, it was revealed that DsCV-1 was present in all *D. septosporum* cultures assessed and the matric stress treatment was unsuccessful in eradicating the virus.

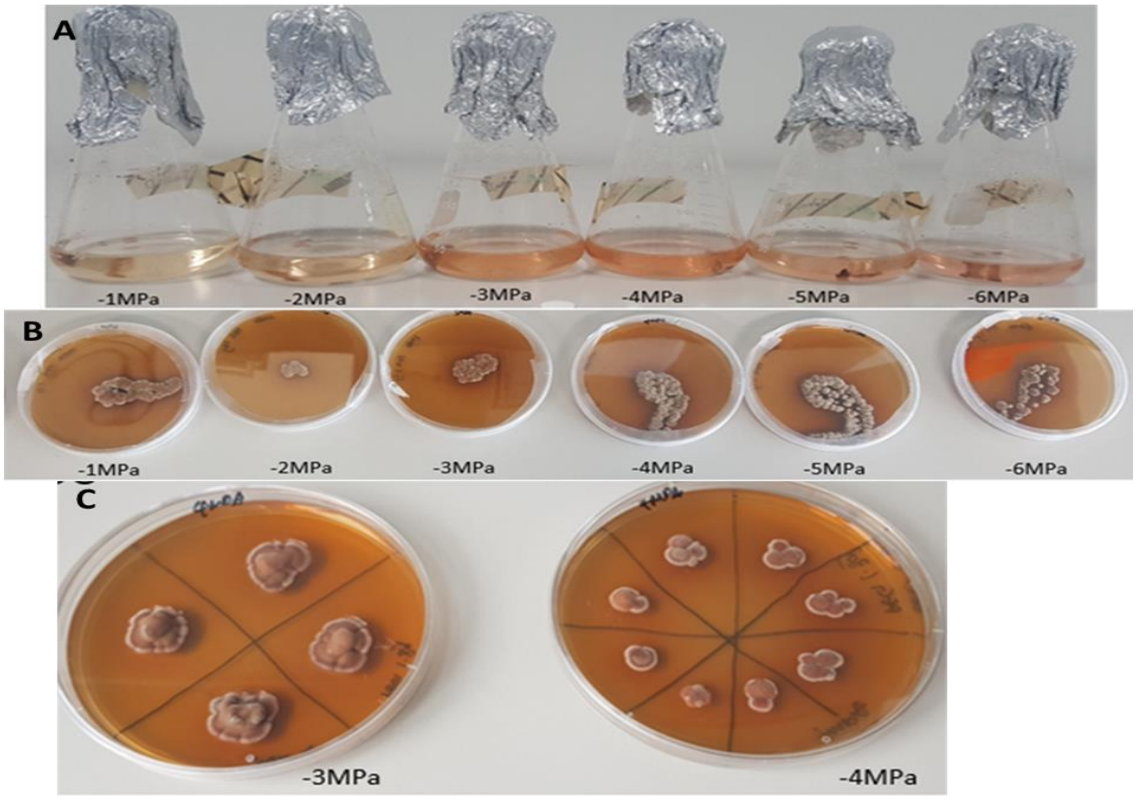


Figure 25. Growth of *D. septosporum* in the presence of PEG.

(A) *D. septosporum* isolate D752.1 grown in SNLM (left to right): -1 MPa, -2 MPa, -3 MPa, -4 MPa, -5MPa, -6MPa. **(B)** As in (A) subcultured on solid SNLM/MEA media. **(C)** Individual colonies grown on SNLM/MEA from single conidia subcultured from (B).

4.3.1.3 Ribavirin treatment.

There were no obvious effects on the growth of *D. septosporum* isolate D752.1 cultured on solid media amended with ribavirin at concentrations of up to 100 µM (section 4.2.1.3), but an increased production of the mycotoxin dothistromin was noted in high ribavirin concentrations (Fig. 26). Following RNA extraction and RT-PCR, it was revealed that the

DsCV-1 specific amplicon was absent from *D. septosporum* grown in the presence of 80-100 µM ribavirin and therefore this treatment was successful in eradicating the virus (Fig. 27). The VI and VF D752.1 isolates were used respectively as positive and negative controls.

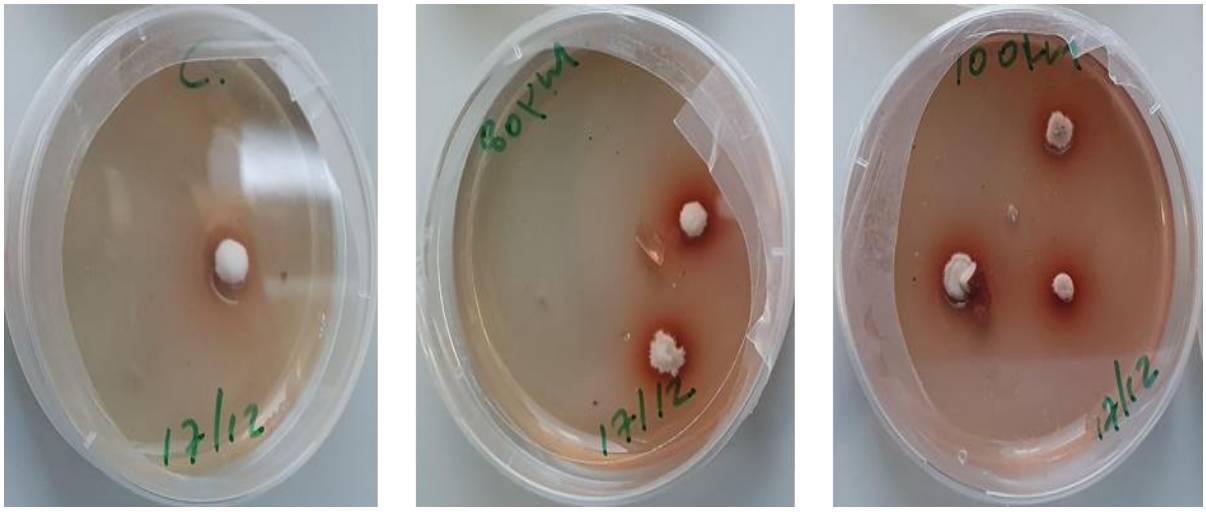


Figure 26. Growth of *D. septosporum* in the presence of ribavirin.

D. septosporum isolate D752.1 grown on (left to right): PMMG (control) without ribavirin, 80 µM ribavirin, 100 µM ribavirin. The red rings around the fungal inocula indicate production of dothistromin.

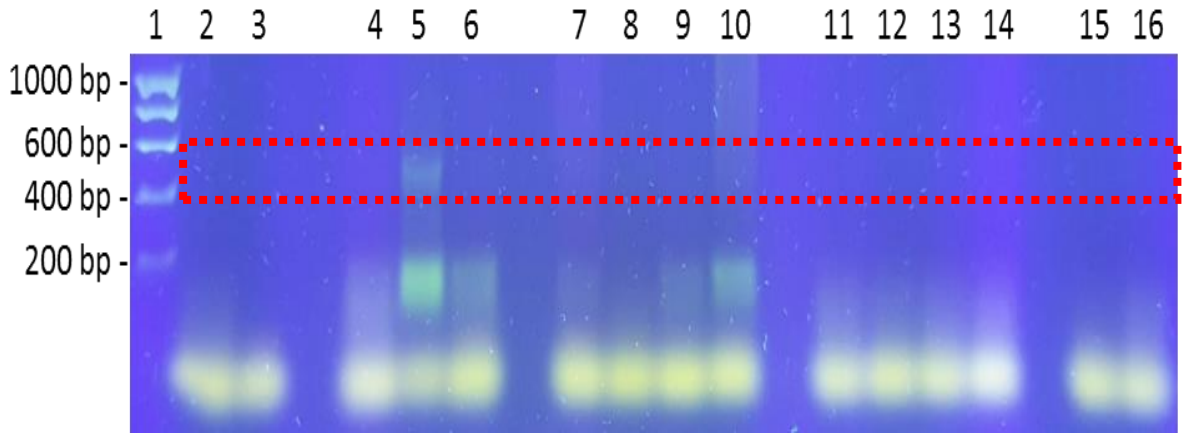


Figure 27. Electrophoresis of DsCV-1 specific amplicons after ribavirin treatment.

Screen of *D. septosporum* isolate D752.1 for the presence of DsCV-1 using RT-PCR amplification of a 465 bp fragment of the DsCV-1 dsRNA 1 RdRp gene following treatment with ribavirin at concentrations of 80 µM (lanes 2, 4, 6-8) and 100 µM (lanes 9-16). Lanes 3 and 5 contain the negative and positive controls, VF and VI D752.1, respectively. Amplicons were displayed on a 0.8% ($^{w}/_{v}$) agarose gel stained with GelRed and electrophoresed in 1 X TAE. Hyper Ladder™ 1kb (Bioline) was used as molecular weight marker and sizes are shown to the left of the gel. The area of the gel where amplicons would be located is indicated with a red box.

4.3.2 Dothistromin synthesis in *D. septosporum* and DsCV-1 infection.

A time course of DsCV-1 RNA accumulation in its parent *D. septosporum* D752.1 isolate was performed over a 20-week incubation period (Fig. 28). Consistent and significant increases in DsCV-1 RNA levels were recorded over the time course validating the selection of D752.1 as the isolate selected for virus eradication and the generation of virus-infected and virus-free isogenic lines.

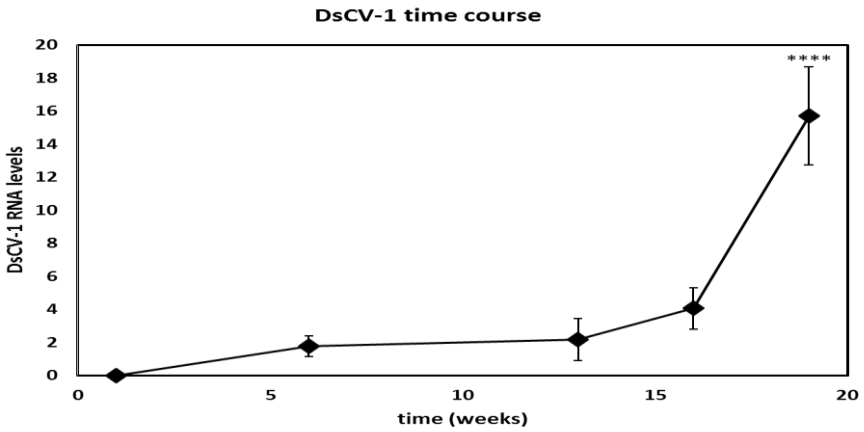


Figure 28. DsCV-1 time course.

Relative RNA levels quantified by qPCR after RNA extraction and cDNA synthesis at different time points. The RNA levels of DsCV-1 were normalized with *D. septosporum* 18S ribosomal DNA. Between six and nine replicates were analyzed for each time point and the error bars represent standard deviation. Asterisks indicate statistical significance (ANOVA; **** *P*-value < 0.0001).

As a further assessment of *D. septosporum* virulence it was intended to use the levels of four genes involved in the synthesis of dothistromin, which is thought to be a virulence factor (Kabir *et al*., 2018). The four genes selected were *dotA, dotC, vbsA* and *pksA* and qPCR primers were designed and standardised (Table 10) to investigate their relative expression in cultures of *D. septosporum* isolate D752.1 grown in SDB and PDB liquid media.

Gene expression levels were normalised with pine 18S ribosomal DNA as a reference gene. Pearson's correlation coefficient (PCC) was calculated for the RNA levels of DsCV-1 and the four *D. septosporum* genes and the results are shown (Fig. 29). The DsCV-1 RNA levels show little or no correlation (|PCC| < 0.35) with any of the genes in the dothistromin production pathway. Conversely, the mRNA levels of all genes in the dothistromin production pathway

demonstrate high linear correlation (PCC > 0.7) with each other. Based on these observations it was decided not to proceed with further investigations concerning the effects of DsCV-1 on dothistromin as a virulence factor in *D. septosporum.*

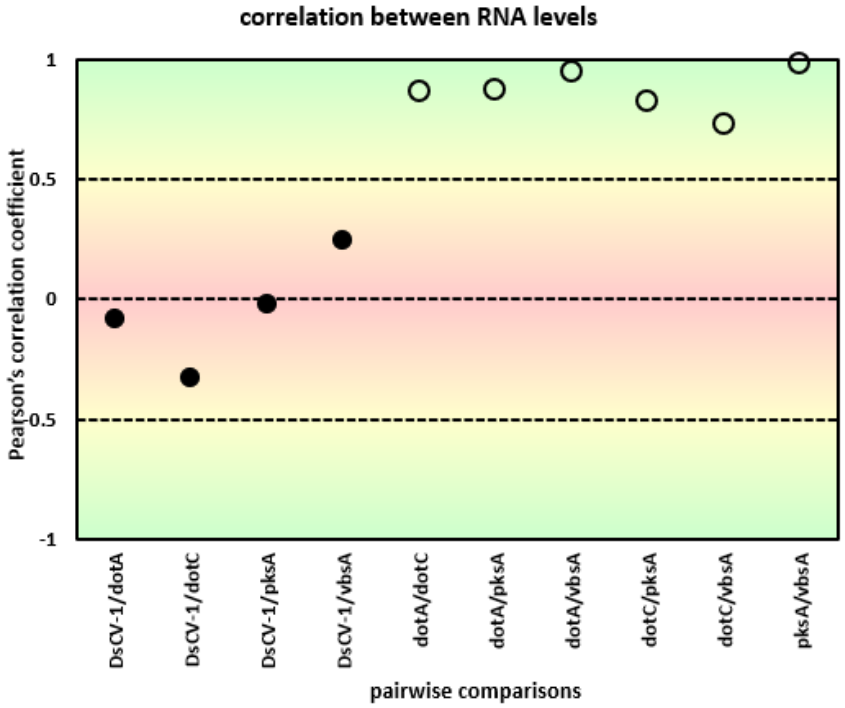


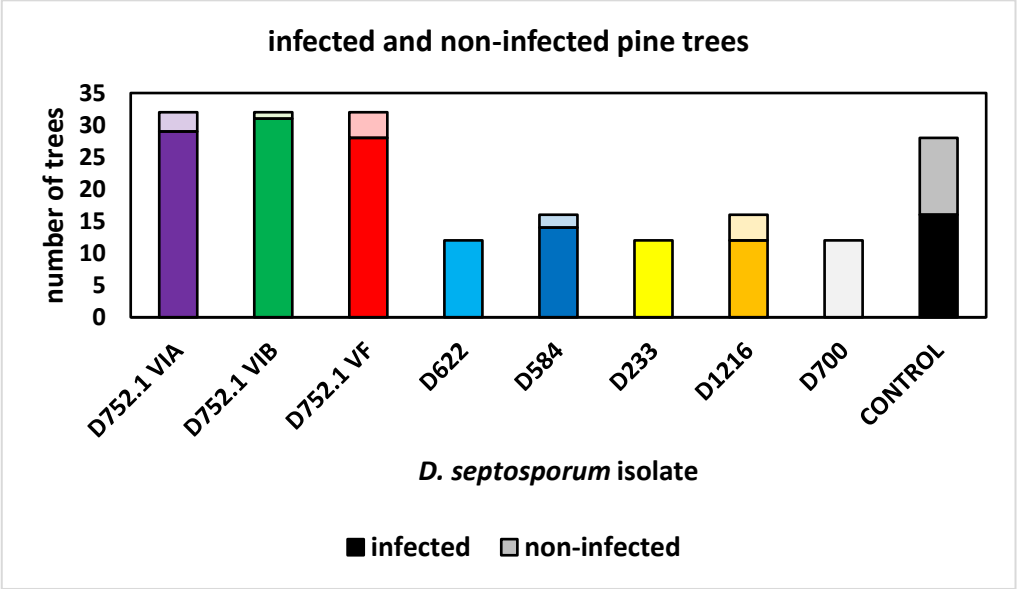
Figure 29. Correlations between RNA levels.

Pearson's correlation coefficient (PCC; PEARSON function in Microsoft Excel) was calculated for the RNA levels of DsCV-1 and four *D. septosporum* genes involved in dothistromin production. The DsCV-1 RNA levels show no or little correlation (PCC| < 0.35) with any of the genes in the dothistromin production pathway (black discs). Conversely, the mRNA levels of all genes in the dothistromin production pathway demonstrate high linear correlation (PCC > 0.7) with each other (black rings).

4.3.3 *D. septosporum* on pine trees and the effects of DsCV-1 infection.

Following the time course of *D. septosporum* infection in pine assessments of DNB infection were made regularly during both trials 1 and 2 at 7, 14, 21, 28, 35, 42, 48 and 61 dpi. Unless otherwise indicated in the figure legends, the results of the two trials were pooled together to maximise statistical robustness. Any pine needles that were not completely green and exhibited colour banding DNB symptoms of DNB and fungal fruiting bodies were scored and subjected to image analysis. As assessed by non-destructive, visual assays not all the pine trees inoculated with the various *D. septosporum* isolates became infected, but most were (Fig. 30 A).

(**A**)



(**B**)

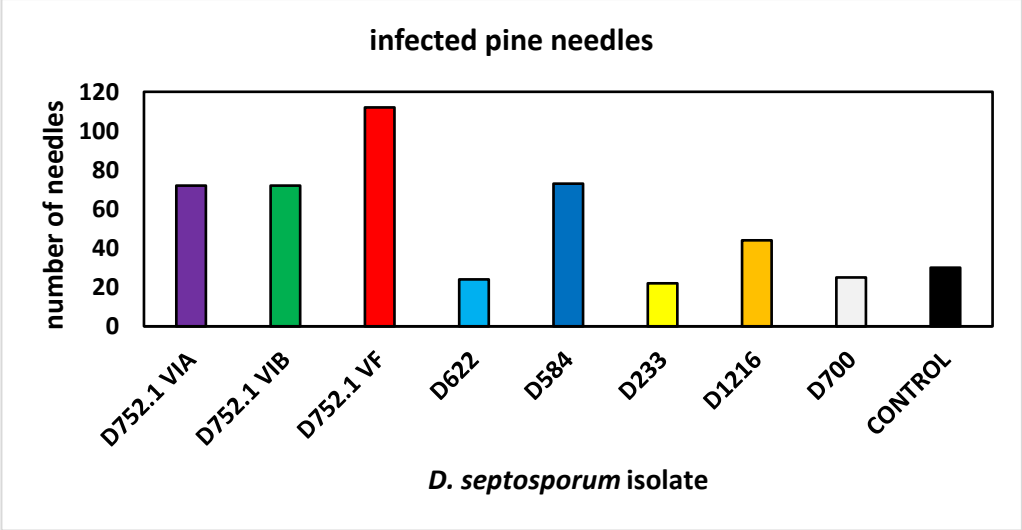


Figure 30. Infection following inoculation with of *D. septosporum*.

(A) Total number of pine trees inoculated with *D. septosporum* isolates. Pine trees that were successfully infected are depicted in solid colors; trees that showed no signs of infection are depicted in transparent colors. **(B)** Total number of pine needles infected.

Two out of the 192 trees used in the trials died, and these were omitted from the analysis. Because of the proximity of trees one to another in the controlled environment chamber a significant number of control trees, inoculated with water, developed symptoms and these were also omitted from the analyses. These observations emphasise the difficulties

associated with performing experiments of the type described here. Non-destructive analysis revealed that there was variability in the numbers of needles exhibiting DNB following *D. septosporum* inoculation and some isolates were more virulent than others e.g., compare isolates D752.1 and D584 with isolates D700 and D233 (Fig. 30 B). The causes of increased virulence of some isolates are unknown. However, no correlation between the geographical source of the *D. septosporum* isolates and virulence can be made since all but one of the isolates available for investigation originated from Scotland (Table 2).

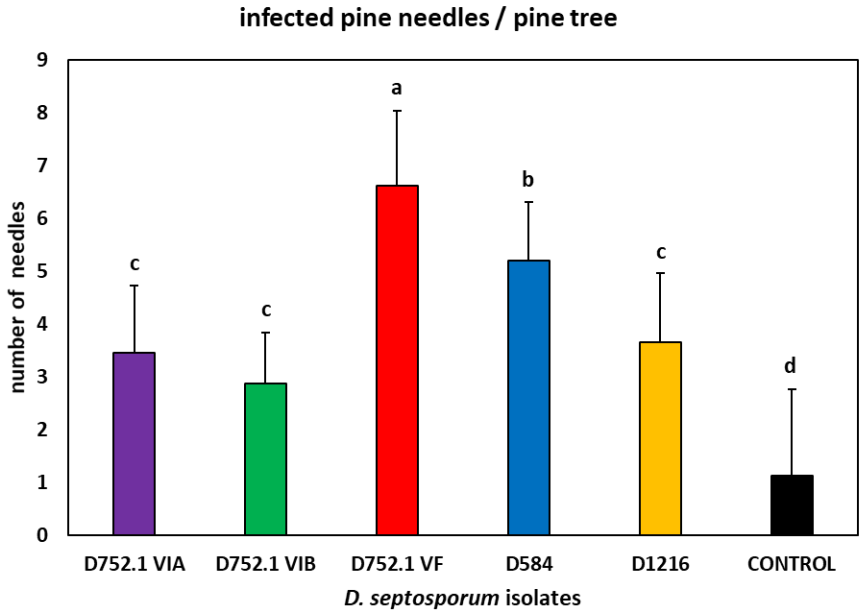


Figure 31. Infected pine needles per pine tree.

The average number of infected pine needles per pine tree following inoculation with *D. septosporum* isolates was calculated. Pine trees showing no signs of *D. septosporum* infection were excluded from the analysis. The error bars represent standard error of the means. Different letters indicate significant differences between treatments (ANOVA; *P*-value < 0.05 at least). This analysis includes only the 5 isolates examined in trial 2.

The exception was isolate D233 VF, which was isolated at Alice Holt, in England and is unusual in that its infection status with DsCV-1 was variable when tested on different occasions (e.g., negative as shown in Fig. 17, section 3.3.3). All the other isolates including the duplicate DsCV-1 infected isogenic lines D752.1 VIA and D752.1 VIB, consistently tested positive for DsCV-1 infection (Fig. 17, section 3.3.3). As anticipated the cured, isogenic line D752.1 VF was virus-free when tested by RT-PCR (Fig. 25). When the average number of infected pine needles per pine tree for 5 *D. septosporum* isolates were assessed (Fig. 31)

the statistically significant differences found mirrored the results shown previously (Fig. 30 B). Examination of the data from both assessments suggests that DsCV-1 infection reduces the virulence of *D. septosporum* since the numbers of infected needles were much higher in trees inoculated with the virus-free D752.1 VF isogenic line as compared to trees inoculated with duplicate, virus-infected lines D752.1 VIA and D752.1 VIB and 2 non-isogenic, virus-infected isolates D584 and D1216. These observations suggest that DsCV-1 might cause hypovirulence of *D. septosporum* in pine. Further statistically significant confirmation of the suspected hypovirulent effect of DsCV-1 infection of *D. septosporum* in pine was obtained when the average percentages of needle images exhibiting characteristic DNB symptoms of red discoloration were quantified using the Fiji/ImageJ software and a custom image analysis script (Fig. 32). Following visual and image analysis infected needles from both trials were harvested and stored at −80°C prior to destructive molecular analysis.

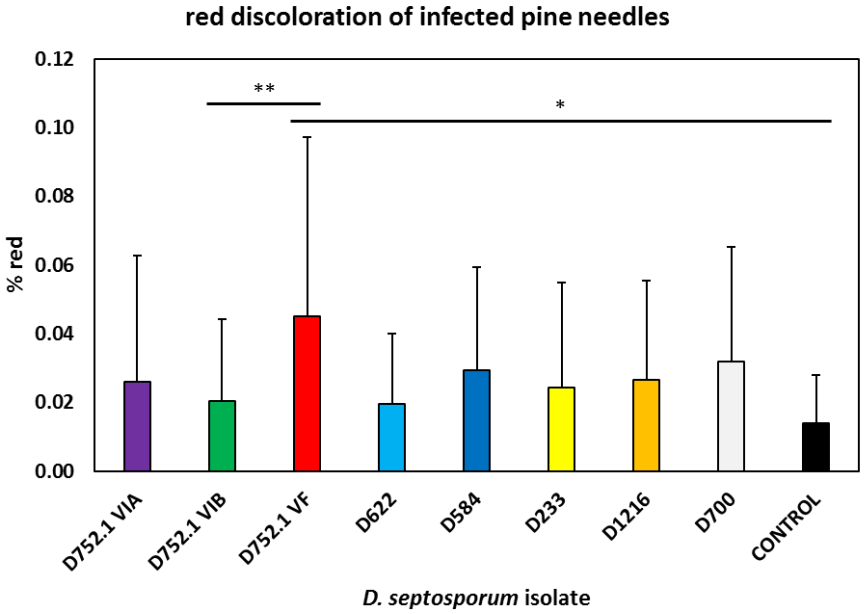


Figure 32. Red discoloration of infected pine needles.

The average percentage of red discoloration per pine needle following infection with *D. septosporum* isolates was quantified using the FIJI/ImageJ software and a custom image analysis script. The error bars represent standard deviation. Asterisks indicate statistical significance (ANOVA; * *P*-value < 0.05, ** *P*-value < 0.01).

The average fungal burden per pine needle following infection with *D. septosporum* isolates was quantified by qPCR following DNA extraction. The levels of the constitutive *D. septosporum* beta-tubulin gene were normalised with pine 18S ribosomal DNA as the

reference gene. Up to seven infected pine needles were analyzed for each *D. septosporum* isolate and all assays were performed in triplicate. The error bars represent standard deviation of the means (Fig. 33). As with the previous non-destructive analyses, molecular analysis of the fungal burden per infected pine needle illustrates a clear trend in that DsCV-1 infection is associated with hypovirulence in *D. septosporum* and that the virus-free isolate D752.1 VF is significantly more virulent than the isogenic lines D752.1 V1A (*P*-value < 0.01) and V1B (*P*-value < 0.001) and other virus-infected isolates including D622 (*P*-value < 0.001), D233 (*P*-value < 0.0001) and D700 (*P*-value < 0.05).

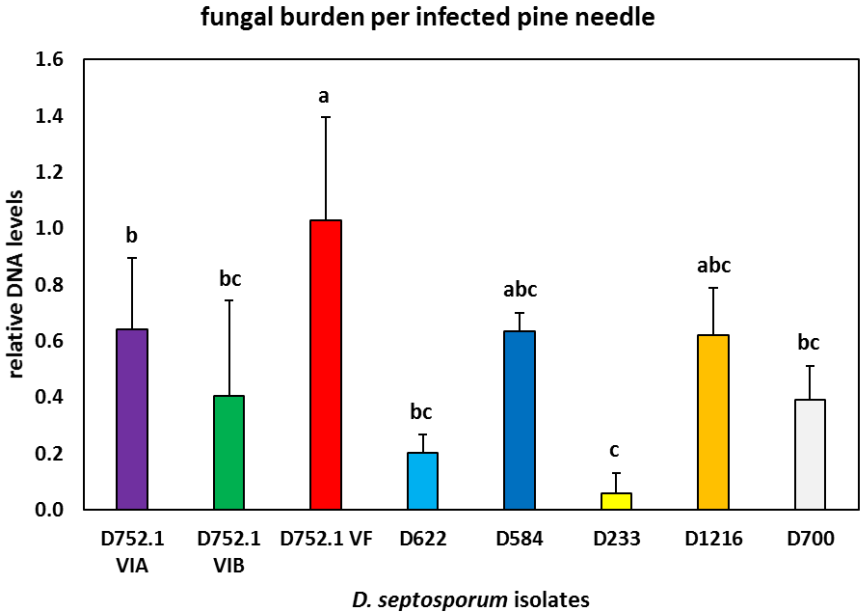


Figure 33. Fungal burden per infected pine needle.

The average fungal burden per pine needles following infection with *D. septosporum* isolates was quantified by qPCR after DNA extraction. The levels of *D. septosporum* beta-tubulin gene were normalized with pine 18S ribosomal DNA. Between three and seventeen samples were analyzed for each isolate and the error bars represent standard deviation. Different letters indicate significant differences between treatments (ANOVA; *P*-value < 0.05 at least).

4.3.4 *D. septosporum* on single pine needles and the effects of DsCV-1 infection.

Unlike the needles identified as infected during traditional pathogenicity trials, the individual needles inoculated with *D. septosporum* isolates did not exhibit the characteristic red banding of DNB. Conversely, dark coloured bands were observed following inoculation with isolates D752.1 VI, D752.1 VF and D622.1 (Fig. 34 A), which were not present on the

non-inoculated needles serving as the negative control. Additionally, isolates D752.1 VI, D752.1 VF, D622.1 and D233 often grew on the surface on the needles and in the case of D752.1 VI clear production of dothistromin was occasionally noted, as illustrated by the dusty pink growth on the left needle (Fig. 34 A).

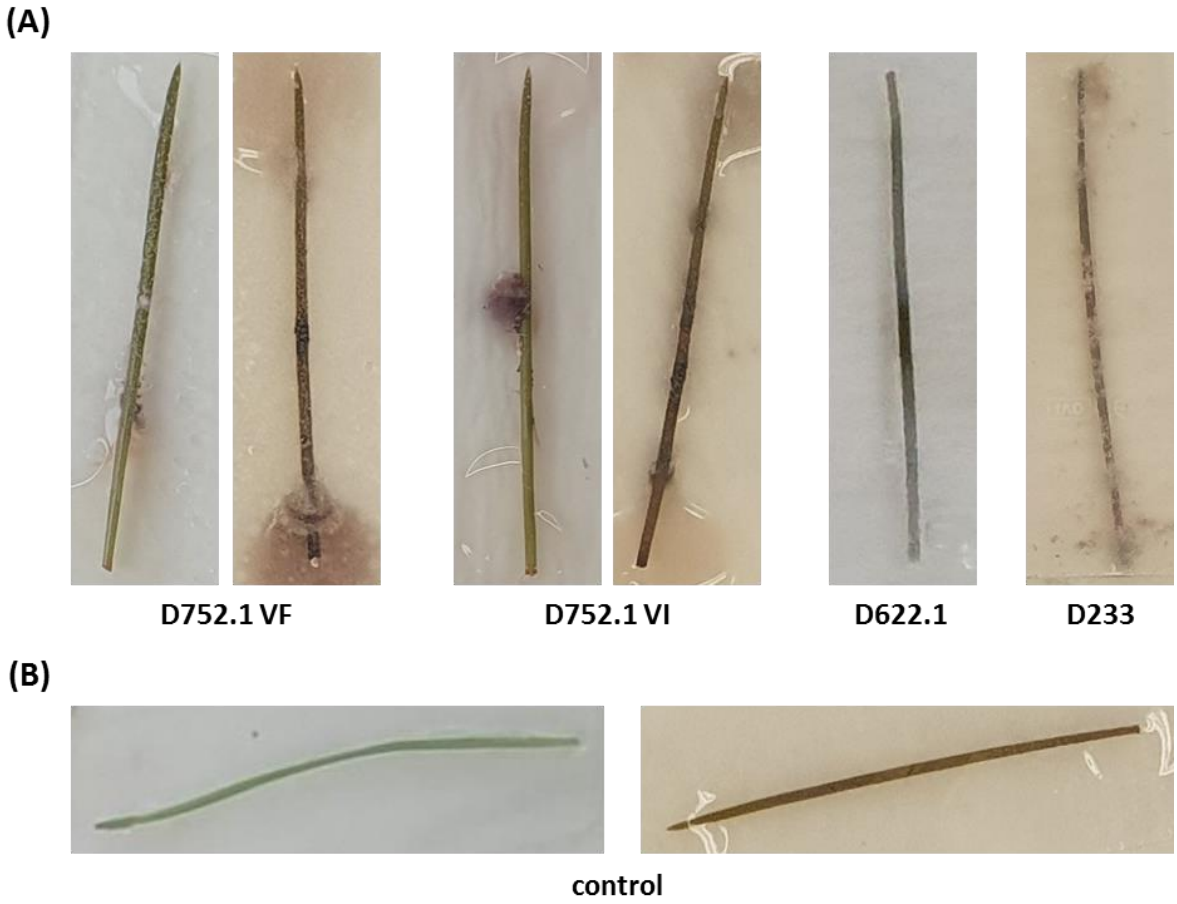


Figure 34. Representative photographs from the single pine needle pathogenicity assay.

(A) Pine needles following inoculation with selected *D. septosporum* isolates D752.1 VF, D752.1 VI, D622.1 and D233. **(B)** Pine needles inoculated with water as a negative control, at the beginning of the assay (left) and at the end of the assay after 5 weeks (right). All needles were immobilised on glass slides by their extremities using tap water agar and inoculated centrally.

Chapter 5.

Chapter 5. Growth and virulence of *B. bassiana* and the effects of virus infection.

5.1. Chapter 5 Introduction.

This project is a continuation of the study on polymycoviruses discovered in the entomopathogenic fungus *B. bassiana* and their potential use as biocontrol agents, published by my supervisory team Dr Ioly Kotta-Loizou and Dr. Robert Coutts in *PLoS Pathogens* in 2017. The use of mycoviruses to manipulate the virulence of entomopathogenic fungi, such as *B. bassiana*, a generic commercially used biocontrol agent, is desirable to control arthropod pest attacks on commercial crops.

The greater wax moth *Galleria mellonella* from the family Pyralidae, order Lepidoptera*,* is a popular infection model (Fuchs *et al*., 2010). Its amenability to infection and its immunity mechanisms render *G. mellonella* a suitable host to study microbial pathogenicity. Fuchs *et al*. (2010) listed the benefits of using *G. mellonella* as an infection model as compared to other invertebrates such as *Caenorhabditis elegans* and *Drosophila melanogaster*: *G. mellonella* larvae can be maintained at a wide temperature range from 25°C to 37°C, facilitating studies on fungi from diverse natural environmental niches or mammalian hosts, while *C. elegans* and *D. melanogaster* are not appropriate for testing at 37°C, the optimal temperature for human pathogens.

Another benefit mentioned by Fuchs & Mylonakis (2006), is the *G. mellonella* infection model multiple options for facile delivery of the pathogen under study. Larvae can be inoculated with a microbe *via* three methods: (1) topical application, (2) oral delivery or (3) injection (Pereira *et al.,* 2020). Fungal delivery can be a drawback for models such as *C. elegans*: while genetically more tractable than *G. mellonella*, the infection process relies on ingestion of fungi, which at times can be unpalatable to the nematode or physically too large to be ingested. For those pathogens that are ingestible by *C. elegans*, it is uncertain what quantity of inoculum was ingested. *D. melanogaster* constitutes another genetically tractable system but working with it requires experience and there is a need for specialized equipment to infect and house the insects. Moreover, wild-type *D. melanogaster* is resistant to fungal infections necessitating the use of strains with mutations involving the Toll immune response pathway. Conversely, *G. mellonella* larvae are housed in Petri dishes and are kept in an incubator post-infection.

Hygromycin B (HmB) is an aminoglycosidic antibiotic that inhibits protein synthesis by disrupting peptidyl-tRNA translocation (Punt *et al.*, 1987). Genes that confer resistance to hygromycin B (HmB) have been successfully isolated and characterised from *Streptomyces hygroscopicus* and *Escherichia coli*. These genes inactivate the antibiotic through phosphorylation using HmB phosphotransferase (*hph*; Malpartida *et al.*, 1983). Cloning vectors conferring resistance to HmB have been constructed to transform fungal species through protoplast transfection based on the *E. coli hph* gene.

5.1.1 Chapter 5 Objectives.

The main objectives for this chapter were:

- To investigate the effects of virus infection on the growth and pathogenicity of *B. bassiana* through a comparison of virus-infected and virus-free isogenic lines of the fungus.
- To investigate the pathogenic effects of virus free and virus-infected isogenic line on the greater wax moth *G. mellonella* in a controlled environment.
- To investigate the suitability of a plasmid expressing a hygromycin resistance gene as a transfection marker for *B. bassiana.*

5.2 Chapter 5 Materials and Methods.

5.2.1 *B. bassiana* radial growth on solid media.

Spores (n=10^4) from *B. bassiana* wild type, cured or commercially available isolates, (section 2.1) were centrally inoculated onto CDA media and minimal media (MM) agar Petri plates and incubated at 24°C. Growth rate, as defined by the rate of radial expansion, was measured over a period of 17 days for three replicate plates (Fig. 35). 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 edge of the Petri dish. The means of the colony diameters measured were plotted to identify any differences between the isolates.

Figure 35. Radial growth assays of *B. bassiana* isolates.

Radial growth expansion of *B. bassiana* virus-infected isolate EABb 92/11-Dm, its virus-free isogenic line, KVL-03-144, KVL-03-122, and commercially available Botanigard and Naturalis.

5.2.2 The greater wax moth infection model.

Larvae of the greater wax moth (*Galleria mellonella*) in the final stages of development were obtained from Dr Ioly Kotta-Loizou at Imperial College London and were stored before use in wood shavings in the dark to prevent pupation. Only cream-coloured larvae were chosen for inoculation and any grey larvae were excluded from use to assist in observations of characteristic alterations in pigmentation elicited by fungal infection (Fuchs *et al*., 2010). Larvae were used within one week of delivery. Successful larval infection was achieved following direct submersion of larvae for 30 sec into a water suspension of *B. bassiana* spores (n=10^6 spores/mL), allowing the fungus to enter the larvae by penetrating the insect cuticle (Scully & Bidochka, 2005). *B. bassiana* spores were isolated from overnight cultures grown in CD broth at 25°C and diluted in water to the desired concentration for inoculation. After submersion, larvae were transferred into Petri dishes lined with damp Miracloth and wood shavings and stored at 37°C in the dark for 10 days. Over the incubation period larvae were periodically checked for morphological alterations, changes in pigmentation and mortality following physical stimulation with forceps (Fig. 36).

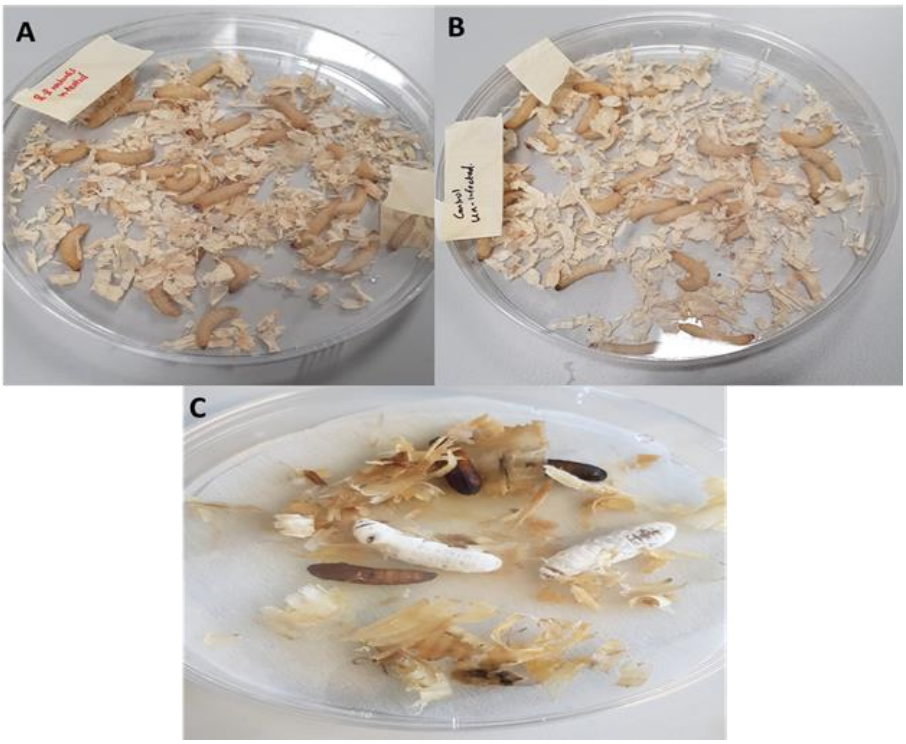


Figure 36. *G. mellonella* larvae infected with *B. bassiana*.

(A) Larvae infected with *B. bassiana* EABb 92/11-Dm VI spores 1 dpi. **(B)** Non-infected larvae (control) 1 dpi. **(C)** Representative changes in larvae morphology and pigmentation 3-6 dpi.

5.2.3 *B. bassiana* hygromycin B sensitivity assays.

B. bassiana isolates, wild-type, commercially available or transfected with plasmid pAN7-1 containing the HmB resistance gene (section 2.1), were screened for sensitivity to HmB following growth for two weeks on CDA plates containing 0 µM (control) or 100 µM, 250 µM, 500 µM, and 1000 µM concentrations of HmB.

B. bassiana isolates were also tested for HmB sensitivity using the agar overlay method described by Punt *et al.* (1987). Spores (n=10^7) were grown on CDA plates amended with different concentrations of HmB (0.001-150 mM). After 16-20 h incubation at 25^{o}C all plates were overlaid with an equal volume of CDA containing the same concentrations of HmB, following further incubation at 25^{o}C for 3-4 days.

5.3 Chapter 5 Results.

The results of chapter 5 include confirmation of virus infection in *B. bassiana* isolates, comparisons of radial growth and pathogenicity using the greater wax moth *G. mellonella* infection model between virus-infected and virus-free isogenic and non-isogenic *B. bassiana* isolates, and investigation of HmB as a potential transfection marker for *B. bassiana*. All objectives were achieved.

5.3.1 Mycovirus containing isolates of *B. bassiana*.

Following small scale dsRNA extraction (section 2.3) and agarose gel electrophoresis of purified dsRNA (section 2.10) from *B. bassiana* isolates EABb 92/11-Dm, IMI 391043, ATHUM 4946, IMI 331273 and IMI 392612, an examination of the profiles displayed confirmed the presence of mycoviruses (Fig. 37).

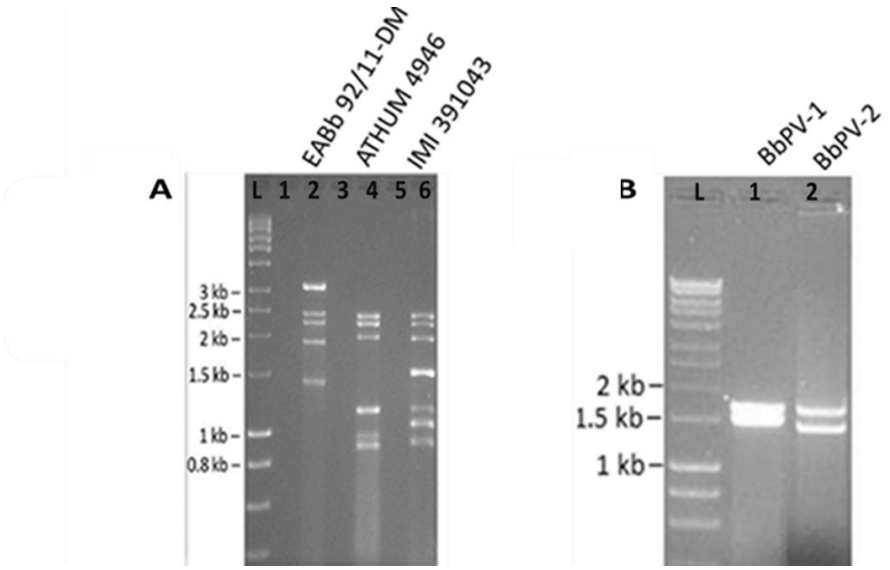


Figure 37. Electrophoretic profiles of *B. bassiana* dsRNAs.

Separation of dsRNA extracted from *B. bassiana* isolates **(A)** EABb 92/11-Dm, ATHUM 4946 and IMI 391043, and **(B)** IMI 331273 and IMI 392612. The dsRNAs were displayed on 1-1.5% ($^{w}/_{v}$) agarose gels stained with GelRed and electrophoresed in 1 X TAE. Hyper Ladder™ 1kb (Bioline) was used as molecular weight marker and sizes are shown to the left of the gel.

The viruses in each *B. bassiana* isolate were examined, fully sequenced and characterised (Kotta-Loizou & Coutts, 2017). The names of putative and known type of viruses the isolates harbour is shown in brackets: EABb 92/11-Dm (unirnavirus and polymycovirus);

ATHUM 4946 (polymycovirus); IMI 391043 (polymycovirus); IMI 331273 (partitivirus), and IMI 392612 (partitivirus). The hosts and countries of the location of these isolates are shown in Table 2. Random clones generated from the second-largest dsRNA element found in isolate EABb 92/11-Dm (Fig. 37, lane 2) once sequenced confirmed the known sequence of dsRNA 1 of a polymycovirus (Kotta-Loizou & Coutts, 2017).

5.3.2 Radial growth comparisons of virus-free and virus-infected *B. bassiana* isolates.

A comparison of virus-infected (VI) and virus-free (VF) EABb 92/11-Dm isogenic lines was conducted, together with non-isogenic KVL-03-144, KVL-03-122, and commercially available Botanigard and Naturalis (Fig. 38).

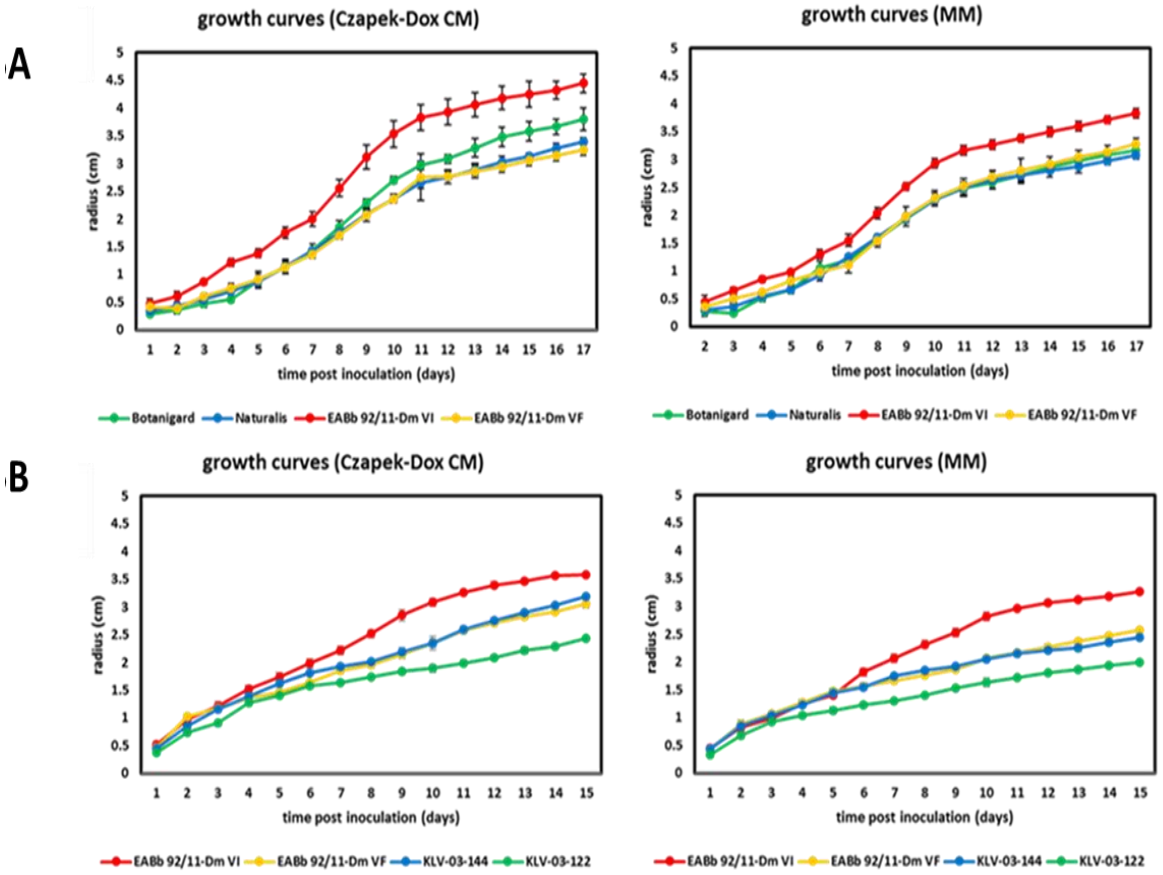


Figure 38. Radial growth comparison of *B. bassiana* isolates.

(A) Radial expansion of *B. bassiana* isolate EABb 92/11-Dm VI compared to the isogenic line EABb 92/11-Dm VF and two commercially available isolates Botanigard and Naturalis; **(B)** isolates KVL-03-144 and KVL-03-122. error bars (where visible) represent standard deviation of the means from three separate experiments.

The radial growth assay showed that the *B. bassiana* isolate EABb 92/11-Dm VI grew substantially faster than the isogenic line EABb 92/11-Dm VF on both CM and MM over a 17-day incubation period (Figs. 38 A and B). These differences were found to be statistically significant (2-way ANOVA; *P*-value <0.0001). There were no significant differences in the morphology between the isogenic lines of *B. bassiana* EABb 92/11-Dm.

Growth comparisons of other virus-free *B. bassiana* isolates, such as the commercially available Botanigard and Naturalis (Fig. 38 A) and the novel, previously uninvestigated, KVL-03-144 and KVL-03-122 isolates (Fig. 38 B), with isolate EABb 92/11-Dm VI revealed that in all cases radial expansion and growth of EABb 92/11-Dm VI on CM and MM was significantly faster and greater as compared to the virus-free isolates. These results mirrored the results found with the *B. bassiana* EABb 92/11-Dm isogenic lines described above but are not directly comparable because of a differing genetic background for the four virus-free isolates tested.

5.3.3 Pathogenicity comparisons of virus-free and virus-infected *B. bassiana* isolates.

The virulence of *B. bassiana* infection on greater wax moth *G. mellonella* larvae was monitored daily following inoculation with fungal spores.

Figure 39. Time course of *B. bassiana* infection and visual pathogenic effects on *G. mellonella* larvae.

Larvae infected with *B. bassiana* EABb 92/11-Dm VI spores were examined at 1, 3, 6 and 9 dpi and representative photographs of the larvae are shown in panels A-D, respectively. **(A)** Larvae begin to darken at 1

dpi and **(B)** this effect increases at 2 dpi. **(C)** Larvae are further reduced in size with increased cuticle darkness at 3-6 dpi. **(D)** The fungal mycelium completely penetrates and covers dead larvae at 9 dpi.

Alterations to the morphology of the larvae were observed 3-6 days post-inoculation (dpi), particularly an increase in cuticle melanisation and mortality (Fig. 39). By 9 dpi the larvae were completely covered in and infiltrated with fungal mycelia and were largely dead (Fig. 39). A comparison of the virulence on *G. mellonella* larvae of *B. bassiana* isogenic lines EABb 92/11-Dm VI and EABb 92/11-Dm VF revealed a small but statistically significant decrease in the survival rates of larvae infected with the VF as compared to the VI isolate (Fig. 40).

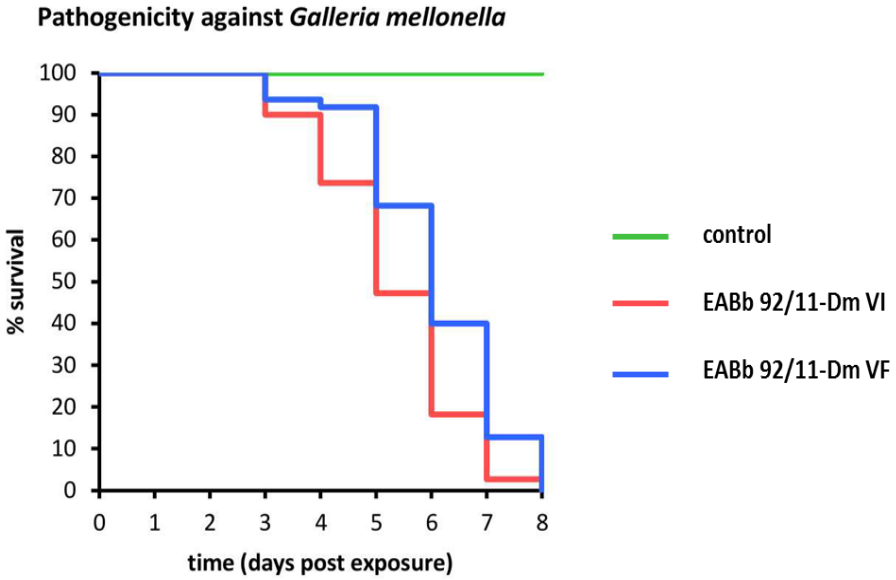


Figure 40. Survival of *G. mellonella* larvae following infection with *B. bassiana*.

Mean survival curves of *G. mellonella* larvae non-infected (green line), infected with *B. bassiana* isolate EABb 92/11-Dm VI (red line) and *B. bassiana* isolate EABb 92/11-Dm VF (blue line). At least three independent repetitions were performed in triplicate (n = 10 larvae per Petri dish). The difference in survival between EABb 92/11-Dm VI and EABb 92/11-Dm VF is statistically significant (Log-rank test, *P*-value < 0.05; Wilcoxon test, *P*-value < 0.05).

5.3.4 HmB as a potential transfection marker for *B. bassiana*.

Transfecting protoplasts of two commercial *B. bassiana* isolates (Botanigard and Naturalis) which are virus-free, with mycoviruses shown to increase growth and virulence is of great interest. As an aid to the selection of successfully transfected fungi it was hoped to use a selectable marker following co-transfection of protoplasts with mycoviruses and a plasmid

containing an antibiotic resistance gene. To this end HmB was selected as a potential selectable marker. Subsequently, the feasibility of using HmB for the selection of mycovirus transfected *B. bassiana* lines was assessed.

Following transfection of *B. bassiana* isolate EABb 92/11-Dm with plasmid pAN7-1 that confers resistance to HmB all transformants grew successfully on solid media containing no antibiotic and on all concentrations of HmB tested (Fig. 41 A). In a similar assay, the non-transfected isolate EABb 92/11-Dm behaved in an identical fashion illustrating that this isolate is naturally resistant to HmB (Fig. 41 B).

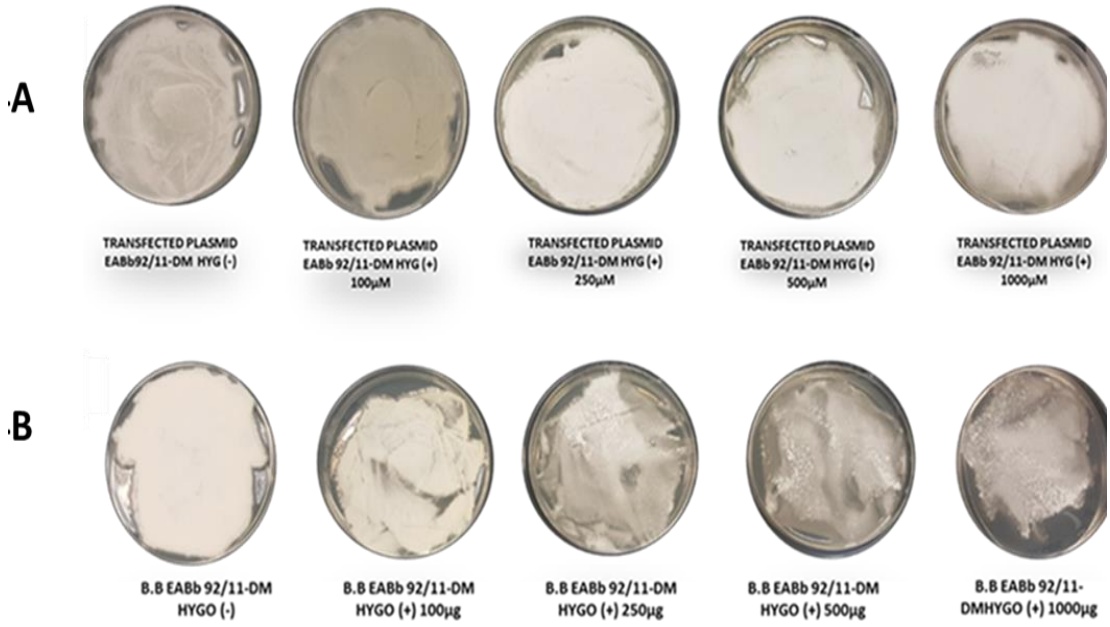


Figure 41. Growth of *B. bassiana* on hygromycin.

(A) *B. bassiana* isolate EABb 92/11-Dm transfected with a plasmid conferring resistance to HmB grown on CDA containing 0 µM (control plate), 100 µM, 250 µM, 500 µM, 1000 µM HmB for two weeks. **(B)** Wild-type, non-transfected *B. bassiana* isolate EABb 92/11-Dm grown on similarly amended media to that in (A).

In confirmation that resistance to HmB might be widespread naturally in *B. bassiana* it was decided to investigate the wild type *B. bassiana* Naturalis and Botanigard commercial isolates. In both cases confluent growth on all plates was observed confirming resistance to HmB. Unfortunately, these results showed that HmB cannot be used as a selection marker for transformation or transfection of *B. bassiana.* Further experiments on this topic were abandoned until a suitable selectable antibiotic for screening transformed *B. bassiana* can be identified.

Chapter 6.

Chapter 6. General Discussion.

6.1 Discussion.

The research objectives of the project were to gain further understanding of the biology of mycoviruses infecting the phytopathogenic fungus *D. septosporum* and the entomopathogenic fungus *B. bassiana*, together with any effects on pathogenicity such as hypovirulence (reduced virulence) or hypervirulence (increased virulence) that would allow use of mycoviruses in biological control.

6.1.1 *D. septosporum*, mycovirus mediated hypovirulence and DNB control.

DsCV-1 is the first mycovirus found in *D. septosporum*. The sequences of its four dsRNA genome segments were fully characterised revealing conserved motifs (Bruenn, 1993; Ghabrial, 1998). DsCV-1 belongs to the established virus family *Chrysoviridae*, one of the four families that accommodate viruses with four dsRNA genome segments, the others being *Quadriviridae* (Lin *et al*., 2012), *Polymycoviridae* (Kanhayuwa *et al.*, 2015, Kotta-Loizou & Coutts 2017) and the proposed family Alternaviridae. However, of these only members of the *Quadriviridae* show any close phylogenetic relatedness to chrysoviruses in the RdRP gene, as also do *Botybirnaviridae, Megabirnaviridae* and *Totiviridae.* The latter have quite different genome organisations (2, 2 and 1 genome segments respectively).

A pair of oligonucleotide primers were designed based on the DsCV-1 nucleotide sequence that encodes the RdRp, the conserved enzyme responsible for the replication of the viral genome. These primers can be used in a diagnostic assay for highly sensitive, highly specific, and rapid detection of DsCV-1 in *D. septosporum* isolates. DsCV-1, originally discovered in the Scottish *D. septosporum* isolate D752.1 from West Argyll, was found to be widespread in the Scottish *D. septosporum* population and present in exemplar isolates from Inverness, Ross & Skye, Moray & Aberdeenshire, and Cowal & Trossachs. Conversely, DsCV-1 was not detected in *D. septosporum* isolates from South England. In the UK, similar natural populations of mycoviruses widespread in a plant pathogenic fungus have been reported previously in *Leptosphaeria biglobosa*, causative agent of phoma stem canker in oilseed rape (Shah, 2017).

The first step for assessing a mycovirus phenotype is the generation of virus-free and virus-infected isogenic lines to allow direct phenotypic, growth and virulence comparisons and to

ensure that any observed difference between these isolates is caused by mycovirus infection. For *D. septosporum*, this was achieved using ribavirin, while treatments with cycloheximide and PEG induced matric stress were unsuccessful. Ribavirin has been used as a treatment against human RNA viruses such as hepatitis C virus, and successfully against plant potyviruses (Mahmoud *et al.,* 2009; Parker, 2005). Although, it was ineffective against a fungal virus infecting *Chalara elegans* (Park *et al.,* 2006), Herrero & Zabalgogeazcoa (2011) successfully eradicated Tolypocladium cylindrosporum victorivirus 1 (TcV1) using ribavirin.

Two independent pathogenicity trials assessing a range of *D. septosporum* isolates were conducted on pine saplings in collaboration with Forest Research UK. A qualitative diagnostics assay for the detection of *D. septosporum* infection in pine needles, already available and routinely used by Forest Research, was converted to a quantitative diagnostics assay. The trials revealed that the DsCV-1-free D752.1 isolate tends to be more infectious as compared to the DsCV-1-infected D752.1 isolate, four other DsCV-1-infected *D. septosporum* isolates from Scotland and one DsCV-1-free *D. septosporum* isolate from Southern England. The trials revealed that the DsCV-1-free D752.1 isolate causes significantly more extended needle damage in the form of red bands and has significantly higher fungal burden as compared to the DsCV-1-infected D752.1 isolate. DsCV-1 appears to decrease infectivity, cause less extended needle damage and lead to lower fungal burden by interfering with fungal host growth and gene expression.

In parallel to the traditional pathogenicity assay, the potential of a single needle pathogenicity assay was investigated but could not fully recapitulate the outcomes of the trials on pine saplings. The differences between the two pathogenicity assays may be attributed to the fact that, in the case of the single needle pathogenicity assay, the detached needles remain without a source of nutrients for weeks. This leads to aging and the initially green needles turn to yellow/brown over the course of the experiment, the difference being clearly visible in the negative control (Fig. 34 B). Despite the diverse manifestations of infection, the single needle pathogenicity assay has potential as an alternative approach to traditional trials and would be a particularly efficient method to investigate different *Dothistroma* spp. isolates on a range of *Pinus* species. Further experiments, such as qPCR analysis of the fungal burden are required, to confirm that the results of the two assays are comparable and the behaviour of *D. septosporum* on single needles is predictive of its behaviour on pine trees.

6.1.2 *B. bassiana*, mycovirus mediated hypervirulence and pest control

Mycovirus infection in *B. bassiana* increases growth and virulence. A similar mild hypervirulent effect coupled with virus infection has been reported for isolates of *A. fumigatus* infected with a polymycovirus (Kanhayuwa *et al*., 2015). However, for *B. bassiana* is not known whether hypervirulence is caused by infection with a combination of a unirnavirus and a polymycovirus in isolate EABb 92/11-Dm (Fig. 35). Hypervirulence has been reported more rarely than hypovirulence in fungi, but the number of mycoviruses that enhance fungal growth and/or virulence is increasing in the literature (e.g. Ahn & Lee, 2001; Özkan & Coutts, 2015; Thapa *et al.*, 2016; Kotta-Loizou & Coutts, 2017).

The greater wax moth *G. mellonella* has been used previously as an infection model to study the pathogenicity and virulence of virus-free and virus-infected isolates of *B. bassiana* (Kotta-Loizou & Coutts, 2017) and *A. fumigatus* (Özkan & Coutts, 2015). The latter is a human pathogenic fungus and main cause of pulmonary aspergillosis in immunocompromised individuals. The versatility of the *G. mellonella* infection model in terms of temperature (Fuchs *et al*., 2010) allows both these species to be tested at 25^{o}C and 37^{o}C respectively. Murine infection models have also been used when studying mycovirus phenotypes (Bhatti *et al.*, 2011).

Filamentous fungi, including some isolates of *B. bassiana*, may be resistant to a wide range of drugs. Proven resistance to HmB as illustrated by *B. bassiana* strains of interest highlights the importance of identifying an alternative drug as a transfection marker, as a prerequisite for further experiments. Potential candidates to be tested in the future are phleomycin, a drug related to bleomycin, and the fungicide benomyl.

Overall, confirmation of the occurrence of hypervirulence in entomopathogenic fungi is an important discovery in the field of biological control (Kotta-Loizou & Coutts, 2017). For instance, mycoviruses might be utilised as enhancers of extant commercially available *B. bassiana* strains, an application that constitutes a viable alternative to the genetic engineering of fungal biocontrol agents to improve their efficacy against insect pests.

Conclusions.

Overall, the aims and objectives of this project were nearly achieved, with only one exception: the 5' terminal region of DsCV-1 dsRNA 3 could not be amplified, cloned, and sequenced and thus this sequence remains incomplete. The research outcomes of the project clearly illustrated that mycovirus infection of the phytopathogenic fungus *D. septosporum* and the entomopathogenic fungus *B. bassiana* results in hypovirulence and hypervirulence, respectively. Therefore, these mycoviruses are promising for use in biological control application in both cases. For *D. septosporum*, virus-infected isolates may be employed in the field to protect pine trees from more aggressive strains; for *B. bassiana*, virus-infected isolates may be employed to improve mycopesticide efficiency. However, more extensive experiments under controlled environment conditions together with field trials are required in both cases.

Future work.

The main focus of this PhD on mycoviruses in *D. spetosporum* raised numerous new directions to be explored in the future. These include steps that will facilitate the development of biological control applications based on DsCV-1 and steps that will lead to better understanding of the molecular mechanisms underpinning the DsCV-1-mediated phenotypes.

1. Extensive population studies of *D. septosporum* isolates from the United Kingdom (Scotland, England, Wales, and Northern Ireland) to fully determine the prevalence of DsCV-1 and potentially other mycoviruses. During the current work, we established a diagnostics assay for highly sensitive, highly specific, and rapid detection of DsCV-1 in *D. septosporum* isolates, which will facilitate and accelerate the screening process.
2. Correlation of the presence of DsCV-1 and the severity of DNB in different *D. septosporum* populations. Since DsCV-1 reduces *D. septosporum* infectivity/virulence and appears to be widespread in Scotland based on our current data, it is possible that Scottish forests are already 'part protected' and damage caused by DNB would be much more extensive and devastating in the absence of the DsCV-1.
3. Additional field trials in collaboration with Forest Research UK, to directly investigate whether the presence of *D. septosporum* isolates infected with DsCV-1 protects the pine trees against a range of more aggressive *D. septosporum* isolates not infected with DsCV-1. This is a crucial experiment that is required prior to any biological control applications.
4. Optimisation of the single pine needle pathogenicity assay, as a quick, rapid, and cheap alternative to the field trials performed in collaboration with Forest Research UK. The establishment of such an assay will be a major breakthrough and will allow us to test a range of different *D. septosporum* isolates, pine species and environmental conditions simultaneously and inform the field trials.
5. Further investigation of potential links between DsCV-1 and dothistromin levels by comparing the expression levels of the genes involved in dothistromin synthesis in virus-infected and virus-free *D. septosporum* isolates at the same developmental stage; quantifying the expression levels of additional genes involved in dothistromin synthesis;

directly measuring dothistromin levels in virus-infected and virus-free *D. septosporum* isolates by colorimetric assays and mass spectrometry; indirectly measuring dothistromin levels in virus-infected and virus-free *D. septosporum* isolates be assessing its toxic effects on fungi, bacteria and other microbes that may compete with *D. septosporum* on pine trees.

6. NGS data analysis *in silico*, including differential gene expression analysis, and verification of selected genes and/or pathways in the laboratory.

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Appendices.

Antibiotics.

Antibiotics were filter-sterilised, and aliquots were stored at -20°C. Media were cooled below 50°C before addition of the antibiotics to prevent heat inactivation.

List of antibiotics and their concentrations:

Antibiotic	Stock concentration	Working concentration
Streptomycin	100 mg/mL	1000 µg/mL
Hygromycin	100 mg/mL	1000 µg/mL

Buffers and media.

All solutions were autoclaved before use.

TE buffer

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

50 x TAE buffer

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

Extraction buffer

EDTA (20 mM) (pH 8.0)

Tris-HCl (20 mM) (pH 7.5)

1% SDS

1% NaCl

Potato dextrose agar (PDA)

Potato infusion can be made by boiling 200 g of sliced (washed but unpeeled) potatoes in *ca.* 1 L distilled water for 30 min and then decanting or straining the broth through cheesecloth. Then 20 g dextrose, 20 g/L technical agar no.3 (Oxoid) and distilled water are added such that the total volume of the suspension is 1 L. The pH is adjusted at 5.6 and the medium is then sterilised by autoclaving at 15 psi for 15 min.

Sabouraud dextrose agar (SDA)

Peptone	10 g
Dextrose	5 g

NaCl	5 g
Technical agar no.3 (Oxoid)	15 g
Distilled water	1000 mL

The pH is adjusted at pH to 7.2 and the medium is then sterilised by autoclaving at 15 psi for 15 min.

Malt extract agar (MEA)

Malt extract (Oxoid)	20 g
Technical agar no.3 (Oxoid)	15 g
Distilled water	1000 mL

Czapek-Dox medium

Sucrose	30 g
KH_2PO_4	0.5 g
KCl	1 g
$NaNO_3$	2 g
$MgCl_2$ $7H_2O$	0.62 g
$FeSO_4$ or $FeCl_3$	0.01 g
Peptone	1.5 g
Yeast extract	1.5 g
Malt extract	1.5 g
Distilled water	1000 mL

Solid Czapek-Dox medium required the addition of 20 g/L technical agar no.3 (Oxoid).

Pine Needle Minimal Medium + Glucose (PMMG)

PMMG Medium (g/L):

$MgSO_4.7H_2O$	0.2 g
K_2HPO_4	0.9 g
KCl	0.2 g
NH_4NO_3	1.0 g
$FeSO_4.7H_2O$	0.002 g
$ZnSO_4.7H_2O$	0.002 g
$MnCl_2.7H_2O$	0.002 g
Asparagine	2.0 g
Glucose	3.0 g

Agar	20.0 g

All chemicals were added to 1 L water in which live pine needles had been freshly soaked (10% w.v) in Milli Q water for 24-48 h at room temperature and adjusted to pH 6.2 The Ph of the medium was adjusted with HCl to 6.5.

1x LB medium

Tryptone (Oxoid) or Peptone	10 g
NaCl	5 g
Yeast extract	5 g
Distilled water	1000 mL

Solid LB medium required the addition of 12 g/L technical agar no.3 (Oxoid).

2x LB medium

Tryptone (Oxoid) or Peptone	20 g
NaCl	20 g
Yeast extract	10 g
Distilled water	1000 mL

Transformation storage solution

2x LB	5 mL
PEG 8000	1 g
Dimethyl sulfoxide (DMSO)	0.5 mL
$MgCl_2$ (1 M)	0.5 mL
Sterilised distilled water	3 mL

Super Optimal broth with Catabolite repression (SOC) 10 mL

Glucose (2 M)	200 µL
$MgCl_2$ (2 M)	50 µL
1x LB	10 mL

SOC solution is freshly prepared every time.

X-gal /IPTG 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 (20 mL).

X-gal solution is freshly prepared every time.

Publications.

Publications in peer-reviewed journals:

Filippou, C., Diss, R.M., **Daudu, J.O.,** Coutts, R.H.A., & Kotta-Loizou, I. (2020). The polymycovirus-mediated growth enhancement of the entomopathogenic fungus *Beauveria bassiana* is dependent on carbon and nitrogen metabolism. *Frontiers in Microbiology*, 12:606366.

Publications in conference proceedings:

Daudu, J.O., Snowden, J., Tubby, K., Coutts, R.H.A., & Kotta-Loizou, I. Studying a mycovirus from *Dothistroma septosporum*, causative agent of pine needle blight. *Access Microbiology*, https://doi.org/10.1099/acmi.ac2019.po0502.

Presented as a poster at the Microbiology Society Annual Conference 2019, International Convention Centre ICC, Belfast, UK.

Publications in newsletters:

Daudu, J.O. Investigations on the effects of a mycovirus on the pathogenicity of *Dothistroma septosporum* the causal agent of pine needle blight. British Society for Plant Pathology (BSPP) newsletter No. 90 Autumn-Winter 2019, pp. 19-20.
https://drive.google.com/file/d/1zBE4-kWxcivIXCil1zOGHoWkaaEDRYsT/view?usp=sharing

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Front. Microbiol., 02 February 2021 | https://doi.org/10.3389/fmicb.2021.606366

The Polymycovirus-Mediated Growth Enhancement of the Entomopathogenic Fungus *Beauveria bassiana* Is Dependent on Carbon and Nitrogen Metabolism

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Polymycoviridae is a growing family of mycoviruses whose members typically have non-conventional capsids and multi-segmented, double-stranded (ds) RNA genomes. *Beauveria bassiana* polymycovirus (BbPmV) 1 is known to enhance the growth and virulence of its fungal host, the entomopathogenic ascomycete and popular biological control agent *B. bassiana*. Here we report the complete sequence of BbPmV-3, which has six genomic dsRNA segments. Phylogenetic analysis of RNA-dependent RNA polymerase (RdRp) protein sequences revealed that BbPmV-3 is closely related to the partially sequenced BbPmV-2 but not BbPmV-1. Nevertheless, both BbPmV-3 and BbPmV-1 have similar effects on their respective host isolates ATHUM 4946 and EABb 92/11-Dm, affecting pigmentation, sporulation, and radial growth. Production of conidia and radial growth are significantly enhanced in virus-infected isolates as compared to virus-free isogenic lines on Czapek-Dox complete and minimal media that contain sucrose and sodium nitrate. However, this polymycovirus-mediated effect on growth is dependent on the carbon and nitrogen sources available to the host fungus. Both BbPmV-3 and BbPmV-1 increase growth of ATHUM 4946 and EABb 92/11-Dm when sucrose is replaced by lactose, trehalose, glucose, or glycerol, while the effect is reversed on maltose and fructose. Similarly, both BbPmV-3 and BbPmV-1 decrease growth of ATHUM 4946 and EABb 92/11-Dm when sodium nitrate is replaced by sodium nitrite, potassium nitrate, or ammonium nitrate. In conclusion, the effects of polymycoviruses on *B. bassiana* are at least partially mediated *via* its metabolic pathways.

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Meeting Report | Open Access

Studying a mycovirus from *Dothistroma septosporum*, causative agent of pine needle blight

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Dothistroma needle blight caused by *D. septosporum* has emerged in the British Isles as a major threat to Corsican pine, lodgepole pine and Scots pine. There is increasing evidence that mycoviruses can reduce the growth and pathogenicity of fungal plant pathogens. The aim of the present study is to characterise a double-stranded RNA virus found in *D. septosporum* and investigatefor putative hypovirulence, a common feature noted for mycoviruses, which might be used for biological control to invasion by more aggressive strains of the fungus. To this endthe viral genome was cloned and sequenced revealing four genomic segments, each one containing a single open reading frame (ORF) flanked by 5' and 3' untranslated regions. The ORFs encode the RNA-dependent RNA polymerase, the capsid protein, a protein of unknown function and a putative protease, respectively. Phylogenetic analysis of the sequences obtained revealed their similarity to members of the established family *Chrysoviridae*, genus *Alphachrysovirus*, which are encapsidated in isometric particles and are known to elicit hypovirulence in their hosts. Subsequently, virus-free and virus-infected isogenic lines were generated to determine any effects of the mycovirus on fungal fitness and pathogenicity. More specifically, the virus-infected isolate is currently being assessed in comparison to the virus-free one in terms of radial growth in solid culture, biomass in liquid culture, pathogenicity in pine trees and production of the mycotoxin dothistromin. In conclusion, this study reports the first mycovirus ever found in *D. septosporum*.

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Nobuhiro Suzuki et al., J Gen Virol

Biological properties and expression strategy of rosellinia necatrix megabirnavirus 1 analysed in an experimental host, Cryphonectria parasitica
Lakha Salaipeth et al., J Gen Virol, 2014

A geminivirus-related DNA mycovirus that confers hypovirulence to a plant pathogenic fungus

Studying a mycovirus from *Dothistroma septosporum*, causative agent of pine needle blight

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Introduction

Dothistroma needle blight (DNB; aka red band needle blight) is caused by the fungus *Dothistroma septosporum*. *D. septosporum* has been found on a range of conifer species and has emerged as a major threat to Corsican pine, lodgepole pine and Scots pine. Defoliation continues annually and gradually weakens trees, significantly reducing timber yields and often resulting in tree death. Mycoviruses (fungal viruses) may cause hypovirulence to their hosts and therefore have largely unexplored potential as biological control agents of plant pathogenic fungi.
The aim of the project is to characterise a novel mycovirus from *D. septosporum* and use potential hypovirulence caused to protect pine trees.

Results

Dothistroma septosporum chrysovirus 1

Agarose gel electrophoresis (left) and schematic representation of the double-stranded (ds) RNA genome of Dothistroma septosporum chrysovirus.

Phenotype and growth of DsCV-1-free and DsCV-1-infected isogenic lines (left). PCR amplification assay for the detection of DsCV-1 (right).

Pathogenicity assays for *D. septosporum*

1. Whole pine tree (*Pinus nigra*) pathogenicity assay.

2. Pine needle pathogenicity assay.

3. Greater wax moth (*Galleria mellonella*) pathogenicity assay.

Summary

- ✓ *D. septosporum* isolates from the UK harbour a dsRNA mycovirus, Dothistroma septosporum chrysovirus 1 (DsCV-1).
- ✓ DsCV-1 has four genomic segments and belongs to genus *Alphachrysovirus*, family *Chrysoviridae*.
- ✓ Alphachrysoviruses are predicted to affect fungal growth and development together with gene expression.
- ✓ DsCV-1-infected and DsCV-1-free isogenic lines have been constructed and their phenotype, growth rate and virulence are being compared.
- ✓ Three distinct pathogenicity assays for *D. septosporum* are currently in progress.

Acknowledgements

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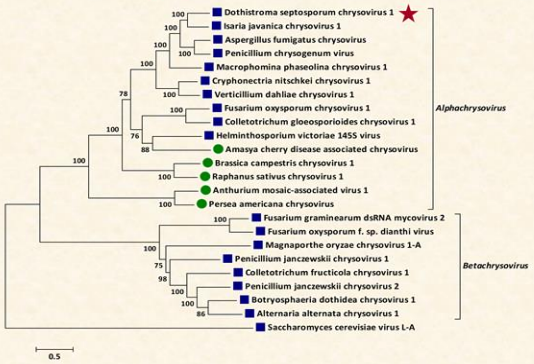
Phylogenetic analysis of DsCV-1



Phylogenetic tree of DsCV-1 and other members of the family *Chrysoviridae* based on the conserved RdRP sequence. At the end of the branches, blue squares indicate that the virus infects fungi; green circles indicate that the virus infects or is associated with plants.

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                1          2          3          4          5          6          7          8
DsCV-1     LVGRS[73]WVTKGGLVQ[64]EKYRTLL[52]DWADFNEQHS[49]GLYSGWRGTTWINTVLNFC[22]HGGDDLDL[33]EFFRN[12]RALAS
IjCV-1     LVGRG[73]WLTKGGLVN[64]RKDRTLL[52]DWADFNEQHS[49]GLYSGWRGTTWINSTLNFC[22]HGGDDLDL[33]EFFRN[12]RALAS
AfuCV      LVGRS[73]WLTKGSLVY[64]NKDRTLL[52]DWANFNEQHS[49]GLYSGWRGTTWVNTVLNFC[22]HGGDDIVL[33]EFFRN[12]RALAS
PoV        LVGRG[73]WLTKGGLVY[64]KKDRTLL[52]DWADFNEQHS[49]GLYSGWRGTTWINTVLNFC[22]HGGDDIDL[33]EFFRN[12]RALAS
MgCV-1     LIGRA[73]WLTKGSLVY[64]GKERTLL[52]DWADFNEQHS[49]GLYSGWRGTSWINTVLNFC[22]HGGDDIDL[33]EFFRN[12]RALAS
CnCV-1     LVGRT[74]WLTKGSLVY[64]RKDRTLL[52]DWADFNEQHT[49]GLYSGWRGTTWINSVLNFC[22]HGGDDVDA[33]EFFRN[12]RALAS
VdCV-1     LIGRT[74]WLTKGSLVY[65]KKDRTLL[52]DWADFNEQHS[49]GLFSGWRGTTWINTVLNFC[22]QGGDDIDS[33]EFFRN[12]RALAS
FoCV-1     LLGKE[73]WLTKGSTVF[64]RKKRALL[51]DWANFNLYHS[49]GLYSGWRGTTWINTVLNCV[22]GGGDDHDG[33]EFFRN[12]RTLTT
CgCV-1     MLGKE[72]WLTKGSTVF[64]RKKRALL[51]DWSNFNLYHS[49]GLYSGWRGTTFINTVLNEV[22]GGGDDHDG[33]EFFRN[12]RALVT
HvV-145B   LLGRR[73]WMTKGSLVS[64]RKDRVLL[52]DWANFNVYQH[49]GLYSGWRGTTWSNTVLNSC[22]QGGDDVDQ[33]EFFRV[12]RGLAT
ACDAcV     LVGRR[73]WLTKGSTVY[64]YKDRTLL[52]DWANFNAFHS[49]GLYSGWRGTSFLNSVLNSC[22]HGGDDIDG[33]EFFRI[12]RALAR
BoCV-1     YTGWQ[72]WVAKGSTVL[60]GKKRALL[52]DWANFNAQHS[49]GLFSGWRGTTWINTVLNFV[22]HGGDDLDV[33]EFYRN[12]RGLAN
RaCV-1     YTGRE[72]WVAKGSTVM[60]GKKRALL[52]DWSNFNAQHS[49]GLFSGWRGTTWINTVLNHV[22]HGGDDLDV[33]EFYRN[12]RGLAN
AMAV       LYGRF[70]WAAKGSTVV[59]AAKRALL[52]DFADFNAQHS[49]GLYSGWRGTTWINSVLNHA[22]GAGDDVDG[33]EFLRV[12]RVLGN
PaCV       LYGRF[70]WAAKGSTVI[59]AAKRALL[52]DFSDFNAQHS[49]GMYSGWRGTTWINSVLNHA[22]GAGDDVDG[33]EFLRV[12]RALGN
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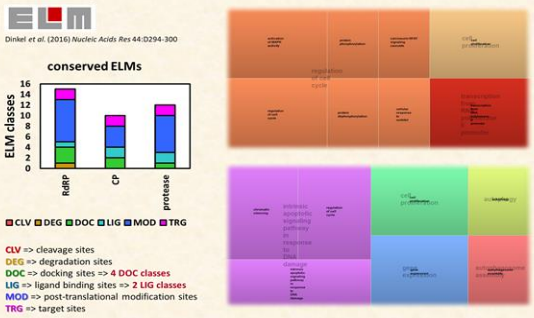
Conserved functional motifs in the RdRP sequence of alphachrysoviruses.

Eukaryotic Linear Motifs in DsCV-1



Conserved ELMs in alphachrysovirus proteins (left). GO analysis and visualisation of DOC (docking sites; right up) and LIG (ligand binding sites; right down) ELMs.

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Investigations on the effects of a mycovirus on the pathogenicity of *Dothistroma septosporum* the causal agent of pine needle blight

I consider myself fortunate to have been awarded a junior fellowship by the BSPP to support my studies on a mycovirus from *Dothistroma septosporum,* the causative agent of pine needle blight. This fellowship has helped me immensely in supporting collaborative work with Dr Katherine Tubby and her colleagues at Forest Research UK.

Dothistroma needle blight (DNB), caused by *Dothistroma septosporum*, has emerged in the British Isles as a significant threat to Corsican pine, lodgepole pine and Scots pine over the past few decades. DNB is endemic on indigenous pine populations in the Northern hemisphere and is a significant world-wide tree health issue. This is particularly true for the UK, where DNB affects the commercial pine tree industry, causing premature defoliation in forest tree nurseries and in some cases tree mortality. It has been reported that over 63 countries and over 82 pine species have been affected by DNB. The economic significance is challenging to evaluate; however, in the UK, an estimated £8.6 million is lost per annum, mainly due to DNB.

Recent studies confirm that mycoviruses (fungal viruses) can reduce the growth and pathogenicity of fungal plant pathogens of their hosts (hypovirulence) and therefore have mostly unexplored potential as biological control agents. One of the most successful mycovirus-mediated biocontrol applications utilised mycovirus infected isolates of *Cryphonectria parasitica*, the causative agent of chestnut blight in chestnut trees (*Castanea spp.*). Cryphonectria parasitica hypovirus 1, a member of the family *Hypoviridae* significantly reduced the growth rate of secondary infections by the host fungus, protecting the chestnut trees.

My PhD project focuses on one *D. septosporum* isolate infected with a tetrapartite mycovirus. My aim was to characterise this novel mycovirus by sequencing its double-stranded RNA genome using genome walking and recombinant DNA technology; to eradicate the mycovirus and assess the effects of infection on fungal phenotype and growth utilising virus-free and virus -infected isogenic isolates and to investigate the pathogenicity of virus-free and virus-infected isogenic isolates on pine tree saplings and pine needles. This last section specifically concerned my BSPP fellowship.

The four viral genomic segments of the mycovirus were cloned and sequenced; each one contained a single open reading frame (ORF) flanked by 5′ and 3′ untranslated regions. The ORFs encode respectively an RNA-dependant RNA polymerase (RdRP) to replicate the virus, the capsid protein, a protein of unknown function and a putative protease. All protein sequences were closely related to members of the family *Chrysoviridae* particularly to Isaria javanica chrysovirus. Phylogenetic analysis of the RdRP sequence showed that the *D. septosporum* mycovirus belongs to the genus *Alphachrysovirus,* family *Chrysoviridae* and was nominated DsCV -1 following the discovery of conserved functional motifs in the RdRP sequence. Further data analysis revealed the presence of conserved Eukaryotic Linear Motifs (ELMs) in the DsCV-1 RdRP sequence. ELMs are small compact protein interaction sites of specific amino acid sequence that play crucial roles in cell regulatory processes and can often be used to manipulate host cellular machinery. Alphachrysoviruses were predicted to affect fungal growth and development together with gene expression based on their conserved ELMs.

Subsequently, DsCV-1 infected and DsCV-1 free isogenic lines were generated using ribavirin to eliminate the virus and their phenotype, growth rate and virulence were compared in terms of radial growth on solid pine minimal media with glucose (PMMG), biomass in liquid PMMG, production of the mycotoxin dothistromin and pathogenicity in pine tree nurseries.

To conduct the pathogenicity assays in pine tree nurseries I have visited the laboratory of Dr Katherine Tubby at Forest Research UK on over 10 occasions, where excellent facilities are available and outstanding work is done on tree and plant pathology (pictured, with myself on the right).

Forest Research is located at Alice Holt Lodge, Wrecclesham, Farnham, Surrey. I took advantage of my visits there to learn new technology, such as growing and maintaining pine trees, inoculation with fungi and sampling, together with quantitative polymerase chain reaction (qPCR) amplification. Additionally, I was granted access to the state-of-the-art growth chambers which mimicked the required conditions for *D. septosporum* pathogenicity assays and allowed the fungus to grow under optimal conditions on pine tree saplings. The DsCV-1 infected and DsCV-1 free isogenic lines and six other isolates of *D. septosporum* are being investigated in two duplicate trials over a period of six months. These experiments are currently ongoing as pine infection of *D. septosporum* takes eight to twelve weeks to be visually confirmed. Following sampling the levels of *D. septosporum* will be quantified using a qPCR protocol devised in the Forest Research laboratories. Any effects of DsCV-1 infection on the saplings will be monitored by PCR comparing virus concentration, fungal growth, fungal toxin levels and pine tree growth.

I wish to acknowledge Drs Ioly Kotta-Loizou (Imperial College) and Avice Hall (University of Hertfordshire) for their support and Dr Robert Coutts (University of Hertfordshire) for his supervisory assistance with the project and for introducing me to Dr Katherine Tubby and James Snowden at Forest Research. Finally, I wish to thank the BSPP for funding this endeavour.

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