A study of La France disease in Agaricus bisporus

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A STUDY OF LA FRANCE DISEASE

IN

AGARICUS BISPORUS

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ABSTRACT

A positive correlation was shown between nine dsRNA segments, 3.6 - 0.78 kbp and La France disease. "Apparently healthy" sporophores and cultures were not found to contain these segments. Curing of these "standard" dsRNA" segments by heat treatment was associated with the return of the healthy phenotype. Transfer of the "standard" dsRNA segments by anastomosis to "apparently healthy" cultures was associated with the appearance and maintenance of the disease phenotype. The majority of the "standard" dsRNAs were encapsidated and appeared to be closely associated with diseased mitochondria. High molecular weight dsRNA segments 15 - 8.8 and 8.6 - 6.6 kbp were also associated with the disease. However, the 8.6 - 6.6 kbp dsRNAs were also found in "apparently healthy" cultures.

DsRNA segment changes evidently induced during culturing were present in both diseased and "apparently healthy" isolates. Loss of the majority of the "standard" and 15 - 8.8 kbp dsRNA segments during culturing did not correlate with any change in the phenotypic expression of the disease. High molecular weight dsRNA segments 8.8 - 6.6 kbp initially absent in diseased sporophores were observed in cultures following the loss of the 15 - 8.8 kbp dsRNA segments. Sequence homology was shown to exist within the "standard" dsRNA segments and between these and the high molecular weight dsRNA segments. No evidence was found for integration of viral sequences into host DNA in diseased and healthy cultures. The 8.6 - 6.6 kbp dsRNA segments were also found occasionally in "apparently healthy" cultures with no phenotypic disease symptoms.

Evidence was not found for the active integration of dsRNA into the host. However, a 20 kbp DNA segment isolated following CF11

chromatography in both diseased and healthy isolates showed homology with the "standard" and high molecular weight dsRNA segments. Although not mitochondrial in origin the exact relevance of this DNA segment remains uncertain. Cell free infectivity studies resulted in initial disease symptoms but these were not, however, maintained during culturing.

ABBREVIATIONS

BSA	Bovine serum albumin.
$CaCl_2$	Calcium chloride.
cDNA	Complementary deoxyribonucleic acid.
CaCl	Caesium chloride.
$CsSO_4$	Caesium sulphate.
dATP	Deoxyadenosine triphosphate.
2,6 DCIP	2,6 - Dichloroindophenol.
dCTP	Deoxycytidine triphosphate.
DEPC	Diethyl pyrocarbonate.
dGTP	Deoxyguanosine triphosphate.
DMSO	Dimethyl sulphoxide.
DNA	Deoxyribonucleic acid.
dsRNA (DsRNA)	Double stranded ribonucleic acid.
EDTA	Ethylene diamine tetra acetic acid.
EM	Electron microscopy.
EtBr	Ethidium bromide.
EtOH	Ethanol.
FDA	Fluorescein diacetate.
g	Gravity.
g	Grams.
gfwt	Grams fresh weight.
HAc	Glacial acetic acid.
Hepes	N- [2-Hydroxyethyl] piperazine-N [2-ethanesulfonic acid].
H_2O	Water.
kbp	Kilobase pair.
KCl	Potassium chloride.
Liquid N_2	Liquid nitrogen.
M	Molar.

mA	Milliamperes.
ml	Millilitre.
mg	Milligram.
$MgSO_4$	Magnesium sulphate,
mM	Millimolar.
MOPS	3- [N-Morphopline] propanesulfonic acid.
mtDNA	Mitochondrial deoxyribonucleic acid.
NaAc	Sodium acetate.
NaCl	Sodium Chloride.
Na_2HPO_4	Disodium hydrogen phospate.
NaOH	Sodium hydroxide.
um	Micromolar.
ul	Microlitre.
ng	Nanogram.
nm	Nanometre.
°	Degrees.
°C	Degrees centigrade.
%	Percentage.
OD	Optical density.
OLB	Oligolabelling buffer.
PEG	Polyethylene glycol.
RFLP	Restriction fragment length polymorphism.
RNA	Ribonucleic acid.
RT	Room temperature.
SDH	Succinate dehydrogenase.
SDS	Sodium dodecyl sulphate.
ScVs	Saccharomyces cerevisaie viruses.
TEMED	N,N,N',N'- Tetramethylethylenedimine.
Tris	Tris [hydroxymethy] amino methane.
TE	Tris [hydroxymethy] amino methane ethylene dismine tetra acetic acid EDTA.

UV	Ultra violet.
v/v	Volume/volume.
VLPs	Virus-like particles.
w/v	Weight/volume.

1. INTRODUCTION

Most fungal viruses have segmented double-stranded RNA (dsRNA) genomes that persist indefinitely in their host cells (Bozarth, 1979; Buck, 1986). Uncertainties surrounding the taxonomy and replication of mycoviruses are paralleled by doubts concerning their precise biological significance. Ecologically mycoviruses are exceptional, possibly unique for their existence appears to be entirely intracellular. Transmission is by hyphal anastomosis and dispersal occurs within spores. This contrasts sharply with extracellular modes of dispersal which predominate in the majority of plant and animal viruses.

Viruses or virus like particles (VLPs) have been described in over 150 fungal species. A variety of morphological particle types have been observed, although many have only been detected by thin section electron microscopy of infected cells and their true viral nature awaits confirmation. In a few fungi dsRNA occurs not in isometric particles but in membrane-bound club shaped particles or in as yet undetermined sub-cellular locations. Most isometric dsRNA mycoviruses do not exert deleterious effects on their host. However, a number of fungal viruses or virus-like agents associated with A.bisporus and certain plant pathogens have been shown to have possible deleterious effects on their host (van-Zaayan, 1979; Buck, 1987)

1.1 LA FRANCE DISEASE OF A. BISPORUS

1.1.1 SYMPTOMS

In the early 1950 s a disease causing poor mycelial growth, reduction of fruiting bodies and premature cap opening in A. bisporus was first reported by Sinden and Hauser (1950). Initially a number of diseases were attributed to the above symptoms: watery stipe, La France, Brown, mummy disease and X-disease, all of which can show degeneration of mycelium, sporophore abnormalities and waterlogging of the stipe (Gandy, 1959). The one condition which sets La France disease apart from the others is the symptom known as "die-back" where following rapid mycelial degeneration cropping ceases (Hollings, 1962; Gandy, 1962). A number of conditions which lead to watery stipe, long stalks in relation to cap size and drum stick formation, can be avoided by correcting physical conditions in the crop (Hollings et al., 1963). La France diseased crops, however, cannot be restored to normality by physical changes (Gandy, 1960 a; 1960 b) and once initiated the die-back condition prevails. Infected mycelial cultures from diseased sporophores show appressed growth with few aerial hyphae on malt agar as opposed to the more vigorous aerial growth of apparently healthy cultures (Hollings et al., 1963; Schisler, et al., 1967; van Zaayan, 1979). By adding infected mushroom compost or diseased agar cultures to healthy spawn, Hollings (1962) showed the disease to be transmissible. The time of infection was important, the earlier the infection in the crop cycle the greater the severity of the disease (Hollings, 1962). If infection

occurs at the initial spawning stage symptoms and yield losses will become progressively worse during the cropping cycle (Table 1.1).

TABLE. 1.1 THE EFFECT OF LA FRANCE DISEASE ON FLUSH YIELD

	FLUSH NO.		
	1st	2nd	3rd
Diseased	28.0	7.6	1.2*
Healthy	49.3	59.1	29.9 Kg

From Van-Zaayan (1979). * Mean weight per flush

However, once established on solid media diseased isolates although showing typical disease symptoms do not appear to degenerate further (Last et al, 1967). Symptoms and the severity of infection also depends on the strains used (Sinden et al,., 1950; Gandy, 1962; Schisler et al., 1967; van Zaayan, 1976). Particular strains sometimes appear immune to infection (van Zaayan, 1976).

One symptom believed to be associated with the disease is that of early maturity, causing spore release. In fact some of the most destructive outbreaks of La France disease in the 1950s - 1960s occurred when for some reason the mushrooms had been allowed to open prior to harvesting, releasing spores into the growing rooms (van Zaayan, 1979). Diseased spores were shown by Schisler et al., (1963; 1967) and van-Zaayan (1979), to germinate as readily as healthy spores and in most instances give a greater percentage

germination. Diseased spores sprayed onto compost during spawning elicited the typical disease symptoms (Schisler et al., 1967).

1.1.2 ASSOCIATION OF LA FRANCE DISEASE WITH VIRUS PARTICLES

The first person to associate virus particles with La France disease was Hollings (1962)who discovered three particles, two spherical particles 25 and 29 nm in diameter and bacilliform particles with a length of 50 nm and width of 19 nm. Later work by Hollings (1967) showed that two further spherical particles of diameters 34-35 nm and 50 nm were also present in diseased sporphores. Similar particles were observed in several countries (van-Zaayan 1979; Marino et al., 1976; Nair, 1972), the most common particles being the bacilliform and spherical particles of diameters 25 and 34-35 nm. Mushroom stalks were shown to be a better source of virus than caps and young sporophores better than old. One important observation was that apparently healthy mushrooms surrounding areas containing obviously diseased mushrooms were found to be symptomless carriers and were a rich source of virus particles (Hollings et al., 1963).

It was established early on that La France disease was not an all or nothing response and that varying levels of the disease could occur. A number of efforts to implicate one or a number of particle types as causal agents have been attempted. Last et al., (1967), took mycelial isolations from mushrooms at different distances from the site of disease inoculation. Cultures from stunted and abnormal sporophores from near the inoculation site grew slowly, were

brown and appressed and lacked mycelial strands; they contained high levels of 29 nm particles and few 25 nm particles. The concentration of 25 nm particles remained relatively constant with increasing distance from the inoculation site but that of the 29 nm particle progressively decreased. As the concentration of 29 nm particles diminished cultures grew faster and produced more coarse strands; this implied a role for the 29 nm particles in the disease. Later work by Last et al., (1974), appeared to show an inverse correlation between concentrations of 25 nm and 29 nm particles in sporophores and levels of mycelial growth on malt agar. Van Zaayan (1972) found 34 nm particles moving through dolipores in hyphal cross sections implicating cell to cell translocation of at least these particles. Nair (1972) on the other hand found some diseased Australian isolates which failed to follow Last et al's inverse correlation of growth rate and particle number, and showed increases in the growth of particle containing isolates during culturing. In some cases workers did not find any particles in mushrooms showing symptoms generally associated with virus infection (Nair, 1972).

Cell free transmission of virus particles remains a problem. Hollings (1962) showed a reduction in cropping after injecting a crude preparation of virus particles into apparently healthy sporophores. van Zaayan et al., (1968) also found virus particles in apparently healthy material injected with crude particle isolates. Doubts on the purity of these cell free preparations, failure to repeat this early work and the later finding of virus particles in healthy material has meant that the majority of evidence

implicating virus particles as the causal agent of La France disease remains circumstantial.

One strange phenomenon that occurred concerning the particles found took place during the late 1960s and 1970s. In England in 1968 the 25 nm and 29 nm particles were common while other particles remained relatively rare. However, in two years this pattern changed with 35 and 25 nm particles being common and 29 nm and 50 nm particles less common (Hollings & Stone 1971). It is believed that this change may have been brought about by changes in purification procedures (van-Zaayan, 1979), but particle variation cannot be ruled out. By 1979 Barton and Hollings again found 25 and 35 nm particles to be the most prevalent and this applied to diseased mushrooms of other countries also. Recently in the U.S.A. Koons et al., (1983) and Wach (1983) have indicated that spherical particles of 19, 25 and 34 nm diameters are more consistently associated with the disease than bacilliform particles.

Improvements in purification (Barton & Hollings, 1979; Passmore & Frost, 1974; 1979; 1980) and electron microscope techniques (Del Vecchio, 1978) allowed the detection of virus particles at very low levels. Initially particles were only found in diseased material (Hollings, 1962, Schisler et al., 1967; Last et al., 1967). Nair (1972) was the first to find a variety of particle types in apparently healthy or non-symptomatic mushrooms collected from high yielding crops. Anastomosis between diseased and healthy isolates although leading to the transfer of particles did not always produce symptomatic mushrooms, Nair (1972).

Passmore & Frost (1979) looking at 23 strains of A. bisporus found no consistent difference between the particle content of "apparently healthy" or abnormal sporophores from over 500 sporophore samples. Neither normal or abnormal sprophores were free of virus-like particles, with apparently healthy sporophores sometimes containing high concentrations of particles.

1.1.3 DOUBLE STRANDED RNA

As with the majority of other fungal viruses and VLPs mushroom viruses are also associated with dsRNA (Buck, 1986) The bacilliform particle which appears to have a ssRNA genome being an exception (Molin et al., 1973; Lapiere 1973; Tavantiz et al., 1980). Initially (Barton & Hollings, 1979; Marino et al., 1974; Wach and Romaine, 1983) dsRNA was only found in diseased sporophores and apparently normal sporophores surrounding the periphery of the infected area (Hicks & Haughton 1986). DsRNA has now been detected in a large number of healthy spawn, sporophore and cultured isolates tested (Deahl et al., 1986; Harmsen et al., 1989; Romaine et al., 1989). The mere presence of dsRNA does not necessarily lead to malformed fruit bodies but certain combinations of dsRNA segments appear closely linked with the onset of disease symptoms. A "standard" dsRNA pattern consisting of nine segments, 3.6-0.78 kbp (Hicks & Haughton, 1986; Wach et al., 1987; Harmsen et al., 1989; Romaine et al., 1989) has come to be associated with severely diseased sporophores. This pattern seems indicative of severely infected isolates in U.S.A, U.K. and Holland. In the crop, Ross et al., (1987) showed yield losses to be

associated with a characteristic 5 band pattern of dsRNA possibly consisting of the 3.6, 3.0, 2.8-2.7, 2.5 and 1.35 kbp dsRNA segments of Harmsen et al., (1989) and Romaine et al., (1989). The degree of crop loss appeared to correlate with the concentration of bands. Variations within the standard pattern have also been noted with additions of 6.8, 0.516, 0.5, 0.425, and 0.41 kbp dsRNA segments (Wach et al., 1987) and a 2.35 kbp dsRNA segment (Harmsen et al., 1989) also found in severely diseased isolates. Cases have also been found where some dsRNA segments have been absent from the "standard" pattern and the sporophores still appear diseased Wach et al., (1987). Controversy also exists over dsRNA being the causal agent as the "standard" pattern has been found in apparently healthy sporophores at the edge of infected regions in mushroom trays (Harmsen et al., 1989), at levels comparable to those found in severely diseased material. Also, Deahl et al.,(1987) found the "standard" pattern in sporophores of A. bisporus from healthy crops showing no apparent symptoms. It has transpired that very few strains of A. bisporus do not contain dsRNA (Harmsen et al., 1989; Romaine et al., 1989). These authors never found mushrooms from healthy high yielding crops without any dsRNA. DsRNA found in healthy material appears to show extensive variation, for example, Harmsen et al., (1989) and Romaine et al., (1989) showed healthy sporophores to contain a 2.35-2.4 kbp dsRNA fragment, dsRNA segments from the standard pattern, and several faint high molecular weight dsRNA segments. Romaine et al., (1989) isolated the 2.4 kbp fragment in 70% of healthy but only in 30% of diseased basidiocarps, this segment does not appear to be associated with a particular

phenotype. Particle associations do not appear to have been carried out on healthy sporophores containing this segment. Earlier work by Hicks and Haughton (1986) may have also picked up this 2.4 kbp segment in apparently normal sporophores but the electrophoresis system used did not allow a clear determination of where the band originated in relation to the standard pattern.

1.1.4 ASSOCIATION BETWEEN dsRNA AND VIRUS PARTICLES

The mixed infection system which exists in A. bisporus has posed problems in purifying particular particles and characterising the dsRNA's they contain. The bacilliform particle 50 x 19 nm is the best characterised of the viruses, it is a non-envelope particle with a capsid made up of a single capsid polypeptide species of Mr 24,000 daltons (Tavantiz and Smith 1980). Lapiere (1973) detected two species of ssRNA in a crude preparation of bacilliform particles. Tavantiz and Smith (1980) using a highly purified preparation showed particles to contain a ssRNA species of 4 kb. When translated in-vitro this gave rise to a major polypeptide of molecular weight 77,000 daltons and several minor polypeptides. The bacilliform particle has never been found alone in A. bisporus and is usually accompanied by the isometric viruses 25 and 35 nm in diameter.

Barton and Hollings (1979) felt that as diseased isolates contain 25 and 35 nm spherical particles and bacilliform particles in random mixtures they probably represent separate entities and are not part of a multicomponent

system. The 35 nm particles were shown to contain two dsRNA species 2,276 bp and 2,123 bp and 25 nm particles two dsRNA species 2,124 bp (Barton & Hollings 1979). How these segments fit into the standard dsRNA pattern is unclear as current dsRNA molecular weights appear to differ from earlier estimates, this may represent segment variation but is more likely due to technical differences. Wach and Romaine (1983) detected nine dsRNA segments in diseased sporophores, two of these 6.3 kbp and 1.9 kbp were apparently associated with 19 nm and 25 nm particles, whereas the other seven 2.5, 2.2, 2.1, 1.3, 1.1, 0.73 and 0.63 kbp dsRNA s were apparently associated with 35 nm particles. Later work by Wach et al., (1987) found purified 35 nm particles to contain two dsRNAs 6,525 bp and 2,124 bp. The majority of dsRNAs previously associated with 35 nm particles by Wach et al., (1983) were not found encapsidated and probably represent intermediates in dsRNA replication or have some functioinal role in the particle life cycle. As well as the 5 major particles described so far rod shaped particles (Lapiere et al., 1971; Luisoni, 1972) and club shaped particles (Lesemann et al., 1972; Atkey et al., 1978) have also been found in infected material. The club shaped particles consisted of a double membrane envelope surrounding an electron dense core. Similar particles have also been isolated in hypovirulent strains of the horse chestnut blight fungus Endothia parasitica (Dodds 1980) and shown to contain dsRNA; the presence of dsRNA in these particles of A. bisporus was not ascertained.

1.2 EFFECTS OF FUNGAL VIRUSES ON OTHER FUNGI

Virus-like particles (VLPs) and virus-like nucleic acids have been found in over 50 plant pathogens. Associations have been established between VLPs and a number of degenerative/hypovirulent phenomenon in several plant pathogens. In some pathogens evidence for dsRNA/VLPs being the causal agents for these conditions is good while in others it remains circumstantial. However, in R.solani (Zanzinger et al., 1984) dsRNA appears to be associated with virulence rather than hypovirulence.

1.2.1 HYPOVIRULENCE AND DEGENERATIVE DISEASES IN PLANT PATHOGENIC FUNGI

(i) Hypovirulence

Endothia parasitica is the fungal plant pathogen responsible for Chestnut blight. Hypovirulent forms of E. parasitica were first seen as a reduction in damage caused by the pathogen on chestnut plantations (Biraghi 1953). Hypovirulent isolates were shown to be dominant when mixed in a tree with normal virulent isolates (Grente & Sauret, 1969). Dominance was found to be due to transmission of cytoplasmic factors from hypovirulent to virulent isolates (Van Alfen et al., 1975). Moffit & Lister (1975) detected dsRNA in hypovirulent but not virulent isolates. In 1977 Day et al., reported the dsRNA found in hypovirulent isolates to have a segmented genome and that dsRNA segment sizes tended to vary from strain to strain.

Although evidence is strong that dsRNA is responsible for hypovirulence in E. parasitica it is not yet conclusive. When the hypovirulence factor is transferred dsRNA is always transmitted (Anagnostakis et al., 1979). Curing dsRNA from hypovirulent strains resulted in the strain reverting to the normal phenotype (Fulbright 1984). DsRNA segment variations can be found within a particular strain but hypovirulence was still shown in all cases (Van Alfen 1986; Anagnostakis, 1982; Dodds, 1980; 1980; L'Hostis, 1985).

DsRNA in E. parasitica invariably appears to affect the expression of virulence and sporulation. Recent studies by Powell & Van Alfen (1987) have shown that dsRNA affects the accummulation of specific Poly(A)(+)RNAs. Products of vir1 and vir2 genes readily found in virulent strains were found in much lower concentrations in hypovirulent strains. However, Eliston (1978) has proposed that some dsRNA containing and presumably hypovirulent strains can grow in stems at rates comparable to normal isolates.

Isometric virus particles with genomes of dsRNA occur commonly in field isolates of the wheat-take-all-fungus, Gaeumannomyces graminis var. tritici (Ggt) (Buck, 1986). Populations of G. graminis generally contain large numbers of vegetative compatability groups, virus particles and dsRNA segments within such groups show extensive variation (Frick & Lister, 1978;

Buck et al., 1981; Jamil et al., 1984).

Whether or not dsRNA plays a role in Ggt pathogenicity has still to be determined. Natural Ggt isolates appear to vary in pathogenicity from strongly invasive to hypovirulent (Asher 1981). LeMaire et al., (1970) found that an ascospore isolate 911, containing levels of virus particles too low to be detected was more pathogenic than its weakly pathogenic virus infected parent. However, Rawlinson et al., (1973) found that some apparently virus-free ascospore isolates were much less pathogenic than their virus infected parents. Recent work by Stanway (1985) looking at levels of pathogenicity according to sizes of dsRNA segments indicated that no size class of dsRNA either alone or in combination positively reduced pathogenicity. DNA plasmids have also been found in Ggt (Honeymann, 1983) but their role in pathogenicity has yet to be investigated.

Rhizoctonia solani is a soil borne pathogen which causes root decay in over 130 plant species (Baker, 1970). Hypovirulent strains were found showing a reduction in pigmentation, irregular morphology and reduced growth rates (Castanho et al., 1978). Hypovirulence was shown by Castanho et al., (1978) to be associated with dsRNA. However, later work by Zanzinger (1984) reported that 49 of 50 isolates ranging from highly virulent to essentially avirulent contained dsRNA. This was supported by Finkler (1985) who reported that native hypovirulent strains were

devoid of dsRNA and that in fact the virulent strains carried dsRNA. Finkler (1985) using one of Castanho's original disease free virulent strains showed it to contain dsRNA. According to Finkler (1985) hypovirulent and virulent strains showing no difference in growth rate on laboratory media could only be distinguished by pathogenicity tests. Discrepancies in the pathogenic state of cultures in the early experiments could account for the discrepancies in the dsRNA content of virulent and hypovirulent strains. Koltin and fellow workers at the University of Tel-Aviv feel that much of the confusion concerning the association of dsRNA with hypovirulence is due to non-dsRNA artifacts isolated along with dsRNA. These artifacts, seen after CF11 chromatography as high molecular weight bands during electrophoresis, are probably DNA. Recent work by Hashiba et al., (1984) has indicated the presence of DNA plasmids in weakly pathogenic isolates. It is clear that further investigations are required to resolve the roles of dsRNA and DNA plasmids in virulence attenuation or virulence expression in R. solani.

(ii) Degenerative diseases.

Dutch elm disease is caused by a combination of the vascular wilt pathogen Ceratocystis ulmi and vector Elm bark beetles of the family Scolytidae. A recently discovered cytoplasmically transmissible genetic determinant (d-factor) of C. ulmi is capable of exerting a deleterious effect on fungal growth and

reproductive fitness (Brasier, 1983). Analysis of d^2-infected isolates, initially isolated by Brasier (1983), revealed ten dsRNA segments ranging from 2.4 - 0.3 X 10^6 Daltons (Rogers et al, 1986). Following infection with the d-factor by anastomosis the infected isolate will eventually show a reduction in growth rate (Brasier, 1986). Loss of the d^2 factor correlated well with loss of multiple dsRNA segments and recovery of normal growth (Rogers et al., 1986).

Pathogenicity of C. ulmi is assessed on the basis of the degree of wilting that results after spore inoculation into the xylem. Generally d-infection is lost as the fungus spreads throughout the tree (Brasier 1986). The overall mean defoliation caused by d-infected isolates was very close to that of non d-infected isolates. Occasionally d-infection actually promoted pathogenic ability and growth adding further confusion to an already complex behavioural picture.

Helminthosporium victoriae is a highly specialised pathogen that only inflicts damage on oat varieties with the victoriae type of resistance to the crown rust, Puccinia coronata (Litzenberg, 1949). Isolates of H. victoriae were shown to carry a transmissible disease, which following an initial period of normal growth, produced sectors at colony margins with the collapse and lysis of existing aeriel mycelium (Linberg, 1959). Diseased isolates contained two serologically and electrophoretically distinct viruses,

190S and 145S. The 190S virus contains a 4412 bp dsRNA fragment and the 145S virus four dsRNAs ranging from 3,530-2,941 bp (Sanderlin & Ghabrial, 1978). Disease severity appears to correlate with levels of the 145S virus (Ghabrial et al., 1979; Sanderlin & Ghabrial, 1978). However, phenotypic differences have been shown to correlate with levels of 190S particles (Ghabrial, 1986).

Pathogenicity of H. victoriae depends on the production of the host specific toxin "victorin" (Luke & Wheeler, 1955, Scheffer et al., 1967). "Victorin" produced in culture was 2-10 times higher in normal than diseased isolates (Linberg, 1960). Sanderlin (1977) showed that the presence or absence of virus was not directly related to toxin production and that it is growth rate changes that result in decreased toxin production.

1.2.2 Beneficial effects of dsRNA mycoviruses

In at least two fungi the presence of dsRNA mycoviruses appears to bestow a selective advantage on the host. The plant pathogen Ustilago maydis and Saccharomyces cerevisiae contain dsRNA viruses that encode for a killer toxin that kills cells of the same species lacking virus immunity. Nuclear genes also appear necessary for the replication of virus particles (Koltin, 1986; Ball et al., 1984; Wickner 1974; 1981). In some aspects the killer systems are analogous to the secretion of bacteriocins by certain strains of coliform bacteria, where toxin production is mediated by a plasmid.

(i) Killer viruses of Saccharomyces cerevisiae

The yeast viruses comprise an excellent model system for studies of the relationship between a host eukaryotic cell and its resident dsRNA viruses. Most S. cerevisiae strains have resident viruses (ScVs) (Brier, 1980). ScV are exclusively cytoplasmic and not associated with mitochondria or other known yeast plasmids (Wickner, 1980; Fink & Styles, 1972; Al-Aidroos, 1973). All known ScVs have one virus species with a dsRNA(L) of about 4.8 kbp (Herring & Bevan, 1974). Some strains also have a second satellite dsRNA of 1.9 kbp (M) encapsidated separately in particles of the same diameter (ScV-M) (Young & Yagin, 1978). Many of these strains produce an extracellular toxin (killer toxin) which kills cells not harbouring a resident ScV-M virus. Both L and M satellite dsRNAs differ in different viral sub-types encoding different toxin and resistance specificities (Young & Yagin, 1978; Wickner, 1980). There are at least 13 killer toxins and immunity specificities among yeast genera (Young & Yagin, 1978; Philliskirk & Young, 1975).

(ii) Killer systems of Ustilago maydis

Ustilago maydis is the heterobasidiomycete pathogen that causes boil smut of corn. Puhalla (1968) showed that some sporidial cultures known to be vegetatively compatible showed inter-strain inhibition during plate assay. Inhibition was found to be due to a proteinaceous substance (Hankin & Puhalla, 1974).

Toxin production was shown to be a cytoplasmic phenomenon (Koltin & Day, 1976) but nuclear genes were involved in encoding resistance (Day & Anagnostakis, 1973). Wood & Bozarth (1973) isolated virus particles but their presence was not directly related to the inhibitory function or immunity. DsRNA segments 2.87 - 0.44 x 10^6 daltons (light, medium, and heavy) were found in strains coding for the inhibitory substance (Bozarth et al., 1981). Evidence suggests that viruses encapsidate the dsRNA individually (Bozarth, 1978), the different sizes of dsRNA accounting for the different densities of 41 nm particles in density gradients. All strains that contain dsRNA have at least one heavy segment, medium and light segments were never found without the heavy segments (Koltin, 1987). The medium class of dsRNA appear to code for the inhibitory substance (Koltin, 1987) and the light segments are involved in the expression and immunity to the toxin (Koltin, 1978, Peery, 1982).

1.3 OBJECTIVES

Although extensively studied over the last 40 years the underlying causes of La France disease in A.bisporus remain poorly understood (van-Zaayan, 1979; Buck, 1986). DsRNA and VLPs have been associated with the disease but their presence at comparable concentrations in "apparently healthy" material has led to confusion over their significance. Also attempts to establish Koch's postulates with VLPs/dsRNA have proved difficult and inconclusive.

The objectives of this study were to extensively characterise all dsRNA segments and VLPs associated with the disease. To determine the cellular locaisation of dsRNA/VLPs within diseased hyphae in an attempt to understand how debilitating effects may be caused.

In order to gain a greater understanding of the role of dsRNA segments, chemical and physical treatments were applied to diseased cultures in an attempt to cure them. The possibility that dsRNA can exist in an integrated form was assessed in cultures that appear to have lost the majority of dsRNA but maintain the disease phenotype. Finally, attempts were to be made using cell free methods to establish Koch's postulates for the disease.

2. MATERIALS AND METHODS

2.0 BUFFERS & MEDIA

Djuskista Scheffers and Wiken liquid media.

2% malt extract (oxoid), 0.5% soluble white light caesin and 0.2% di- potassium hydrogen orthophosphate.

Deharts (x 100)

10g ficoll (Pharmacia),, 10g polyvinylpyrrolidone, 10g BSA and distilled water to 500 ml. The solution was filter sterilised and dispensed into 25 ml aliquotes and stored at -20°C.

DNA extraction buffer.

200 mM Tris - Cl pH 8.5, 250 mM NaCl, 25 mM EDTA (disodium salt), 0.5% (w/v) SDS.

Elution buffer (Polyacrylamide gel extraction)

0.5 M ammonium acetate, 0.01 M $MgCl_2$, 0.1 mM EDTA (disodium salt), 0.5% (w/v) SDS.

Hae III restriction buffer

6 mM Tris HCl, 50 mM NaCl, 6 mM $MgCl_2$, 7mM 2 - mercaptoethanol and 0.01% (w/v) triton x -100 pH 7.6.

Loading buffer (x 6)

0.25% bromophenol blue, 0.25% Xylene cyanol, 30% glycerol in H_2O, stored at 4°C..

Malt Agar (MA)

1% malt extract (oxoid), 1.2% Agar (lab. No. 2).

Oligolabelling buffer (OLB)

Solution A :- 625 µl 2M tris HCl (pH 8.0), 25 µl 5M $MgCl_2$, 350 µl H_2O, 18 µl 2 - mercaptoethanol, 5 µl each of 0.1M, dATP, dTTP, dGTP (Pharmacia)

(each triphosphate was dissolved in 3 mM Tris - HCl (pH 7.0), 0.2 mM EDTA 0.1 M)

This was stored at - 20°C.

Solution B :- 2M HEPES pH 6.6 stored at 4°C.

Solution C :- Hexadeoxyribonucleotides (pharmacia) suspended in 3 mM Tris - HCL (pH 7.0), 0.2 mM EDTA at 90 OD units/µl stored at -20°C.

Solutions A, B and C were added together in the ratio 2:5:3.

Osmotic medium (OM).

0.5M $MgSO_4$, 0.05M maleic acid pH 6.75

Sau 3a restriction buffer

10 mM Tris - HCl, 60 mM NaCl, 7 mM $MgCl_2$. pH 7.5.

Sorbitol MOPs media (SM)

0.5M sorbitol, 10 mM MOPs, 10 mM NaOH, pH 6.75.

20 X SSC

175.3g NaCl, 88.2g sodiumcitrate adjusted to pH 7 with NaOH and made up to 1 litre with distilled water.

20 X SSPE

3.6M NaCl, 0.2M sodium phosphate pH 7.7 and 0.002M EDTA (disodium salt).

10 X STE

0.1M Tris - Cl, 1M NaCl, 10 mM EDTA (disodium salt), pH 8.0.

TE buffer

10 mM Tris - Cl, 1 mM EDTA (disodium salt) pH 7.4 or pH 7.6. (The above concentration was used in all cases unless otherwise specified.)

50 X TAE

2M Tris - acetate. 0.5M EDTA pH 8.0.

50 X TBE

4.5M Tris - acetate, 0.1M EDTA pH 8.3

TAQ I restriction buffer

6 mM Tris - HCl, 100 mM NaCl, 6 mM $MgCl_2$.
6 mM 2 - mercaptoethanol, pH 7.4.

Yeast malt dextrose agar (YMDA).

1% yeast extract (lab M), 1% malt extract (oxoid).
2% dextrose, 2% No. 2 agar (lab. M).

All reagents were of analar grade from sigma unless otherwise stated.

2.1 CULTURING

2.1.1 Production and maintenance of diseased and apparently healthy cultures from sporophores (strain U3).

Following surface flame sterilization, isolations were made on malt agar (MA) from the internal cap (pileus) tissue. Subcultures were taken and during a 2-4 week incubation at 23°C, the following characteristics were assessed: (i) growth rate, (ii) level of sectoring, and (iii) colour. The above parameters were monitored routinely throughout the duration of the project.

Cultures were generally maintained on vented MA plates at 23°C and on MA slopes at 4°C. In order to assess the dsRNA and viral particle content of cultured material, 5 x 4 mm plugs were taken from colonies grown at 23°C and aseptically transferred to shaking cultures containing Djuskista Scheffers and Wiken liquid media.

Depending on the severity of the disease, cultures were left for 6-8 weeks at room temperature (RT) (20°C-25°C) before harvesting the mycelia by vacuum filtration. The origins of diseased and "apparently healthy" cultures used are shown in table 2.1.

TABLE 2.1

Origins of cultures established from diseased and "apparently healthy" sporophores.

CULTURE	STRAIN	SOURCE
SD12	S22	Country Kitchens
H6F3	U3	Country Kitchens
H9F2	D	Country Kitchens
H5F3	U3	Country Kitchens
Culture F	U3	Country Kitchens
Culture H	U3	Country Kitchens
Culture D	U3	Country Kitchens
"Apparently healthy"	U3	Country Kitchens

2.2 DOUBLE STRANDED RNA (dsRNA).

2.2.1 Double stranded RNA Extraction

The method used was a modification of the method of Morris & Dodds (1979) and involves the preferential binding of dsRNA to a CF11 cellulose column in 16.5% ethanol (EtOH). Mycelia from sporophore or cultured material were ground in liquid nitrogen and transferred to a 250 ml flask. The following reagents were added: 2 ml/g fresh weight (f.wt.) of 1 x STE buffer containing 0.01% 2-mercaptoethanol, 2 ml/g(f.wt) of STE saturated phenol, 1 ml/g(f.wt) of chloroform: pentanol (25:1) and 0.1% (w/v) SDS. The mixture was shaken gently for 20-30 minutes at RT.

The homogenate was centrifuged for 30 minutes at 12,000 g, the aqueous phase collected and adjusted to 16.5% EtOH. The CF11 chromatography column was prepared by pouring 30 ml of STE, 16.5% EtOH containing 2.5 g of Whatman CF11 powder into a sterile 30 ml disposable syringe containing a Whatman No.

1 filter paper plug. The sample was passed through the column and the column washed with 80 ml STE 16.5% EtOH. The dsRNA was eluted with 3 x 5 ml aliquots of STE the first 5 ml of eluate was discarded as it contained little or no dsRNA. The final 10 ml was collected and ethanol precipitated:

3 volumes of EtOH and 0.1 vol of 3M sodium acetate pH 5.5 was added and precipitated overnight at -20°C. The sample was centrifuged for 30 minutes at 8,000g the pellet dried under vacuum, and resuspended in the desired amount of TE buffer pH 7.5.

2.2.2 Electrophoretic separation of dsRNA and size determination.

(i) 6% polyacrylamide disc and slab gel electrophoresis.

Gels were produced from an 18% (w/v) acrylamide stock solution consisting of 4.5 g acrylamide (Sigma) and 0.1g of NN methyl-bis acrylamide (Sigma) made up to 25 ml with distilled water. The stock solution was de-ionised with amberlite monobed resin MB1 (BDH) and the filtered solution used to produce 6% polyacrylamide gels containing 1 x TAE pH 7.8. In 30ml of 6% polyacrilamide gel, 40 ul TEMED (Sigma) and 200µl 10% (w/v) ammonium persulphate (Biorad) were added as setting agents. To produce a flat surface on tube gels a layer of distilled water was added to the top of the 6% polyacrylamide gel before it had set. DsRNA samples were loaded in loading buffer (0.1 X loading buffer per sample) and run in 1 X TAE pH 7.8 for 4 hours at 5mA per tube and 30 volts overnight for slab gels. Gels were stained with ethidium bromide (EtBr) (10 µg/ml)

following electrophoresis.

(ii) 1% Agarose electrophoresis.

Agarose gels were made up with the required amount of agarose (w/v), 1 x TBE pH 8.3 and boiled until all the agarose had melted. EtBr (0.5 μg/ml) was added and the gel cooled to 50°C before pouring into the gel mould. Gels were run in 1 x TBE pH 8.3 containing EtBr (0.5 μg/ml) at 30 Volts overnight. DsRNA samples were loaded in loading buffer as above.

For both gel systems Lambda DNA molecular weight markers were used. Lambda DNA (0.9 μg)(NBL) was digested with Hind III (NBL) and Hind III/EcoRI (NBL) to give molecular weight makers of 23 Kbp - 2.0 Kbp and 21 Kbp - 0.8 Kbp respectively.

2.2.3 Determination of the nature of nucleic acid bands from diseased and apparently healthy material.

(i) Following electrophoresis.

Adapted from the method of Hamilton (1980). Tube gels or sections cut from agarose/polyacrylamide slab gels were digested for 1-2 hours at RT with 50 μg/ml DNase I (Sigma) in 30 mM $MgCL_2$. Gels were also digested with boiled RNase A. (Sigma) 50 μg/ml in high salt (0.3M Nacl) and distilled water for 1-2 hours. Single stranded RNA is digested by RNase A in high salt but dsRNA remains intact until placed in a solution containing low (0.1 SSC) or no salt where it is digested (Marino, et al., 1976).

(ii) Prior to electrophoresis.

Samples were dissolved in TE buffer pH 7.4 containing 30 mM $MgCl_2$ for DNase digests and 0.3M NaCl and distilled water for digesting in RNase high and "low salt" (Marino, et al., 1976). DNase I 50 µg/ml and RNase A 50 µg/ml were added to samples and incubated at 37°C for 20 minutes. RNase digestions also involved a phenol : chloroform extraction and ethanol precipitation (method 2.2.1.), samples were run on a 1% agarose gel overnight as previously described.

2.2.4 Extraction of dsRNA segments from agarose and polyacrylamide gels

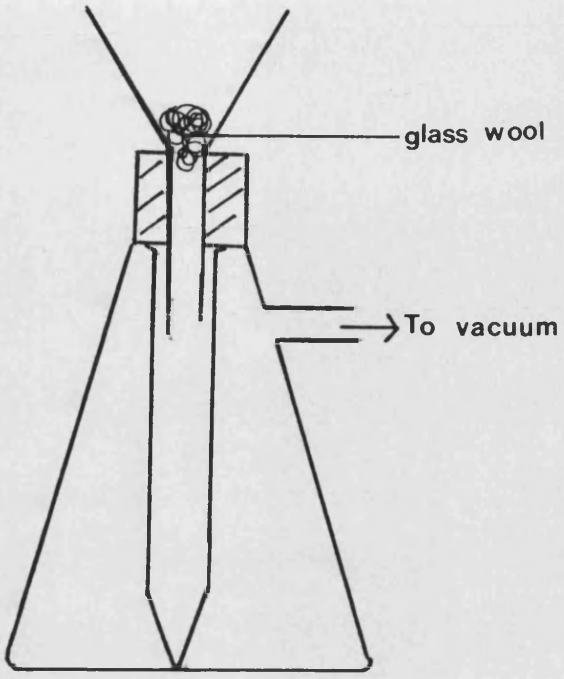
Samples were separated by electrophoresis (section 2.2.2) and dsRNA segements extracted by one of the following methods.

(i) Extraction from polyacrylamide gels.

Adapted from the method of Maxam and Gilbert (1977) and Yang et al (1979). Samples of dsRNA were separated by polyacrylamide tube gel electrophoresis. After running gels were stained with EtBr (10 µg/ml) and bands of interest cut out. Gel fragments were thoroughly squashed in heat sealed plastic bags, 3 ml of elution buffer added and fragments left shaking at 37°C overnight. The buffer and gel fragments were filtered as shown below:

Fig. 2.1

THE REMOVAL OF CONTAMINATING POLYACRYLAMIDE GEL FRAGMENTS FROM dsRNA SEGMENT EXTRACTIONS



the filtered solution was collected ethanol precipitated section (2.2.1) and stored at -20°C until required.

(ii) Extraction from agarose gels using Whatman DE 81 paper.

Adapted from the method of Paterson (1988), dsRNA segments were separated by 1% agarose electrophoresis and electroblotted on to 1 cm squares of DE 81 paper. Slits were cut alongside tracks and individual bands electrophoresed onto DE 81 paper by rotating the gel through 90°. Once transferred DE 81 squares were homogenised with 100 µl 1.5M NaCl, 50 mMTris, 1mM EDTA pH 7.0 and heated to 65°C for 10 minutes and cooled slowly. The paper was removed from the aqueous solution by centrifugation and the aqueous phase

ethanol precipitated.

(iii) Low melting agarose gels.

Adapted from a method initially described for DNA by Weislander (1979). DsRNA segments were separated on a 1% low melting point agarose gel (Sigma) overnight at 20 Volts. Individual segments cut from the gel were melted in "Oakridge" style phenol resistant centrifuge tubes with 2 ml of TE pH 7.4 at 65°C. Samples were mixed thoroughly until all the gel fragments had dissolved, 2ml of pre-heated TE equilibated phenol : chloroform (2:1) (65°C) was added and mixed for 10 minutes. Samples were spun for 10 minutes at 9,000g after which the supernatant was removed and ethanol precipitated.

2.2.5 DsRNA denaturation and transfer to Hybond N membranes (Amersham).

DsRNA transfer requires an initial denaturation step prior to denaturating electrophoresis and Northern blotting. Two methods were used formamide and formaldehyde denaturing gels and denaturation of dsRNA with glyoxal (Thomas, 1980) prior to running on non-denaturing gels.

(i) Formaldehyde/formamide.

Formamide (50% final concentration) was added to all dsRNA samples (20 µg) together with 6% formaldehyde and 1 x Hepes/EDTA pH 7.0 and heated to 100°C for 2 minutes before cooling on ice. Samples were run on 1.2% agarose gels containing 1 x Hepes/EDTA pH 7.0 and 6% formaldehyde; (the agarose was melted in buffer and cooled to 55°C before adding formaldehyde). Gels were run for 22 hours at 2V/cm in recirculated 1 x Hepes /

EDTA pH 7.0 containing 6% formaldehyde.

Following electrophoresis the gel was soaked for 5 minutes in several changes of sterile distilled water. The RNA was further fragmented by soaking in an excess of 50 mM NaOH, 10 mM NaCl for 45 minutes at RT before neutralizing in 0.1 mM Tris - Cl pH 7.5 for 45 minutes The gel was soaked for 1 hour in 1 M Tris 1.5 M NaCl prior to transfer as in section 2.3.2.

(ii) Glyoxal/DMSO.

Adapted from McMarster & Carmichael (1977) and Thomas (1980). DsRNA (20 µg) was denatured in a solution containing 2.7 ul 6M glyoxal (BDH), 8.0 µl DMSO (Sigma) and 1.6 µl O.1M NaH_2PO_4 pH 7.0 and incubated at 50°C for 60 minutes in a tightly closed tube. Loading buffer (0.1 X) was added to samples which were electrophoresed on 1.2% agarose gels poured and run in 0.01 M Na-H_2PO_4 pH 7.0 at 3-4 V/cm; (because glyoxal reacts with ethidium bromide the gels were run in the absence of the dye). Constant recirculation of the buffer was required in order to maintain the pH within acceptable limits (glyoxal disassociates from RNA at pH values >8.0). At the end of the run the gel was stained with ethidium bromide (0.5 µg/ml.)

To transfer glyoxalated RNA to Hybond N (Amersham) no further treatment is required and blotting takes place as in section 2.3. *

* Note: All solutions and pieces of apparatus used in section 2.2.5 were initially treated with 0.01% diethyl pyrocarbonate (Sigma) and autoclaved or left standing

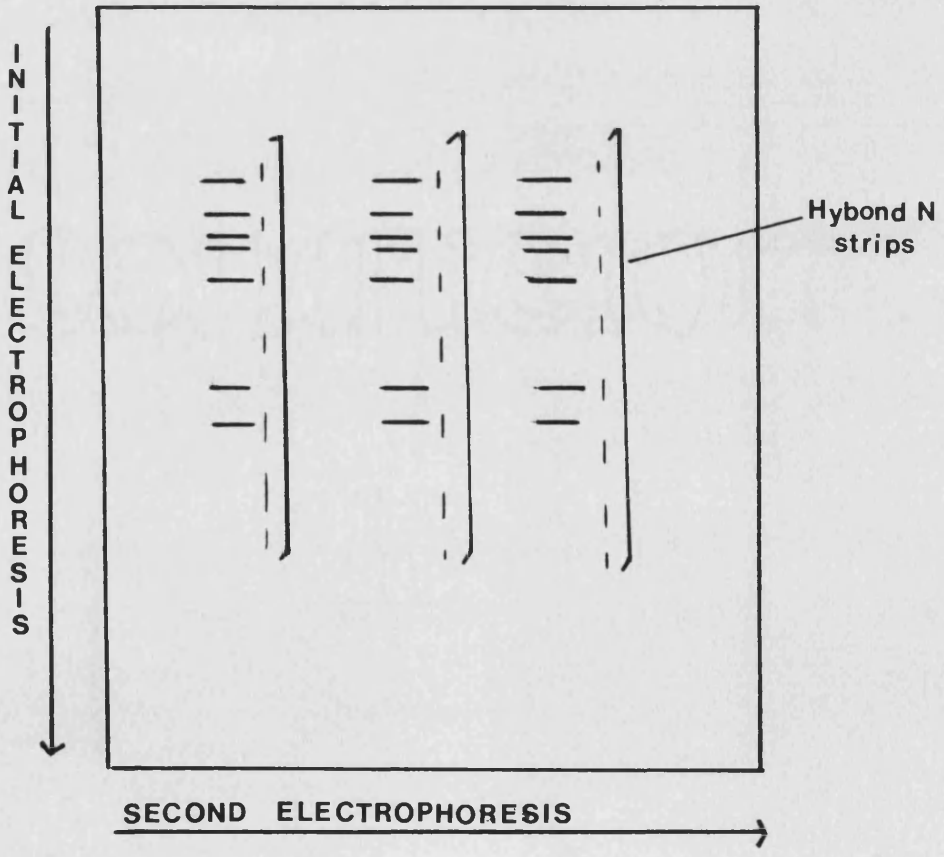
overnight.

(iii) Electroblotting.

DsRNA samples were separated overnight on a 1% agarose gel run at 30 Volts. Strips of Hybond N Membrane (Amersham) were inserted in slits cut alongside each track Fig. 2.2. DsRNA segments were electroblotted onto the membrane strips by passing between 50-100 Volts across the gel. Once transfer had occurred blots were washed briefly in 2 x SSC, dried and RNA irreversibly bound by 5 minutes exposure to U.V.

FIG. 2.2

ELECTROPHORETIC TRANSFER OF NON-DENATURED dsRNA ONTO HYBOND N STRIPS



Denaturation of dsRNA on Hybond N strips.

To find the minimum level of denaturation required to render the dsRNA accessible to compatible probing a range of NaOH concentrations were used. Hybond N strips were soaked in 10, 50, 100, 250 and 500 mM NaOH for 20 minutes at RT. 100% Formamide at 70°C for 2 minutes was also used as a denaturant. Following denaturation membranes were washed twice for 5 minutes in neutralizing solution which consisted of 1.5M NaCl, 0.5M Tris, 0.01M EDTA. Denaturation was assessed using Northern hybridisation (section 2.4.3) and a cDNA probe to total dsRNA (section 2.4.1).

(iv) Dot blotting.

Individual dsRNA segments were extracted (section 2.2.4) and the concentration of each segment estimated by electrophoresis using known concentrations of markers. Between 20-30 ng of each segment (smaller amounts were used with segments in very low concentration) were taken for each dot blot. Samples were made up to 50 ul in TE buffer pH 7.4, boiled for 5 minutes and cooled on ice. Negative controls of 20 ng E coli transfer RNA (Sigma) were treated in the same way. Samples were spotted on to Hybond N membranes and allowed to dry before binding with UV as previously described.

2.3 DNA

2.3.1 DNA extraction from diseased and apparently heathly material

Adapted from the method of Raeder & Broda (1985). The diseased/apparently healthy mycelium was powdered by

grinding in liquid N_2 and 50 mg placed in an eppendorf to which 500 µl of extraction buffer, 350 µl of phenol (pre-equilibrated in extraction buffer) and 150 µl of chloroform was added and mixed thoroughly. Samples were spun at 12,000g for 15-60 minutes, the supernatant removed and 1 volume of chloroform added. After mixing, samples were spun for 10 minutes at 12,000g, the upper phase removed and 250µl of isopropanol added and left overnight at 4°C. Precipitated DNA was pelleted, washed in 70% ETOH, vacuum dried and resuspended in 100ul TE pH 7.4.

2.3.2 Mitochondrial/total DNA digests and Southern blotting

(i) DNA digests.

The possible integration of viral dsRNA into the host genome and genetic changes induced during culturing were investigated using restriction fragment length polymorphism (RFLP) analysis (Ainsworth & Sharp, 1989). Total DNA and mitochondrial DNA from different diseased and healthy cultures was cut with the four base pair cutters; Hae III Sau 3a and Taq I (NBL). Each reaction used between 10-40 ng DNA, 30 enzyme units of Hae III, Taq 1 or Sau 3a and 60 ng of boiled RNase A (Sigma) in the appropriate enzyme buffer. Reactions were carried out at 37°C (Hae III and Sau 3a and 65°C (Taq I) for 4 hours.

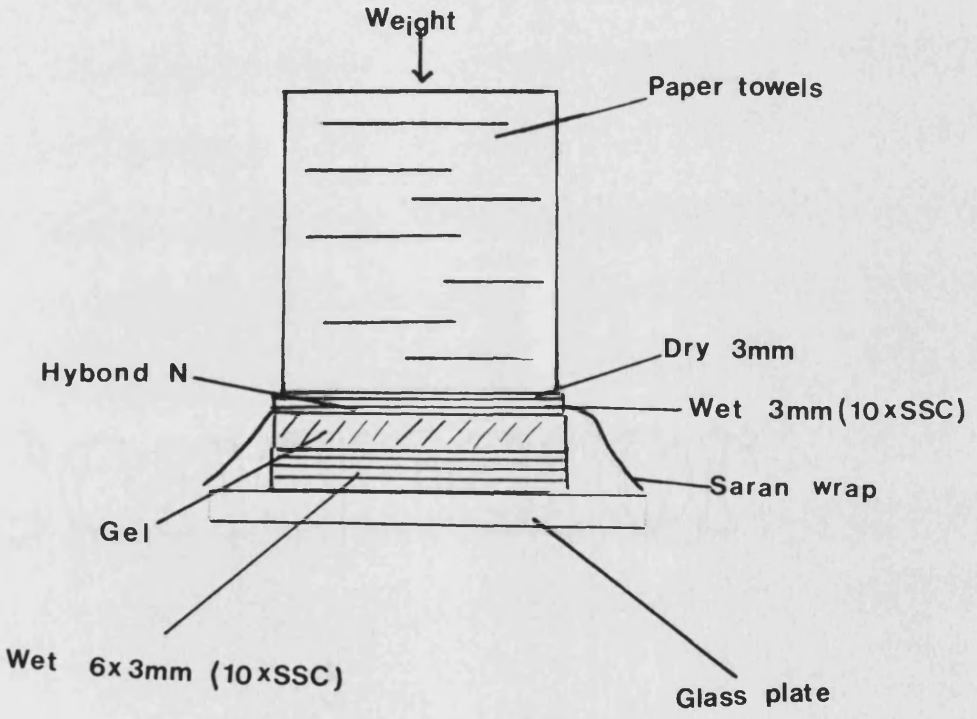
(ii) Southern blotting.

Restriction digested DNA samples were electrophoresed at 30V overnight on 1.5% agarose gels. The gel was pre-treated prior to Southern blotting by de-purinating in 0.2M HCl 20 minutes and denaturing with

0.5M NaOH, 1.5M NaCl for 30 minutes. The alkali was neutralized in 1M Tris 1.5M NaCl pH7 for 30-60 minutes. Blotting was as shown in Fig. 2.3 and carried out overnight. Following transfer the Hybond N membrane was rinsed briefly in 2 x SSC to remove gel fragments, dried and exposed to UV for 5 minutes to fix the DNA irreversibly to the membrane.

FIG. 2.3

SOUTHERN BLOTTING OF DNA ONTO HYBOND N



2.4 HYBRIDISATION

2.4.1 cDNA probes to dsRNA

Adapted from the method of Palukaitis (1986).

(i) Preparation of random primers.

Herring sperm DNA (5 mg) was dissolved in 1ml of 25mM Tris-HCl pH 7.4, 10mM $MgCl_2$ and digested with 70 μg of DNase I (Sigma) for 2 hours at 37°C. DNase I was inactivated by boiling for 10 minutes ; RNases which may contaminate DNase were removed by 2-3 extractions with an equal volume of phenol : chloroform [1:1]. DNase I fragments were precipitated with ethanol, resuspended and reprecipitated. The final DNA pellet was resuspended in 1 ml of 25mM Tris-HCl pH 8.3, 10 mM $MgCl_2$. The solution should contain DNA fragments 10-20 nucleotides in length, fragment sizes were determined using Orange G (Sigma) and Bromophenol blue (Sigma) markers on a 1% agarose gel.

(ii) cDNA synthesis.

The final reaction volume (60 μl) contained the following reagents:-

:- 1-20 ng of dsRNA (RNA strands separated by boiling for 5 minutes).

:- 20 μl of random primer solution (See section 3.4.1 (i)).

:- 50 mM Tris - HCL pH 8.3.

:- 13 mM Mg CL_2.

:- 100 mM KCL

:- 20 mM Dithiothreitol (Sigma)

:- 0.5 mM of each of d-GTP, d-TTP, d-ATP (Pharmacia)

:- 10 μ Ci α ^{32}P-CTP (Amersham)

:- 4 mM Tetrasodium pyrophosphate (Sigma)

:- 20 units of reverse transcriptase (Pharmacia).

The above reagents were incubated for 1 hour at 37°C and the reaction stopped by boiling (20 seconds). Boiled RNase A (300 ng) and RNase T_1, (125 enzyme units)(Sigma) was added and incubated for 20 minutes at 37°C. 100 ul of TE pH 7.4 was added and the cDNA phenol extracted three times with an equal volume of phenol : chloroform (1:1). The level of radiolabel incorporated into cDNA was monitored at this stage by taking 1 ul of the final supernatant and following method 2.4.4. The remaining supernatant was ethanol precipitated with 1 ul of denatured salmon sperm DNA (10 mg/ml) as a carrier. Precipitated cDNA was pelleted, freeze dried and resuspended in 10 µl TE pH 7.4.

(iii) Production of cDNA from lower concentrations (<1-5ng) of dsRNA segments.

With very low concentrations (<1-5 ng) of dsRNA segments attempts were made to increase the levels of radiolabeled cDNA produced. Using the property of reverse transcriptase to make copies from DNA as well as RNA strands provided free 3' ends are available (Efstratiadias et al., 1976 and Rougeon et al., 1976. Random primers present in the reaction bind to homologous regions of cDNA providing free 3' ends and allowing synthesis of a second DNA strand to occur in 5' - 3' direction. The synthesis reaction was the same but following the initial 1 hour incubation at 37°C the reactants were heated to 70°C for 5 minutes, rapidly cooled on ice, 20 units of reverse transciptase added

and left for a further hour at 37°C. This procedure was repeated three times and the reaction stopped by boiling as before.

2.4.2. Oligolabelling DNA

Taken from the method of Feinberg & Vogelstein (1984). Variable amounts of mitochondrial DNA and the 20 Kbp DNA segment isolated during dsRNA extraction (5-20 ng) were oligolabelled as follows. The DNA in a volume of 1-8 µl was denatured by boiling for 5 minutes and once cooled to 37°C the following reagents added, 3.0 µl oligolabelling buffer (OLB), 0.6 µl BSA (10 mg/ml) (pharmacia), 1.5 µl 32p d-CTP (approximately 15 µ Ci ∝ 32p d-CTP, Amersham) and 0.6 µl DNA polymerase "Klenow" fragment" (0.6 Enzyme units) (NBL), sterile distilled water was added to give a final volume of 15 ul and the labelling left for 3-4 hours at RT. The reaction was stopped by boiling for 5 minutes prior to the addition of the probe to the hybridisation vessel.

2.4.3. Northern and Southern hybridisation (Amersham (1985)).

(i) Northern

Northern hybridisation was carried out in 5 x SSPE , 50% (v/v) de-ionised formamide (Sigma), 5 x Denharts and 0.5% (w/v) SDS at 42°C. 10 ml of hybridisation solution and 100 ul of denatured sonicated non-homologous salmon sperm DNA (1mg/ml) was added to the RNA blot in a glass hybridisation tube (Hybaid). Pre-hybridisation was carried out in a hybaid rotisserie hybridisation oven for 1 hour at 42°C.

The pre-hybridisation solution was discarded and 10 ml of fresh hybridisation solution, 100 µl of denatured sonicated non-homologous salmon sperm DNA (1 mg/ml)

added together with the denatured DNA probe (boiled for 5 minutes) and left hybridising overnight at 42°C.

Following hybridisation, filters were washed twice in 2 x SSPE, 0.1% (w/v) SDS, 15 minutes at 42°C, once in 1 x SSPE. 0.1% (w/v) SDS 30 minutes at 42°C, and twice in 0.1 x SSPE, 0.1% SDS for 15 minutes at RT. Further washes were also carried out at higher temperatures 65°C and 75° in an attempt to determine whether non-specific binding was a problem with the lower temperature washes.

Blots were dried and autoradiographed at -80°C using FUJI RX medical x-ray film in autoradiography cassettes (Genetic Research Instrumentation Ltd.) fitted with Dupont-Cronex (2) lighting plus intensifying screens. Films were developed for 5 minutes in Kodak D19 developer, rinsed in water and fixed for 5 minutes in Kodak Unifix (Dil 260 g/l) before being thoroughly rinsed in tap water.

(ii) Southern.

Southern hybridisation was carried out in 6 x SSC, 5 x Denharts and 0.5% (W/V) SDS at 65°C. 10 ml of pre-warmed hybridisation solution and 100 µl of denatured sonicated non-homologous salmon sperm DNA (1 mg/ml) was added to the Southern blot in a glass hybridisation tube (Hybaid). Pre-hybridisation was carried out for 1 hour at 65°C in a hybaid rotisserie oven. With large filters or when more than one blot was hybridised nylon mesh sheets (Hybaid) were used in between filters to prevent "shadowing" occurring.

The pre-hybridisation solution was discarded and a further 10 ml of pre-warmed hybridisation solution added with 20 mg/ml of denatured sonicated non-homologous salmon sperm DNA. Boiled (5 minutes) labelled c-DNA or oligolabelled DNA was added, the tube sealed and left hybridising for at least 12 hours at 65°C.

Following hybridisation filters were washed twice in 2 x SSC 65°C for 15 minutes, once in 2 x SSC, 0.1% (w/v) SDS 65°C for 30 minutes. Blots were dried and autoradiographed as in section 2.4.3 (i).

(iii) Probe removal following Southern hybridisation

For probe removal to be effective filters must not be allowed to dry, hence blots to be re-probed are placed in Saranwrap directly after washing. Filters for re-use were boiled for 20-30 minutes in a solution of 10 mM Tris, 1mM EDTA pH 7.5, 1% SDS.

2.4.4. Measurement of radiolabel incorporated into c-DNA or oligolabelled DNA

Samples from oligolabelling and cDNA reactions (1 µl) were diluted with 60 µl of sterile H_2O and a known volume spotted onto two DE 81 discs (A & B). Disc B was washed six times, 5 minutes per wash in 0.5M Na_2HPO_4 pH 7.0 twice with steril H_2O 1 minute per wash and twice in 95% EtOH 1 minute per wash. The level of ^{32}P in discs (A) & (B) was estimated by Cerenkov counting, the count for disc (B) relative to (A) showing the amount of radiolabel incorporated into nucleic acid.

2.5 VIRUS PARTICLES

2.5.1 Initial crude virus preparation

Adapted from the methods of Passmore & Frost (1974) (1979) and Wach (1983). Purifications took place in 0.05 M potassium phosphate pH 7, 3 ml of phosphate buffer containing 0.1% 2-mercaptoethanol was added for each gram of hyphal material and homogenised in a blender. The homogenate centrifuged 6,000 g for 20 minutes and the supernatant collected. The supernatant was adjusted to 0.6M NaCl, 10% PEG (6,000 M.W) and stirred for 2.5 hours at 4°C. The precipitate was collected by centrifugation 6,000g for 20 minutes and resuspended in 1 ml per gram of buffer overnight. The resuspended pellet was subjected to two cycles of differential centrifugation, 6,000g for 20 minutes followed by 66,000g for 3.5 hours. The pellet was resuspended overnight in 0.5 ml of buffer.

2.5.2 Further purification

(i) $CsSO_4$ density gradient.

The resuspended pellet from section 2.5.1 was layered onto a 10-50% (w/v) $CsSO_4$ gradient. Gradients were centrifuged to equilibrium, 112,400g for 22-24 hours at 10°C and virus containing factions collected by piercing the base of the tube. Samples of each fraction were taken for particle (EM) and dsRNA analysis, the remainder dialysed overnight and concentrated by centrifugation at 66,000g for 3-5 hours. Pellets were resuspended in 0.5 ml of 0.5M Phosphate buffer pH7 and stored at 4°C.

(ii) Sepharose CL 2B Chromatography.

Further particle separation was attempted using Sepharose CL 2B chromatography. Samples were taken directly from $CsSO_4$ gradients and added to the pre-equilibrated/cooled column (0.05M phosphate buffer pH7) The void volume was eluted and fractions collected in a cooled fraction collector. Samples of each fraction were taken for dsRNA and particle (EM) analysis.

2.5.3 A rapid method for detecting particles in either crude or purified preparations

Composite electrophoresis using 2% polyacrylamide, 0.5% agarose gels (Makhouk et al.,1988) gels of partially purified (section 2.5.1.) or purified (section 2.5.2(i)) particle preparations, was carried out on a vertical 8 x 14 cm slab gel. Gels were prepared with 2% polyacrylamide (from an 18% stock solution section 2.2.2), 0.5% agarose in 0.65M Tris, 0.2M boric acid buffer pH 8.5. Electrophoresis was carried out overnight at 100V in 0.6g Tris, 2.88g glycerine per litre electrophoresis buffer.

Following electrophoresis, gels were stained with 0.1% Coomassie blue (Sigma) in a 12% trichloroacetic acid solution for 1-2 hours and then destained overnight with distilled water. A more sensitive staining method, silver staining (Sammons 1981) was used for particle levels below the detectability of the Coomassie stain (<30 ng.). Gels were initially fixed in 50% EtOH, 10% Hac overnight. Then washed twice in 25% EtOH, 10% Hac for 1 hour and twice in 10% ETOH, 0.5% HaC for 1 hour. Staining took place in $AgNO_3$ (1.9 g/l) for 2 hours followed by a 10 second rinse in double distilled water. The silver was reduced with

formaldehyde (7.5 ml/l) in 0.75N NaOH for 10 minutes. The gel can be enhanced if required in Na_2CO_3 (7.5 g/l) for 2-10 minutes and particle/protein bands visualised on a light box.

2.5.4. Electron Microscopy

(i) Crude preparations.

Samples from section 2.5.1 (100-200 µl) were placed onto formvar (Agar Scientific Ltd.) carbon coated 100 mesh grids and stained with phosphotungstic acid (PTA) pH 7. Grids were examined using a Joel 100 CX, electron microscope the bacilliform particle diameter 19 nm was used as a size marker.

(ii) Samples direct from $CsSO_4$ gradiets

Due to the high salt content of $CsSO_4$ preparations precipitating the PTA an adaption of the above method was used. The sample was dried down on to the grid and then washed 2-3 times with distilled water prior to staining with PTA. 0.01% bacitracin (Sigma) was sometimes used as a spreader.

2.6 DISEASE TRANSFER

2.6.1 Production of protoplasts

(i) Method 1

Adapted from the method of Munoz Rivas, et al., (1986). "Apparently healthy" mycelia from (1-3 grams) liquid culture were pelleted at 1,000g 10 minutes in a fixed angle rotor at 4°c. Samples were resuspended in 3 ml of filter-sterilized osmotic medium (OM) containing 10 mg/ml of Novozyme 234 and shaken gently for 2 hours at 30°C. Samples were taken at different times to check

for protoplast production. Protoplasts were mixed with 5 ml of CM and gently overlayed with 2 ml of SM and harvested by centrifuging 1,500g 20 minutes at 25°C in a swing out rotor. Protoplasts, which form a band at the interface of the two solutions (protoplasts float in CM but not in SM) were aspirated with SM and kept at RT while additional protoplasts were recovered by resuspending the remaining material.

Pooled protoplasts were mixed with an equal volume of SM, pelleted by centrifugation, resuspended in 4 ml of SM and adjusted to 10 mM $CaCl_2$. The number of protoplasts was determined using a haemocytometer and phenol red staining. Protoplast viability was assessed using fluorescein diacetate (FDA), FDA was made up in acetone at 5mg/ml and added to protoplasts to give a final concentraction of 0.01%. After a 30 minute incubation in the dark viable protoplasts were seen as fluorescent objects under the fluorescence microscope (Olympus BHS).

Protoplasts were plated out using the agar overlay technique. An equal volume of protoplast suspension was mixed with 0.4% agar (lab. No.2) in SM, 0.5 ml of this was poured onto each MA petri dish, sealed with parafilm and incubated at 23°C.

(ii) Method 2

Adapted from the method of Buxton et al., (1985), "apparently healthy" hyphae from liquid culture were homogenised in a sterile blender for 2 seconds and used to seed liquid cultures grown overnight at 23°C. Mycelia were harvested by filtering through a Whatman

0.45μm filter and washed with sterile water. The mycelia was added to 50 ml of filter sterilized 0.6M $MgSO_4$, 10 mM potassium phosphate pH 5.8 to which was added 20 mg of Novozyme 234 and 3mg BSA per gram of mycelia. Digestion was allowed to proceed at 30°C with gentle shaking for 4 hours. Large mycelial fragments were removed by filtration through nylon mesh and protoplasts harvested by centrifuging at 2,500g for 10 minutes.

Protoplasts were washed twice by resuspending in 10 ml 0.6 KCL pH 7.0 and centrifuging 2,500g for 10 minutes. Protoplasts were finally resuspended in 4 ml 1.2M Sorbitol 10 mM Tris-HcL, 10 mM $CaCl_2$ pH 7.5 and their viability assessed before plating out by the agar overlay method (method 1).

2.7 MITOCHONDRIA

2.7.1 Preparation of mitochondria from fungi

Adapted from Rogers et al (1987). Mycelia (10g) were mixed with 20 ml of 15% (w/v) sucrose in TE (100 mM Tris - Cl, 0.2mM EDTA) pH 7.5, 20g glass beads (Braun glasperlen 0.45 - 0.5 mm diameter) and shaken in a Braun MSK homogeniser at 0-5°C. The homogenate was mixed with an equal volume of 1% (w/v) sucrose in TE pH 7.5 and nuclei and cell wall debris (fraction i) pelleted by centrifugation at 1,300g for 10 minutes at 4°C. The supernatant was collected and centrifuged at 14,000g for 30 minutes at 4°C to produce a high speed supernatant (fraction ii) and a crude mitochondrial pellet which was resuspended in 4 ml of 20% (w/v) sucrose in TE pH 7.5. Intact mitochondria were purified using step gradients consisting of a bottom layer of 60% (w/v) sucrose in TE pH 7.5, a middle layer of 35%

(w/v) sucrose in TE pH 7.5 and a top layer the crude mitochondrial fraction in 20% (w/v) sucrose in TE pH 7.5. Gradients were centrifuged at 64,000g for 110 minutes in a swing out rotor at 4°C. Following centrifugation the gradient was divided into the 20% sucrose layer (fraction iii), the 35% sucrose layer (fraction iv) and the mitochondrial fraction which forms a dense white band at the 35-60% interface. The mitochondrial fraction was diluted slowly with 3 volumes of 10 mM Tris - Cl, 0.2 mM EDTA, pH 8.0 and centrifuged at 14,000g for 30 minutes at 4°C. The pellet was resuspended in 50 mM Tris - Cl, 5 mM EDTA, 60 mM NaCl, pH 8.0 (1ml) to give the purified mitochondria (fraction vi). Pellets which form at the bottom of the 60% layer were resuspended in 1 ml of TE pH 7.5 and designated fraction v). dsRNA was extracted from each fraction using method 2.2.1.

2.7.2 Succinate dehydrogenase (SOH) assay for mitochondria

The following reagents were added to 100 µl of each gradient fraction and changes in absorbance monitored at 620 nm, 500 ul 0.3M phosphate buffer pH 7.0, 50 µl 0.5M $MgSO_4$, 100 µl 0.1M KCN, 50 µl 5% (v/v) HCl, 20 µl 1.7 x 10^{-3}M 2.6 DCIP, 2 mls of H_2O and 200 µl 0.25 sodium succinate.

2.7.3 Preparation of mitochondria for E.M analysis

Mitochondrial preparations were fixed for 1 hour in 5% glutaraldehyde, spun down and resuspended in resuspending buffer. Pellets were removed and placed in a glass tube with 1% osmium tetroxide (Agar Scientific Ltd) and left for 1 hour. The Osmium was removed and samples washed with buffer, tannic acid was added and left for 1 hour. Acetone washes 15 mins each wash at 30, 50, 70 and 100% were carried

out twice at each concentration. The acetone was decanted and resin (Taab Laboratories Equip. Ltd.) added at a concentration of 1:1 and left rotating overnight. The resin was removed and more added in a 3:1 ratio and left rotating overnight. Specimens were placed in embedding moulds so that they were at the top of the block and left to set at 60^{o}C overnight.

2.7.4 Cutting sections for EM

(i) Preparation of grids

A thoroughly cleansed slide was placed in a separating funnel and 1% formvar in chloroform added to cover the slide. The 1% formvar/chloroform was slowly allowed to drain coating the slide with a thin layer of formvar. The formvar film was removed by gently prising it off in a beaker of distilled water, 200 square mesh grids were added to the film which was picked up on a piece of paper.

(ii) Cutting Sections

Fine sections were cut using a glass or diamond knife and picked up with the formar coated grids.

(iii) Staining

Grids were placed section side down on drops of uranyl acetate and left in the dark for 5 minutes. Excess stain was washed off by placing grids on five drops of water. The grid was then placed in a saturated 1N NaOH environment in a petri dish and a drop of 0.1% lead citrate added and left for 5 minutes. Excess stain was washed off and the specimen allowed to dry before viewing under the electron microscope.

3. LA FRANCE DISEASE, EFFECTS IN THE CROP AND IN CULTURE

3.1 Disease symptoms

La France diseased and apparently healthy sporophores (strain U3) were obtained from Country Kitchens (CK), Mushroom Farm, Langford, Avon. Diseased sporophores were taken from crops showing disease symptoms, which included long stalks in relation to cap size, drum stick appearance and extensive yield losses. Sporophores were also taken from crops where yields were down but without apparent symptoms. "Apparently healthy" sporophores were taken from high yielding trays showing no apparent disease symptoms. Examples of diseased and "apparently healthy sporophores used in the following experiments are shown in (Plate 3.1 & 3.2) and were cultured as indicated in Chapter 2.1.

On MA/YMDA diseased cultures A-I and H6F3 always showed growth rates lower than that of "apparently healthy" cultures (Table 3.1). Diseased cultures showed extensive browning on YMDA and generally formed "solid" colonies with extensive sectioning and few aerial hyphae (Plate 3.3). On YMDA "apparently healthy" cultures produced rapidly growing white aerial hyphae with little browning or sectoring (Plate 3.4). On MA the growth of diseased and apparently healthy isolates differed from YMDA. Diseased cultures did not show as much browning but formed slow growing appressed colonies with extensive sectoring and few aerial hyphae (Plate 3.5 (ii) (iii)). "Apparently healthy" isolates produced less aerial hyphae, but tended to show faster growth rates (Plate 3.5 (i)).

PLATE 3.1 LA FRANCE DISEASED SPOROPHORES (STRAIN U3)

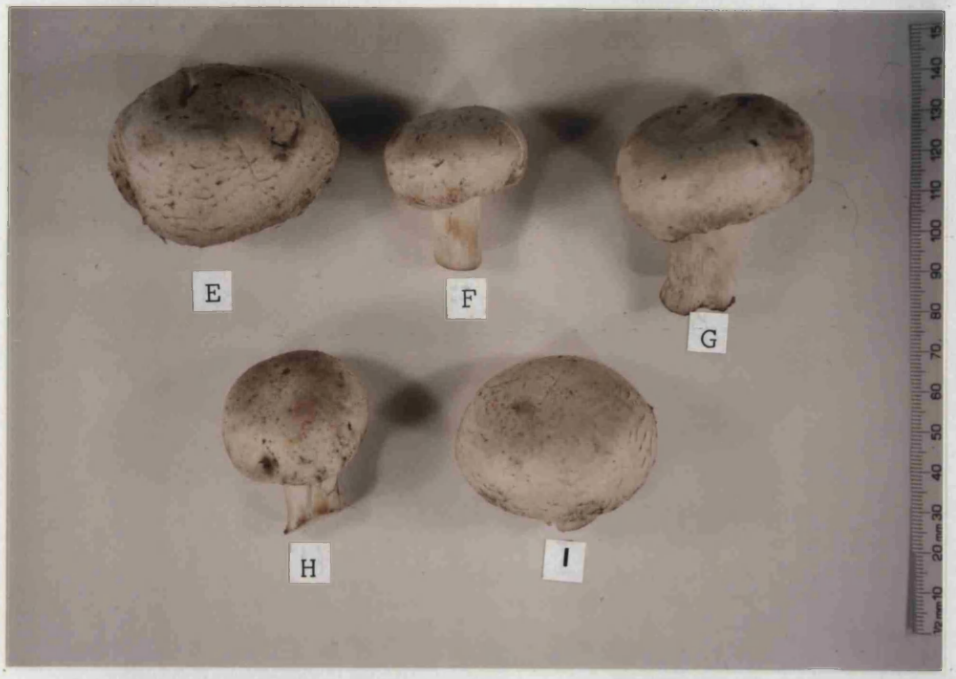
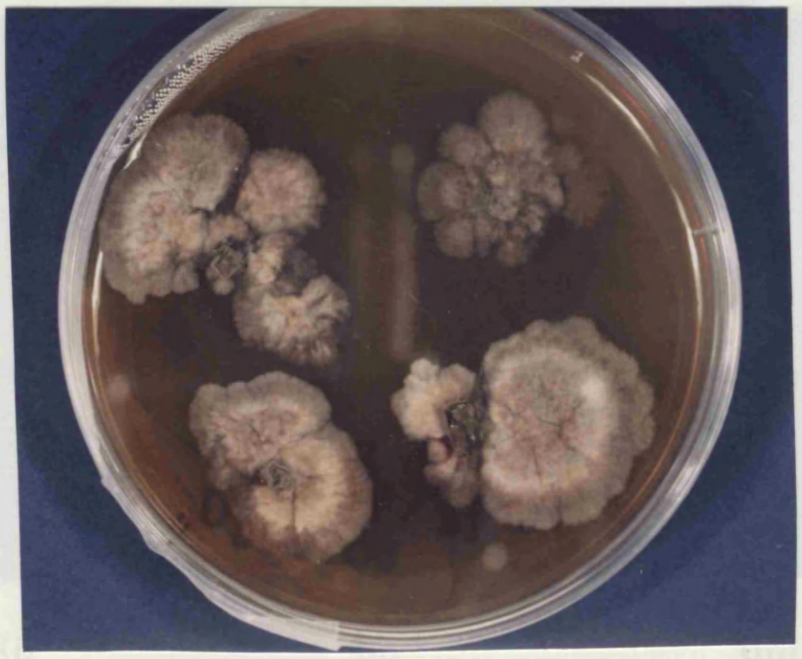


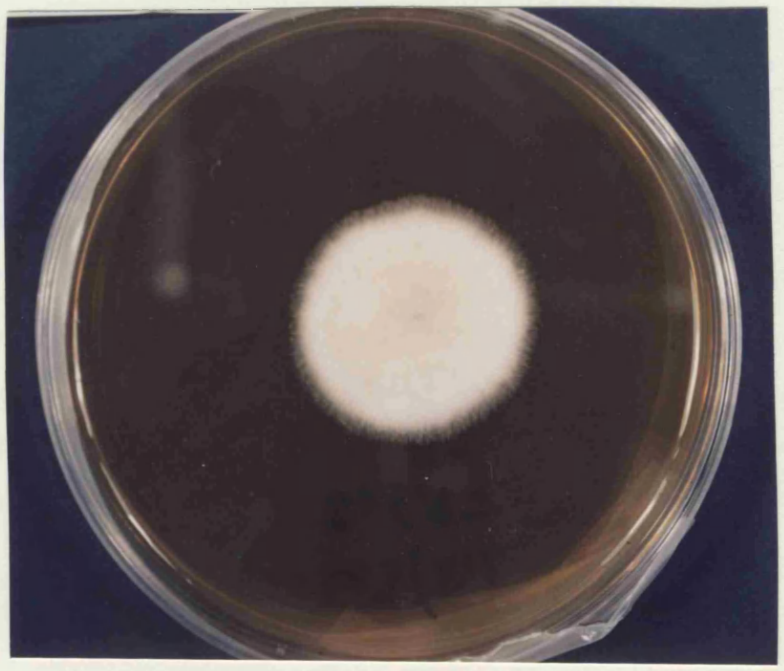
PLATE 3.2 "APPARENTLY HEALTHY" SPOROPHORES (STRAIN U3)

PLATE 3.3 DISEASED CULTURE F (STRAIN U3) ON YMDA



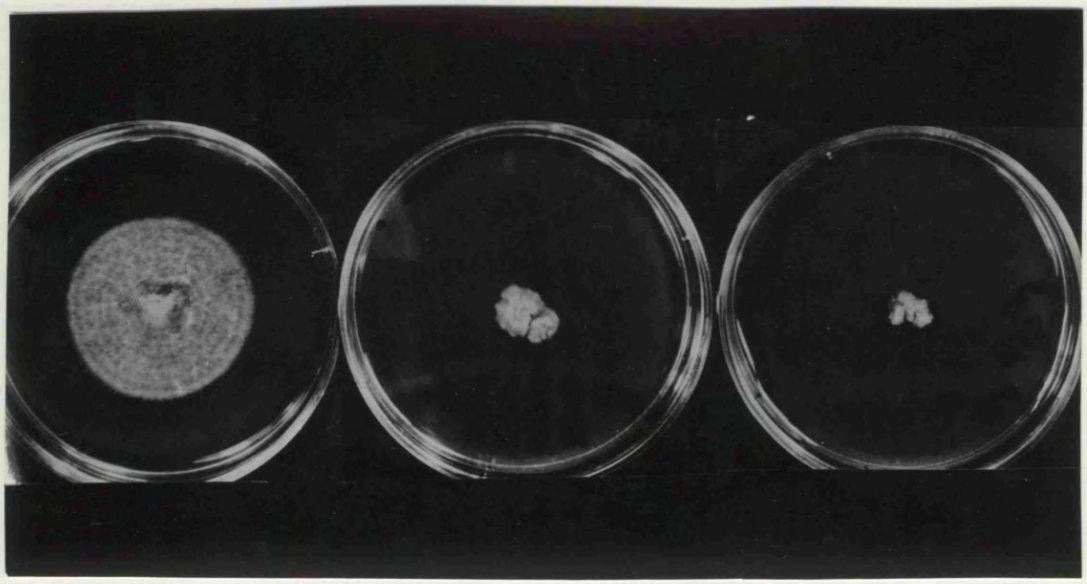
Six weeks growth

PLATE 3.4 "APPARENTLY HEALTHY" CULTURE (STRAIN U3) ON YMDA



Ten days growth

PLATE 3.5 MYCELIAL ISOLATES AFTER 3 WEEKS ON MA



(i) (ii) (iii)

(i) "Apparently healthy" (strain U3).
(ii) Diseased H6F3 " "
(iii) Diseased culture F " "

TABLE (3.1) INITIAL GROWTH RATES IN CULTURE

	Strain	Mean growth rates mm per day ± S.D	
		MA	YMDA
DISEASED CULTURES			
SD12	S22	/	0.29 ± 0.04
Culture F	U3	0.2 ± 0.04	0.30 ± 0.05
Culture H	U3	/	0.31 ± 0.03
Culture E	U3	/	0.70 ± 0.08
Culture D	U3	/	0.46 ± 0.04
H6F3	U3	0.26 ± 0.08	0.2 ± 0.04
INTERMEDIATE			
H9F2	D	0.59 ± 0.15	/
H5F3	U3	1.2 ± 0.08	/
"APPARENTLY HEALTHY"			
	U3	2.7 ± 0.54	2.0 ± 0.04

Severely diseased cultures from an S22 strain initially cultured by Hicks & Haughton (1986) and stored at 4°C were also investigated. On YMDA/MA S22 diseased isolates produced similar symptoms to diseased U3 strains but generally showed more aerial growth than diseased U3 isolates.

3.2 DsRNA segments /virus particles associated with diseased and "apparently healthy" sporophores and initial cultures.

3.2.1 DsRNA

Diseased sporophores A-I and H6F3 used in this study were initially tested by CK for the presence of dsRNA. Although crude (nucleic acid samples were not further purified following phenol extraction) a positive result was ascribed to sporophores containing 5 or more dsRNA segments (Page, 1987). Sporophores A-I and H6F3 all with a positive dsRNA rating from CK were also found in the present study to contain high levels of dsRNA (1-4 µg/g fresh wt).

DsRNA extracted (chapter 2.2.1.) from 5-10g of diseased sporophores A-I showed them all to contain the 9 dsRNA segments of the "standard", pattern (3.6, 3.0, 2.8, 2.7, 2.6, 1.5, 1.3, 0.8, and 0.78 kbp) plus several minor dsRNA segments, including a 4-5 kbp and 3-4 high molecular weight segments (sizes not accurately determined) (Plate 3.6). All segments were shown to be dsRNA using RNase high and low salt digestions (Chapter 2.2.3). A DNA segment at approximately 20 kbp was also seen following CF11 chromatography and this remained following RNase digestion.

DsRNA extracted from 10g of diseased sporophores H6F3 also contained the 9 dsRNA segments of the "standard" pattern (3.6 - 0.78 kbp). Minor dsRNA segments 4-5, 2.5, 2.4 kbp and high molecular weight dsRNA segments 15, 12, 11.2 and 8.8 kbp (Plate 3.7 (i)) were also detected. All segments were confirmed as dsRNA using RNase digestion (Chapter 2.2.3). A DNA segment approximately 20 kbp was again present following CF11 chromatography.

PLATE 3.6 POLYACRYLAMIDE TUBE GEL ELECTROPHORESIS OF dsRNA EXTRACTED FROM DISEASED SPOROPHORES A-I

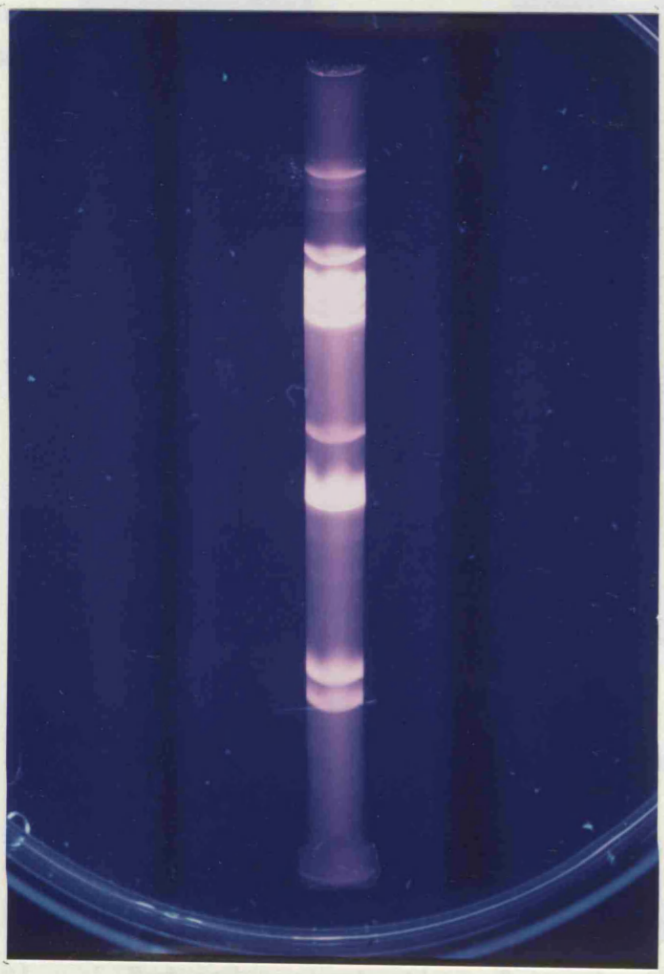
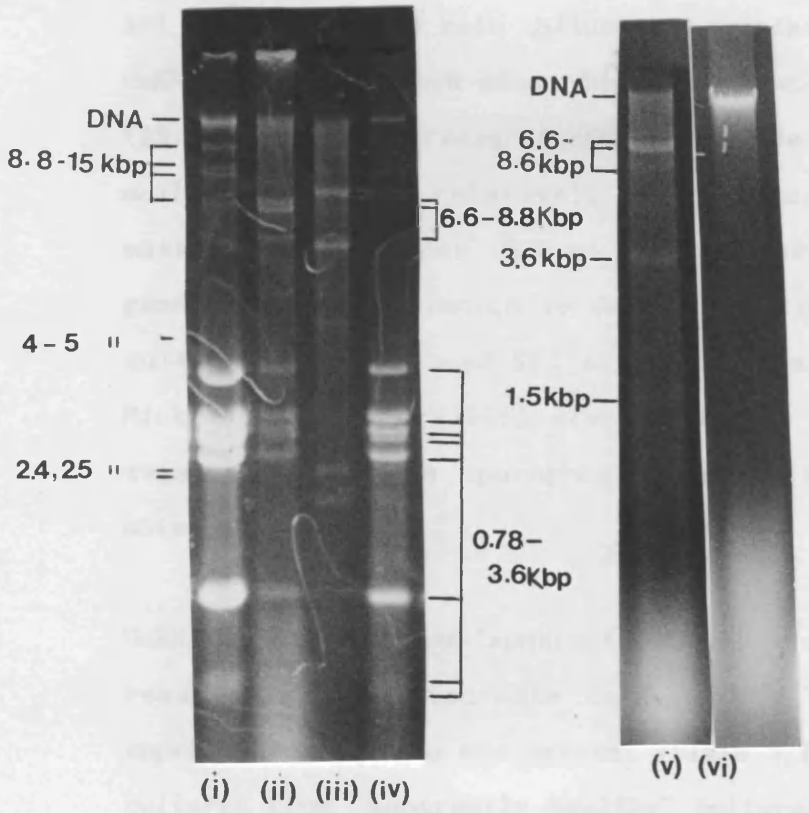


PLATE 3.7 AGAROSE GEL ELECTROPHORESIS OF dsRNA EXTRACTED FROM DISEASED AND HEALTHY SPOROPHORES AND CULTURES



(i) Diseased H6F3 sporophores ("standard" pattern plus 15-8.8 kbp dsRNAs)

(ii) A mycelial culture of H6F3 five months after culturing.

(iii) Lambda DNA Hind III digest (300ng).

(iv) Reduced concentration of sample (i).

(v) A mycelial culture of Culture F twelve months after culturing.

(vi) "Apparently healthy" sporophores (no dsRNA detected).

On initial culturing dsRNA extracted from 3-5g of cultures A-I and H6F3 showed both cultures to maintain the "standard" dsRNA segment pattern plus the high molecular weight bands (15 -8.8 kbp in the case of H6F3). Because liquid culturing methods produced relatively small amounts of diseased material 1 - 2g per 250 ml flask, dsRNA levels were generally not high enough to detect minor dsRNA segments in culture. The diseased S22 strain initially cultured by Hicks and Haughton (1986) also showed the "standard" dsRNA segment pattern in sporophores and initially cultured material.

DsRNA extraction from "apparently healthy" sporophores (30g) resulted in no detectable dsRNA but a DNA segment of approximately 20 kbp was present (Plate 3.7 (vi)). Initial cultures from "apparently healthy" cultures also showed no dsRNA segments to be present.

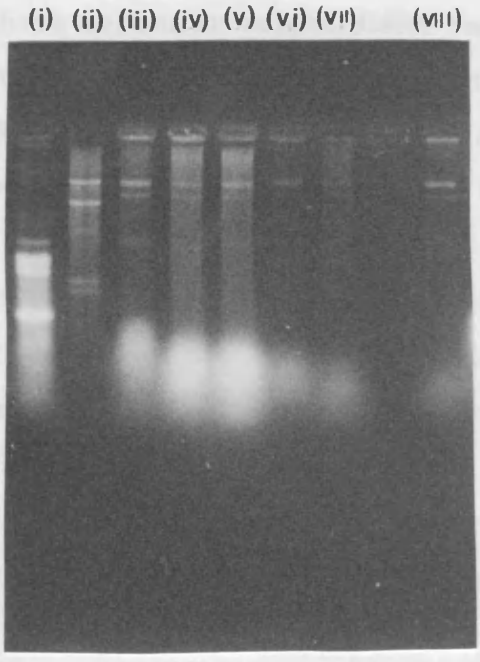
3.2.2 Virus-like particles (VLPs)

Crude particle preparations (Chapter 2.5.1) from 20 - 30g of diseased sporophores A-I and H6F3 indicated bacilliform particles and a range of spherical particles to be present. Accurate size determination was not attempted but at least 3-4 different types of spherical particles approximately 24, 29, 35, 44 nm in diameter were present. "Apparently healthy" sporophore material (30g) did not contain any VLPs. A crude virus preparation from 30g of culture D also yielded low levels of a number of spherical particles.

3.3 DsRNA segment changes in diseased and apparently healthy cultures

Early experiments were carried out on the diseased S22

PLATE 3.8 AGAROSE GEL ELECTROPHORESIS OF dsRNA EXTRACTED FROM SIX S22 DISEASED ISOLATES



(i) DsRNA segments of the "standard" pattern.

(ii) Lambda DNA Hind III digest (300ng).

(iii) S22 diseased isolate (A)

(iv) " " " (B)

(v) " " " (C)

(vi) " " " (D)

(vii) " " " (E)

(viii) " " " (F)

NB DsRNA extracted from 10-15g of diseased mycelium.

strain initially cultured by Hicks and Haughton (1986). Frozen mycelial samples harvested in 1985 (10-20g) yielded high levels of dsRNA segments of the "standard" pattern plus several high molecular weight dsRNA segments (sizes were not determined). However, dsRNA extracted from the current diseased S22 culture stock (>30g) did not contain the complete "standard" dsRNA pattern but contained dsRNA segments 8.6, 8.1 and 6.6 kbp and low concentrations of the "standard" pattern dsRNA segments 3.6 and 1.5 kbp (Plate 3.8). Extensive analysis over a 12 month period failed to show any change in the above dsRNA profile with no reappearance of the standard pattern.

Diseased cultures A-I, initially showed the "standard" dsRNA pattern plus several high molecular weight dsRNA segments (sizes were not determined). Culture F, a severely diseased isolate from this group was studied over an eight month period to compare growth rate changes with dsRNA content. Over an eight month culture period on YMDA, culture F lost the "standard" dsRNA pattern but retained high molecular segments 8.6, 8.1 and 6.6 kbp and also the 3.6 and 1.5 kbp dsRNA segments of the "standard" pattern (Plate 3.7(v)). Not only culture F but all diseased cultures of the A-X series showed these dsRNA segment changes, in all cases cultures maintained the disease phenotype and initial growth rates (Table 3.1).

Why these dsRNA segment re-arrangements occurred is unclear. It is possible that YMDA media which has a high osmotic potential, due to a high concentration of glucose may be putting further stress on diseased cultures already under severe stress from the disease . Later cultures isolated from diseased sporophores , including H6F3 , used MA with a far lower osmotic potential as a growing

source. Culture F was grown on MA for the next five months during this period growth rate and morphology remained relatively constant with no change in the 8.6, 8.1, 6.6, 3.6 and 1.5 kbp dsRNA profile.

H6F3 diseased sporophores containing the "standard" dsRNA pattern plus additional very high molecular weight segments (VHMS) approximately 15, 12, 11.2 and 8.8 kbp were cultured on MA. On initial culturing the standard dsRNA pattern and the 15, 12, 11.2 and 8.8 kbp segments were found routinely in 3-5g of diseased mycelia for the first 1-3 months (Plate 3.7(i)). Between 4-6 months a change in the dsRNA segments profile occurred resulting in the loss of the VHMS segments and the sudden appearance of the 8.6, 8.1 and 6.6 kbp dsRNA segments seen in previous diseased cultures, the "standard" dsRNA segments remained (Plate 3.7(ii)).

Growth rates of H6F3 cultures over a 17 month culture period remained relatively constant. The "standard" dsRNA pattern and 8.6, 8.1 and 6.6 kbp segments also remained at approximately the same concentration throughout the culture period. However, after 17 months without a significant change in growth rate, a similar change occurred to the dsRNA segments profiles as had occurred in the earlier diseased cultures. The 8.6, 8.1 and 6.6 kbp segments remained but the "standard" pattern was absent apart from the 3.6 and 1.5 kbp dsRNA segments at low concentrations.

"Apparently healthy" sporophores were shown to contain no detectable dsRNA or virus particles. When the dsRNA segment changes previously described in culture F occurred "apparently healthy" cultures were transferred from YMDA to MA, and growth rates increased from 2.0 mm per day to

between 2.7, 2.9 mm per day. Over the nine months following transfer to MA, dsRNA extraction was attempted routinely from between 5-10g of "apparently healthy" mycelia. On a few occasions dsRNA was detected in low concentrations; segments found correlated with the 8.8, 8.1 and 6.6 kbp dsRNAs isolated in diseased cultures. Towards the end of the nine month period the proportion of healthy isolates containing low levels of dsRNA segments had risen to 60-70% of all isolates tested. The presence of these high molecular weight segments appeared to have no effect on the growth rates of "apparently healthy" cultures.

3.4 Moderately diseased sporophores and cultures

Other sporophores examined did not fit into the "apparently healthy" or diseased category. A D-strain (virus breaker strain) H9F2 showed slight decreases in yield in the crop but was diagnosed as dsRNA negative by CK. Following dsRNA extraction from 10-20g sporophore material several dsRNA segments were identified, four high molecular weight dsRNA s 15. 12., 11.2 and 8.8 kbp plus 4.0, 2.4 and 2.0 kbp dsRNA segments. Particle preparations yielded solely bacilliform particles and contained only one dsRNA segment, approximately 2.0 kbp. On MA, culture H9F2 gave an initial mean growth rate, 0.59 mm per day and a morphology that would indicate a diseased culture. As "apparently healthy" D strain isolates not containing dsRNA were not available it was difficult to be positive that H9F2 was in fact a diseased culture. Growth rates of cultures analysed 2 months later showed a slight increase in growth rate to 0.8 mm per day. In 5g of cultured mycelia the 4.0, 2.4 and 2.0 kbp dsRNA segments were no longer present and only the 15, 12, 11.2 and 8.8 kbp dsRNA segments were detected (Plate 7.2 (15 & 17)).

Moderately diseased H5F3, U3 strain sporophores were also studied. Sporophores from two first flush crops designated positive and negative for dsRNA by CK, were both designated negative by CK on examination of second flush material. Studies in this laboratory have demonstrated that both second flush samples contain dsRNA segments, 3.5 and 2.0 kbp in length. Insufficient sporophore material was available for particle content to be assessed. Mean growth rates of the two cultures on MA were 1.1 and 1.2 mm per day and culture morphology was intermediate between diseased and "apparently healthy" material (Table 3.1).

3.5 Virus-like particle characterisation

Large scale particle purification was attempted on 300-600g of frozen infected U3 sporophores from CK, using methods described in Chapter 3.5.1 and 3.5.2. Experiments were designed so that initial crude preparations would be used as an indication of the types and relative amounts of virus particles present. Further purification using $CsSO_4$ gradients would enable separation into individual particles for dsRNA assessement.

3.5.1 Crude preparations

Crude preparations were found to contain large amounts of host debris, making determination of the relative amounts of particular particle types prior to $CsSO_4$ density gradients impossible. A 10 minute spin, on the final high speed pellet (10,000g at $4^{o}C$) was given to remove excess host debris, prior to loading on to $CsSO_4$ gradients.

3.5.2 $CsSO_4$ density gradients

Determination of particle size.

Following $CsSO_4$ centrifugation (Chapter 2.5.2) six distinct bands were seen in the gradient (Plate 3.9), bands 2 and 3 were shown to contain virus particles which were associated with 3.6, 3.0, 2.7, 2.6 and 1.3 kbp dsRNA segments. Although present in the crude particle preparation (Plate 3.10 (i & ii)) 1.5, 0.8 and 0.73 kbp dsRNA segments did not appear to be associated with particles in the gradient fractions (Plate 3.10 (18-27)). Composite gel electrophoresis (Chapter 2.5.3) of bands 1-6 showed bands 2 and 3 to differ in protein composition from all other fractions (Plate 3.11). Also protein profiles produced from fractions 2 and 3 resembled those found in crude diseased but not healthy particle preparations.

Particle sizes were determined from electron micrographs (Plate 3.12) using the bacilliform particle of mean width 19 $\pm$ 0.76nm, as a size marker (Hollings (1962); Van-Zaayan (1967); (1968); Nair (1969); (1972); Lapiere (1971); Molin and Lapiere (1973); Mayer (1976)) Approximately 1,000 particles were measured on 13 electron-micrographs spread throughout the virus containing region. Five spherical particles 24 nm, 27 nm, 32 nm, 38 nm and 44 nm in diameter were detected plus a bacilliform particle 50 X 19 nm (Plate 3.12, Table 3.2). It was not possible to compare relative amounts of particular particles from crude preparations, but particles found in $CsSO_4$ gradients were in the following proportions: 38-44 nm - 37%; 27 nm - 9%; 50 X 19 nm - 36%; 32 nm - 18%; and 24 nm - 0.5%. (See appendix). A 17 nm spherical particle was also found at very high concentrations in the gradient (Plate 3.12). Because of doubts as to the origin of the 17nm particles they were

PLATE 3.9 BANDS PRODUCED FOLLOWING THE SEPARATION OF A CRUDE VLP PREPARATION ON A $CsSO_4$ DENSITY GRADIENT

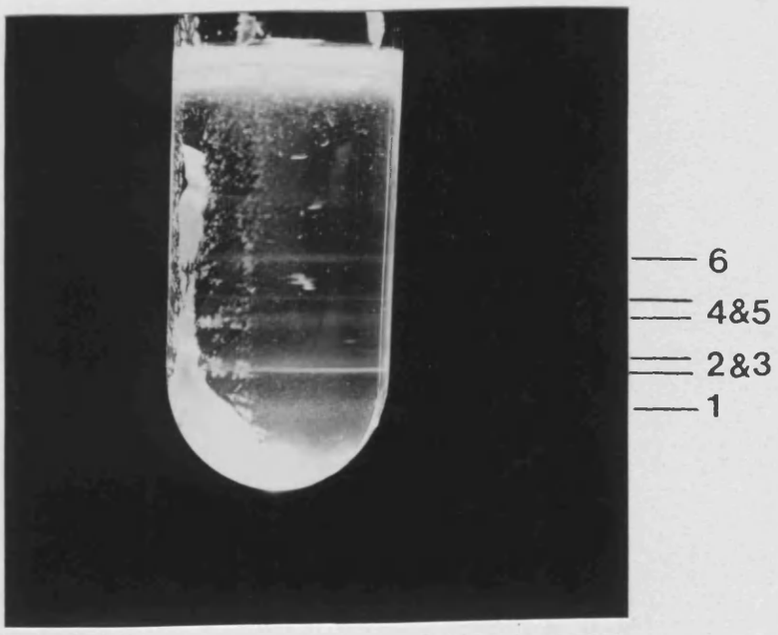
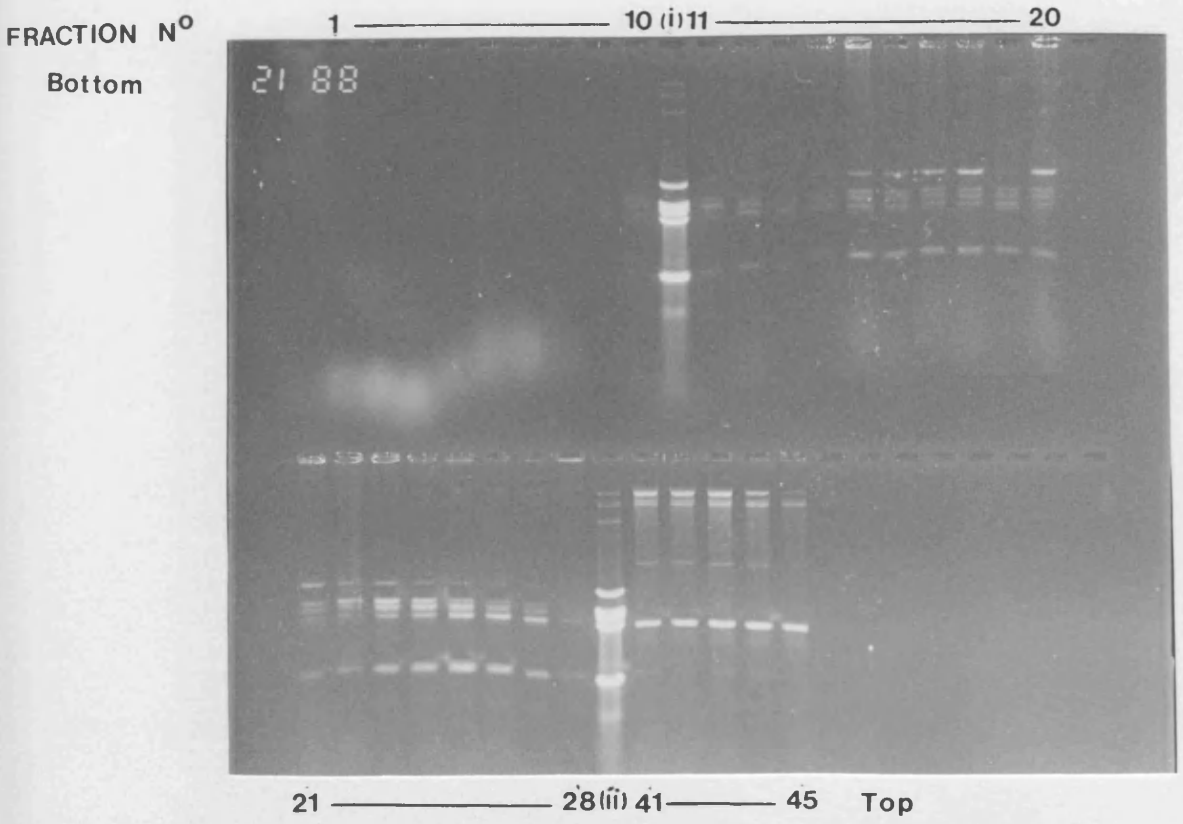


PLATE 3.10 DsRNA ASSOCIATED WITH FRACTIONS OBTAINED FROM A $CsSO_4$ DENSITY GRADIENT OF A CRUDE VLP PREPARATION



NB Fractions 29-40 (not shown) contained no detectable dsRNAsegments. Fractions (i) and (ii) contained dsRNA extracted from the crude preparation prior to loading onto the gradient.

PLATE 3.11 NON-DENATURING ELECTROPHORESIS OF PROTEIN BANDS 1-6 FROM THE $CsSO_4$ GRADIENT (PLATE 3.9) ON A COMPOSITE AGAROSE POLYACRYLAMIDE GEL

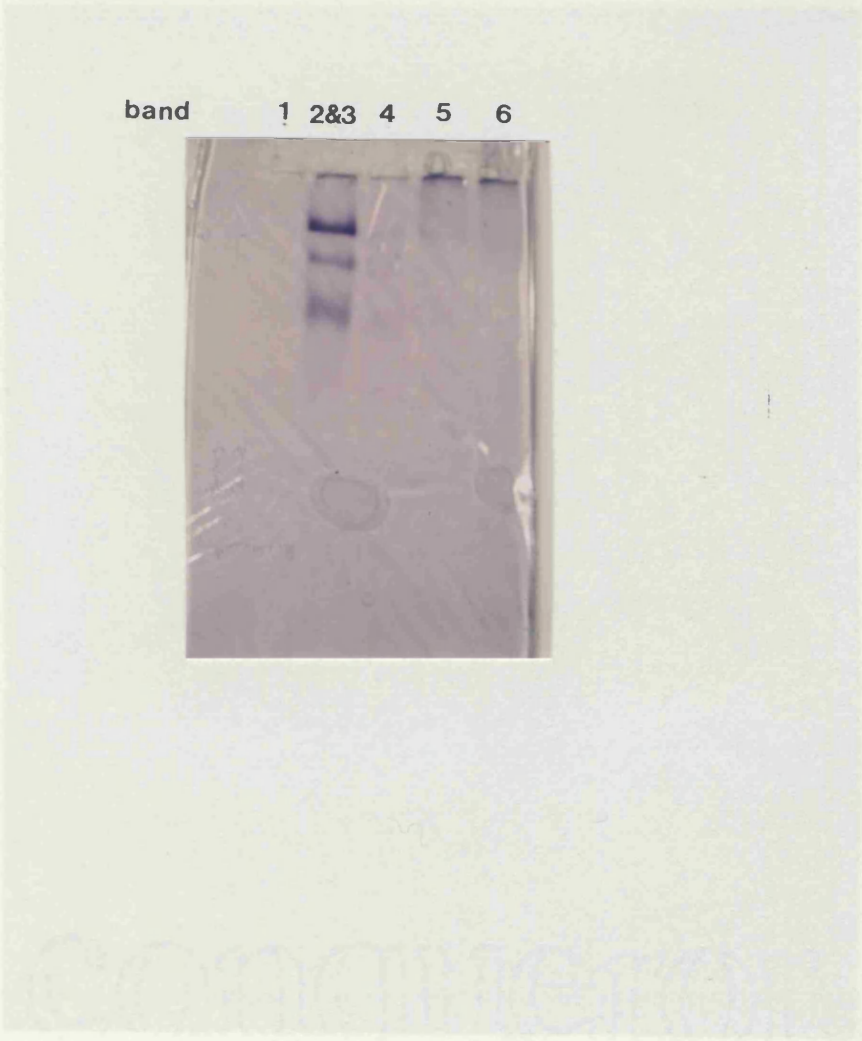
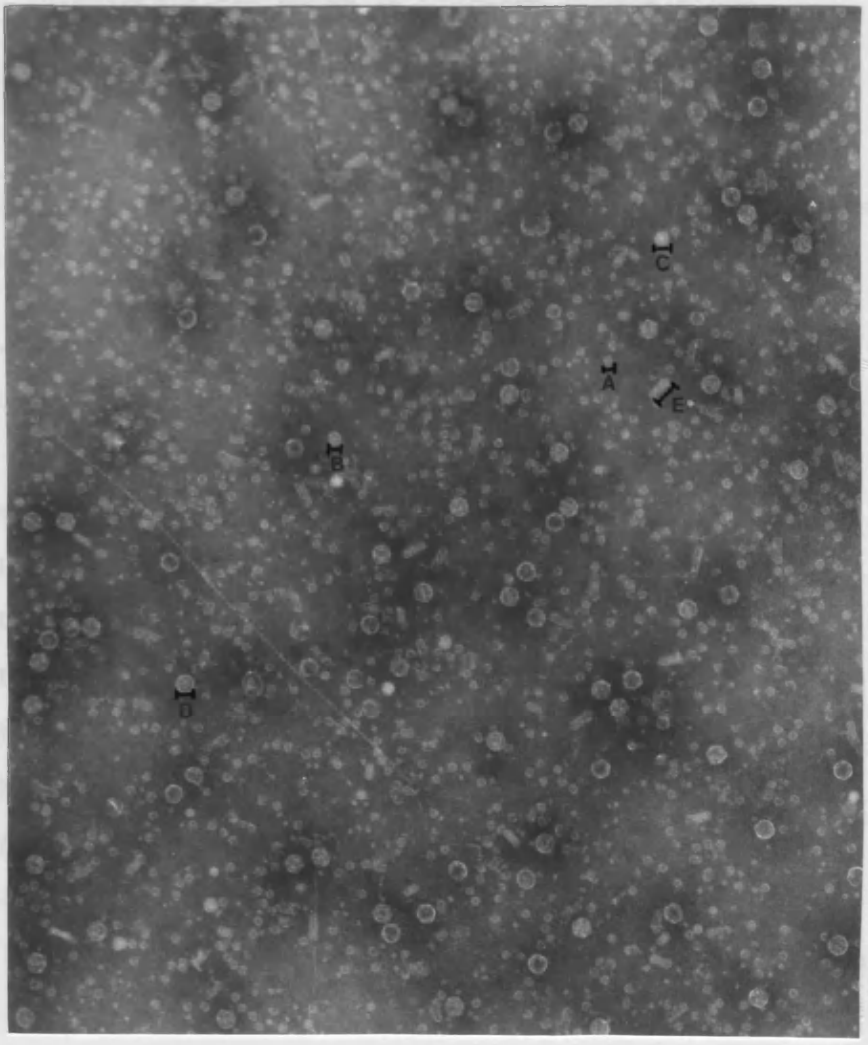


PLATE 3.12 VLPs DETECTED IN PARTICLE FRACTIONS OF $CsSO_4$ DENSITY GRADIENTS



SCALE 0.5 cm = 95nm

SPHERICAL PARTICLES

A = 17nm
B = 24nm
C = 27nm
D = 38-44nm

BACILLIFORM PARTICLE

E = 19-50nm

ommitted from percentage particle calculations.

TABLE 3.2 PARTICLE SIZES FOUND IN DISEASED PREPARATIONS AND COMPARISON WITH VALUES IN THE LITERATURE.

Particle sizes mean diameters and s.d.	Comparable sizes in literature	References
19 ± 0.76 nm (Width of Bacilliform)	19-50 nm	Van-Zaayan (1968)
Spherical particles		
17 ± 1.01 nm *	19 nm	Hollings (1962)
24 ± 1.2 nm	25 nm	Hager (1969)
27 ± 1.2 nm	28 nm	Van-Zaayan (1968)
32 ± 1.14 nm	34-35 nm	
38 ± 2.4 nm 44 ± 2.2 nm	40 nm	Luisoni (1972)

* A number of workers have found 19 nm spherical particles (Passmore & Frost 1979, Hollings & Stone 1979) but controversy exists over their origin.

3.5.3 ATTEMPTED CHARACTERISATION OF VIRUS PARTICLES AND THE dsRNA SEGMENTS THEY CONTAIN

Once the region at which particles banded had been determined by light scattering gradients were separated into 200 ul fractions, 25 ul of each fraction was taken for dsRNA extraction (Chapter 2.2.1, CF11 chromatography was not used). Following electrophoresis (Chapter 2.2.2 (ii)) it was clear that particles had been separated to give a number of fractions containing different and variable concentrations of dsRNA (Plate 3.10). Electron microscopic

analysis of fractions 18, 23, 24, 25 and 26 also revealed differences in particle content, (Tables 3.3 & 3.4)

TABLE 3.3 NUMBERS OF VIRUS PARTICLES PRESENT IN DIFFERENT GRADIENT FRACTIONS

Fraction	Particle Type					
	Spherical					Bacilliform
	44 nm	38 nm	32 nm	27 nm	24 nm	19-50 nm
18	/	/	/	/	4	/
23	5	23	2	3	/	34
	/	25	4	/	/	31
	42	/	4	3	/	41
	/	45	3	/	/	45
24		/	/	10	/	15
	/	/	/	19	/	7
	25	1	/	8	/	37
	22	2	/	9	/	28
25	/	/	94	5	4	36
	/	/	96	/	5	51
26	/	44	/	4	/	25
	/	51	2	3	/	5
	/	47	2	4	/	5
		63	/	9	/	10
TOTAL	95	301	207	77	13	370

The percentages of particular particles in each fraction are given in Table 3.4. As 44 and 38 nm particles were difficult to distinguish on electron micrographs and appear to contain the same dsRNA segments (see later) they were grouped together as one particle size 44-38 nm.

TABLE 3.4 PERCENTAGES OF PARTICLES IN FRACTIONS 18, 23, 24, 25, 26.

Fraction No.	Spherical particles				
	44-38 nm	32 nm	27 nm	24 nm	Bacilliform
18	0	0	0	100%	0
23	45%	4%	2%	0%	49%
24	27%	0	25%	0	48%
25	0%	65%	2%	3%	30%
26	74%	3%	7%	0	16%

4.6 DISCUSSION

Current understanding of the causes of La France disease in A.bisporus are unclear. Early work (Hollings, 1962; 1963; Last et al., 1967) implicated virus particles and the dsRNA they contain as the causal agents of this cytoplasmically transmissible disease. Doubts as to whether the mere presence of VLPs/dsRNA was sufficient to cause the disease came into question following the work of Nair (1972); Passmore & Frost (1979); Deahl et al., (1984); Harmsen et al., (1989); and Romaine et al., (1989). It is now fairly well established that the mere presence of dsRNA or VLPs in sporophores or cultures is insufficient to produce disease symptoms. Recent work by Hicks & Haughton (1986), Wach et al., (1987); Harmsen et al., (1989) and Romaine et al., (1989) has shown that a "standard" dsRNA segment pattern consisting of 9-10 segments (3.6-0.7 kbp) is strongly correlated with disease symptoms. However, Deahl

et al., (1987) and Harmsen et al., (1989) found the "standard" dsRNA pattern in asymptomatic mushrooms indicating other factors including host susceptability may also be required for the expression of disease symptoms.

La France affected sporophores with severe disease symptoms used here were clearly distinguished from "apparently healthy" sporophores with regard to morphology and growth rate on YMDA and MA. However, several diseased isolates, H9F2 (strain d) and H5F3 (strain U3) although producing the browning and sectoring associated with the disease grew at rates between those of severely diseased and "apparently healthy" isolates. These intermediate diseased isolates did not contain the full nine "standard" dsRNA segments, but in both cases contained dsRNA segments which were part of the "standard" pattern. H9F2 sporophores contained four high molecular weight dsRNAs 15, 12, 11.2 and 8.8 kbp plus 4.0, 2.4 and 2.0 kbp segments. Only the high molecular weight segments were detected in subsequent H9F3 cultured material. H5F3 sporophores were investigated due to their changing dsRNA status when examined by CK. First flush samples were designated positive La France disease (at least 5 dsRNAs) and second flush samples negative for dsRNA. Second flush sporophore samples examined in this study were shown to contain a 3.5 and 2.0 kbp dsRNA and have a slightly lower growth rate on MA than the "apparently healthy" U3 strain. In the crop such growth rate differences may not be apparent with morphology and yields probably being little different from "apparently healthy" crops.

The presence of La France diseased isolates with slightly reduced growth rates in culture and several dsRNA segments could be considered evidence for the existence of an

intermediate state of La France disease. Alternatively, such cultures could be considered as recovering from the effects of La France disease on their way to "apparently healthy" growth. Once in culture, H9F2 and H5F3 did not go on to develop full La France disease symptoms. In fact they appeared to become healthier with culturing showing increased growth rates and loss of dsRNA segment. Harmsen et al., (1989) and Romaine et al., (1989) found "apparently healthy" sporophores with no apparent symptoms to contain a 2.35-2.4 kbp dsRNA and a combination of dsRNA segments from the standard pattern. Furthermore, the results of the same authors (Harmsen et al., 1989; Romaine et al., 1989) suggest that "apparently healthy" crops commonly contain dsRNA.

"Apparently healthy" U3 sporophores used in these experiments were initially shown not to contain any detectable dsRNA segments . However, during two years culture on YMDA and MA, low concentration (< 5ng) of high molecular weight dsRNA segments 8.8, 8.1 and 6.0 kbp were occasionally detected. No effect was observed on culture morphology and towards the end of the 2 year period the occurrence of these segments was becoming more frequent. Failure to detect these dsRNAs in some healthy isolates may be due to their low concentration. However, this would not explain the presence of dsRNA in one "apparently healthy" culture but not another when extracted under identical conditions.

All severely diseased sporophores and initial cultures A-I and H6F3 contained the "standard" dsRNA segments 3.6 - 0.78 kbp plus high molecular weight dsRNA segments 15, 12, 11.2 and 8.0 kbp. As with the "apparently healthy" isolate previously described culturing appeared to induce changes in

dsRNA segment patterns, in this case with no obvious effect on disease symptoms. All La France diseased cultures eventually showed a loss of the majority of standard dsRNA segments, only maintaining the 3.6 and 1.5 kbp segments at very low concentrations. Changes were also observed in high molecular weight dsRNA segments 15, 12, 11.2 and 8.0 kbp, which after a 2-6 month culture period were replaced by dsRNA segments 8.8, 8.1 and 6.0 kbp. The type of media used to grow diseased cultures appeared to affect the rate at which segment changes occurred. A rich glucose media such as YMDA effecting more rapid changes in dsRNA segments profiles than MA.

Previous studies concerning changes in the types of VLPs detected in La France diseased isolates between 1960-1990, have indicated possible particle variation to occur (van-Zaayen 1968, Hollings & Stone, 1971; Barton & Hollings, 1979; Wach et al., 1983; and Koons et al., 1983). Also Wach et al., (1987) on several occasions found diseased sporophores where dsRNA segments had been lost from the "standard pattern", but sporophores still appear diseased.

Variation in dsRNA segments in fungi is a common phenomenon and is found, for example, in E.parasitica (Van-Alfen, 1986), O.ulmi (Rogers et al., 1987), G.graminis (Buck et al., 1981), U.maydis (Koltin et al., 1986), Colletotrichum (Dale et al., 1988) and Pyricularia oryzae (Hurst et al., 1986). Segment variation has generally been restricted to different isolates but in a number of cases variable segment patterns have been observed within a single isolate. In U.maydis P1, P2, P3-P4 and P6 isolates showed dsRNA segment changes which resulted in differences in their interactions (Koltin et al., 1986). Strains were shown to

have lost or gained dsRNA segments previously undetectable in that particlar strain. A P2 strain of U.maydis devoid of cytoplasmic elements for both the production of the inhibitor and resistance functions (Day et al., 1973) was later found by Koltin and Day (1976) to contain virions and dsRNA.

In G.graminis dsRNA segment patterns vary greatly between isolates (Buck et al., 1981). Lapiere et al (1973) reported that an ascospore isolate derived from a virus-infected Ggt isolate 911 was virus free. However, after several years storage viruses re-appeared in this isolate (Ferault et al., 1979). McFadden et al., (1983) observed a similar phenomenon in G.graminis but this time although particle levels had decreased they were detectable by ISEM.

DsRNA viruses in animal systems also show similar dsRNA segment variation, in the cytoplasmic polyhedrosis virus (CPV) changes appear to be induced by laboratory passage (Rubinstein et al., 1978). These segment changes were reproducible, as appeared to be the case in La France disease, with no apparent loss in infectivity after two passages through laboratory-reared larvae of Heliothus armigera. Variant dsRNAs are often associated with the genome of transmission defective isolates of wound tumour viruses (WTV) (Ahmed & Fields, 1981; Brown et al., 1983). Direct physical evidence that the variant RNAs are remnants of particular WTV genome segments was provided by molecular hybridisation studies (Nunn & Summers, 1984). Similar defective elements were also found in S.cerevisiae viruses and have been shown to be derived from viral genome segments by internal deletion events (Bruenn & Brenann, 1980).

It is possible that the lower molecular weight dsRNA segments seen in La France disease isolates could have been derived from larger segments by internal deletion events. This is currently the case in E.parasitica where smaller dsRNA segments are believed to be derived from a large 9 kbp dsRNA segment by internal deletions (Hiremath et al., 1986; Rae et al., 1989). Internal deletion may explain the origin of particular segments but it does not explain the dsRNA segment changes induced during culturing. One explanation for the sudden loss or gain of dsRNA segments was proposed by Ben-Zvi et al., (1984) and Holland (1982). They proposed that not all dsRNA segments were maintained in a steady state with respect to their molar ratios and that some dynamic drift may occur, perhaps as a result of simple genetic alterations. Thus implying that under certain selection pressures dsRNAs are at a base rate below the level of detection but are capable of being induced by an external stimulus.

In the case of the dsRNA segments seen in La France diseased and "apparently healthy" isolates, culturing may provide the stimulus for such a genetic change. A.bisporus is well known for its susceptibility to changes in growing conditions, if conditions are not precisely controlled low yielding poor quality mushrooms are produced (Jodan et al., 1981; Sinden et al., 1962). The induced stress of culturing on the host may induce changes in host physiology which indirectly affect the dsRNA segment profiles.

Sizes of virus particles isolated from La France diseased sporophores compared well with previously published values. Caesium sulphate gradients indicated the 32 nm (34-35*) and 38-44 (40 nm*) spherical and 19-50 nm bacilliform particles

to be the most abundant particles in diseased crops. Spherical particles 17 nm in diameter were also found in high concentration, but their exact nature has yet to be determined. Recent work Passmore & Frost, 1979; Wach & Romaine, 1983 and Koons et al., 1983 showed similar types of particles to be present. It must be noted that relative particle concentrations determined using $CSSO_4$ gradients may not be a true reflection of particles found in the crop; some particles being more sensitive to the chaotropic effects of $CsSO_4$ than others. Virus particles were never detected in "apparently healthy" sporophores using either electron microscopy or composite gel electrophoresis.

Attempts at characterising the dsRNA segments contained within particles encountered similar problems to those in previous studies (Barton & Hollings, 1979; Romaine et al., 1986). Different types of very similar-sized particles did not allow adequate separation by density gradients or column chromatography. However, it was clear from density gradient centrifugation that the 3.6, 3.0, 2.8, 2.7, 2.6 and 1.3 kbp dsRNA segments were encapsidated within virus particles. High molecular weight dsRNA segments 15, 12, 11.2 and 8.0 and a 4.0 kbp segment appeared not to be encapsidated within virus particles.

Electrophoresis of dsRNA extracted from $CsSO_4$ gradient fractions indicated that particles may have been separated (Plate 3.10). However, electron microscopic examination of fractions indicated no clear particle separation. From the data outlined in Tables 3.3 & 3.4 and Plate 3.10 using the relative abundance of virus particles in particular fractions it is possible to make a tentative association between virus particles and dsRNA segments. Fraction 18

appeared to contain only 24 nm particles which correlated with the presence of the 3.6 kbp dsRNA segment. In fraction 18 the 3.6 kbp segment was at a higher concentration than any other fraction. Levels of 24 nm particles in the gradient were very low and it is possible that these particles were disrupted during the purification procedure.

Fraction 23 contained high levels of 38-44 nm particles, with few other particles present except bacilliform particles which have an ssRNA genome of 4.0 kbp (Tavantiz & Smith, 1980). In fraction 23, 3.0, 2.8 and 2.7 kbp dsRNAs appear at a maximum (Plate 3.10). Levels of 38-44 nm particles were also high in fraction 26, however, in this fraction the 38-44 nm particles were probably empty as 3.0, 2.8 and 2.7 kbp dsRNA segment levels were low. As levels of 38-44 nm particles drop in fractions 24 and 25 levels of 3.0, 2.8 and 2.7 kbp dsRNAs also decrease. Fraction 24 contained high levels of 27 nm particles which appeared to correlate with an increase in the concentration of the 1.3 kbp dsRNA segment. However, in fraction 25 the 1.3 kbp dsRNA appears to have reached its optimum with 27 nm particles at a very low level. Fraction 25 does contain high levels of 32 nm particles which appear to correlate with the 2.6 kbp and 1.3 kbp dsRNA segments reaching their optimum. The above situation in fraction 24 and 25 could be explained if two different 1.3 kbp dsRNA segments were associated with virus particles, one with the 27 nm and the other the 32 nm particle. Proposed particle/dsRNA associations are summarised in Table 3.5. Spherical virus particle associations with dsRNA segments (Table 3.5) do not compare well with literature values currently available. Technical problems in sizing and separating virus particles are probably responsible for these differences although the

presence of different strains cannot be ruled out. It is also possible that random packaging of viral dsRNA could occur. From $CsSO_4$ gradients encapsidated dsRNA segments are always found in order of size (Plate 3.10). It is possible that not enough electronmicrographs were taken of each sample to give a true reflection of particles present.

Levels of bacilliform particles within fractions 23-26 remained relatively high, although electrophoresis of gradient fractions failed to associate any genomic RNA segment with these particles. It has been suggested that the 19 nm spherical particle found in abundance in La France disease particle preparations may represent capping regions of bacilliform particles (Hollings & Stone, 1971). Similar sized particles, 17 nm were found in great abundance throughout the gradient and could not be associated with any nucleic acid segment. Suggesting that the 17 nm spherical particles seen in the gradient either represent empty capsids or are perhaps capping regions of the bacilliform. In other particle preparations, including those from H9F3 sporophores purified without $CsSO_4$ density gradients, bacilliform particles detected were associated with a 2-2.5 kbp RNA segment. It is likely that the $CsSO_4$ gradient causes the disruption of bacilliform particles; if this is correct then genome fragments of the bacilliform should be found unencapsidated at the top of the gradient. An unusually high concentration of a 2.6 kbp segment was found at the top of the gradient (Plate 3.10) and found to be susceptible to "high salt" RNase A digestion. It is possible that this ssRNA fragment approximately 4-5 kb could represent the genomic fragment of the apparently empty bacilliform particles.

TABLE 3.5 PROPOSED RELATIONSHIP BETWEEN dsRNA SEGMENTS AND VLPs.

Particle Size	dsRNA Segments	Literature values
17 nm (19* nm)	/	
24 nm (25* nm)	3.6 Kbp	2.1 Kbp (Barton & Hollings, 1979)
38-44 nm (40* nm)	3.0, 2.8 & 2.7 Kbp	/
27 nm (28* nm)	1.3 Kbp	/
32 nm (35* nm)	2.6 & 1.3 Kbp	6.3 & 2.1 (Romaine et al 1987). 2.27 & 2.1 (Barton & Hollings, 1979)

* Comparable literature values.

4. MITOCHONDRIA AND LA FRANCE DISEASE

4.1 INTRODUCTION

Eukaryotic mitochondria are the major sites,in the cell where high energy substrates from glycolysis, fatty acid oxidation and the citric acid cycle are coupled in a process known as oxidative phosphorylation to produce the utilisable energy source ATP. The major ATP "energy yielding step" being electron flow through a series of enzyme complexes in the mitochondrial membranes.

A number of fungal conditions are associated with changes in levels of components of electron transfer chains. Phenotypic changes seen in these fungi sometimes mimic the effects attributed to VLPs/dsRNA in other fungi, such as Agaricus bisporus, Rhizoctonia solani, Helminthosporium victoriae and Ophiostoma ulmi. Examples include poky mutants of Neurospora crassa, containing a mitochondrial mutation which results in reduced levels of cytochrome aa_3, an excess of cytochrome c plus other deficiences in the mitochondrial protein synthesising equipment (Collins et al 1981, Atkins et al 1984). Stopper mutants of N. crassa exhibit a stop start mode of growth similar to the sectoring seen in "La France" diseased A. bisporus and d-factor containing O. Ulmi (Brasier et al, 1987). Abnormalities were reported in these fungi in the electron transfer enzymes with deficiencies of cyt. b and cyt. aa_3 and an excess of cyt. c. The condition is passed on in a non-Mendelian fashion, alterations, deletions and amplifications in mtDNA are believed to be responsible (de Vries et al, 1981). The stop/start growth of the stopper mutant is proposed to be the result of competition between predominately defective mtDNA and

under repressed less defective mtDNA, (de Vries et al, 1981). Ragged mutants of Aspergillus amleodami also produce irregular colonies with a "stop-start" mode of growth and have deficiences in cyt. a and an excess of cyt. c. Such mutants have been shown by Kuntzel et al, (1980) to contain amplified regions of wild type mtDNA.

Laboratory strains of Podospora anserina have a limited capacity for vegetative growth. After a period of linear propagation a progressive decrease in growth occurs resulting in the complete arrest of growth and finally hyphal death (Cummings et al, 1979). Death is associated with a reduction in ariel hyphae, dark pigmentation of the mycelia and a decrease in cytochrome aa_3. P.ansernia differs from previous examples as this pre-programmed cell death appears "normal" and not associated with mitochondrial mutations. Morphogenetic genes acting synergistically have been shown to postpone the onset of senescence, (Esser et al, 1976, 1979) and mitochondrial point mutations have been shown to affect the life span (Belcour & Begel, 1980). Senescence is maternally inherited and can be spread via hyphal fusion. Inhibitors such as DNA intercalating agents or agents affecting mitochondrial functions can postpone senescence (Brockelmann et al, 1987). Treatment of juvenile mycelium with all inhibitors arrested the senescence process, with pre-senescent mycelia only DNA intercalating agents were effective and no inhibitor could reverse senescence in senescent mycelium. Also senescence was only inhibited as long as the inhibitor was present (Tudzynski et al, 1979).

The amplification of small circular (SenDNAs e.g. P1DNA) in Podospora have been correlated with senescence and all appear to

originate from normal mtDNA (Esser et al, 1980). The nucleus has been shown to control at least 2 steps in the onset of senescence, it causes: (a) the liberation of P1DNA from mtDNA and (b) the expression of replicated P1DNA (Esser et al, 1979). No transposition to the nucleus is believed to occur (Koll et al, 1986), the occurrence of circular DNA plasmid amplification in senescent cultures is accompanied by the disappearance of the standard mtDNA (Smith et al, 1973). Some standard mtDNA must be present as cultures can be rejuvenated, also rejuvenated mycelia show the standard mtDNA restriction pattern with no detectable SenDNA (Koll et al, 1984). A second type of DNA molecule similar to the P1DNA of Podospora is "Kailo" DNA a linear mitochondrial DNA plasmid found in some N. Crassa strains (Bertrand et al, 1980). "Kailo" DNA is believed to cause mtDNA re-arrangements and cessation of growth. Like the mobile intron of Podospora "Kailo" DNA may be causatively involved in re-arranging the fungal genome.

The effects of dsRNA and fungal viruses on mitochondria is a poorly studied area. DNA plasmids resembling viruses rather than plasmids have been found in mitochondria. Linear DNAs of Ascobolus immersus are localised within the mitochondria but show no homology with high molecular weight mtDNA (Kempken et al, 1989). In A. immersus evidence for a virus like protein primer replication system exists, similar to that found in adenoviruses (Carusi, 1977) and Bacillus phage (Inciarte et al, 1976), the plasmid was also shown to code for a viral RNA polymerase. In this case the linear plasmid is cryptic having no deleterious effect on the host. Other circular DNA plasmids found in mitochondria of N. crassa " Mauriceville", "Labelle" and "Fiji" (Collins et al, (1981) and Stohl et al, 1982) show no homology

with mtDNA or each other. However, sites are present for homologous recombination with standard mtDNA though integration has not been observed so far. As recombination with mtDNA is a possibility re-arrangement of the mitochondrial genome by such plasmids cannot be ruled out.

DsRNA has been found in the mitochondria of S. cerevisiae (Beilharz et al, 1982), O. ulmi (Buck et al, 1987) and stem and leaf rust fungi (Kim & Klassens, 1989). In yeast a 14Kb dsRNA molecule was discovered that contained long transcripts derived from most regions of yeast mtDNA. It is proposed that prolonged transcription of both strands of yeast mtDNA can occur and that mtdsRNA arises from hybridization of these long complimentary transcripts. DsRNA was hypothesized to play some role in the transcription process of mtDNA (Beilharz et al, 1982).

Initial work with the d-factor in O. ulmi (Braiser, 1986), showed the d-factor to be cytoplasmically located but not in mitochondria. Chloramphenicol resistance marked mitochondria were not transferred with the d-factor during anastamosis. The d-factor has now been linked with dsRNA segments and it is believed that the passage of essential segments during anastomosis is required for d-factor transfer (Rogers et al, 1986). Rodgers et al, 1987) showed the mitochondrial fraction of dsRNA containing isolates of O. ulmi to contain more dsRNA than any other cellular fraction. The lower levels of dsRNA found in other cellular fractions could explain d-factor passage without mitochondrial transfer during anastamosis (Hintz et al, 1988). A deficiency in cyto aa_3 was seen in d-infected mitochondria but homology was not observed between the dsRNA segments and the

mtDNA (Rogers et al, 1987). Mitochondrial DNA rearrangements in d-infected and normal isolates have not been studied though it is possible that dsRNA may affect cytochrome levels if it could cause such re-arrangements. The presence of dsRNA in mitochondria has also been observed in stem and leaf rusts of cereals, (Kim & Klassen, 1989) though no deleterious effects of dsRNA on fungal pathogenicity have been observed, (Newton and Caten, 1985)

The only system where dsRNA has been associated with mitochondrial DNA re-arrangements is with cytoplasmic male sterility (CMS) in maize (Leaver & Gray,1982) and Vicia faba (Grill & Garger, 1981). In maize sterile plants are unable to produce fertile pollen. In all examples examined cytoplasmic male sterility is determined by genes present in the mitochondria and can be modified by nuclear restorer genes (Rf). Four general types of cytoplasm N, T, C and S exist. N (normal) gives rise to functional pollen, T, C and S are male sterile and have cytoplasms which can be distinguished by mitochondrial restriction mapping and restoration of fertility by different nuclear Rf genes.

Restriction digested mitochondrial DNA from CMS and normal plants show extensive differences. Normal and CMS T mtDNA genomes are 570kb and 540kb in length respectively with approximately 500kb of sequences in common. However, considerable variation in sequence organisation is present (Fauron et al, 1989). The CMS C genome is more closely related to CMS T than N, (Fauron et al, 1989), while CMS S exhibits a genomic organisation different from any of the others (Schardl et al, 1984).

Cytomplasmic male sterility also occurs in V. faba, 70nm membranous particles, cytoplasmic spherical bodies (CSB) are present in the cytoplasm of male sterile plants but not fertile plants (Edwardson et al, 1976). Purified CSBs contained a 16.7kbp dsRNA Scalla et al, 1981). Individual plants that have spontaneously reverted to fertility lack detectable levels of CSB and dsRNA. Nuclear restorer genes can suppress the ability of the dsRNA to cause CMS. Fertile and sterile lines show mitochondrial restriction profiles that differ and remain unchanged when fertility is restored by nuclear restorer genes (Tupens et al, 1988). Further generations are permanently fertile even with segregation of nuclear restorer genes. Homology was shown between CMS associated dsRNA and the genome of both sterile and fertile lines, but showed no homology to organelle DNA.

The following chapter examines the association of dsRNA with mitochondria of diseased sporophores and cultures of Agaricus bisporus. Mitochondrial DNA re-arrangements within diseased isolates were also investigated as potential causes of aberrantgrowth rates observed in infected tissue.

4.2 ASSESSMENT OF THE dsRNA CONTENT OF "APPARENTLY HEALTHY" AND DISEASED MITOCHONDRIA FROM SPOROPHORES AND CULTURE

Mitochondria from diseased and "apparently healthy" material were purified as shown in Chapter 2.7.1, the dsRNA content of the different cellular fractions compared. Fractions were made up of the following cellular components:

FRACTION i nuclei, cell wall debris and intact cells.

FRACTION ii cytoplasmic components including non-membrane bound viral particles (without mitochondria).

FRACTION iii the remains of the crude mitochondrial sample layer following density gradient centrifugation.

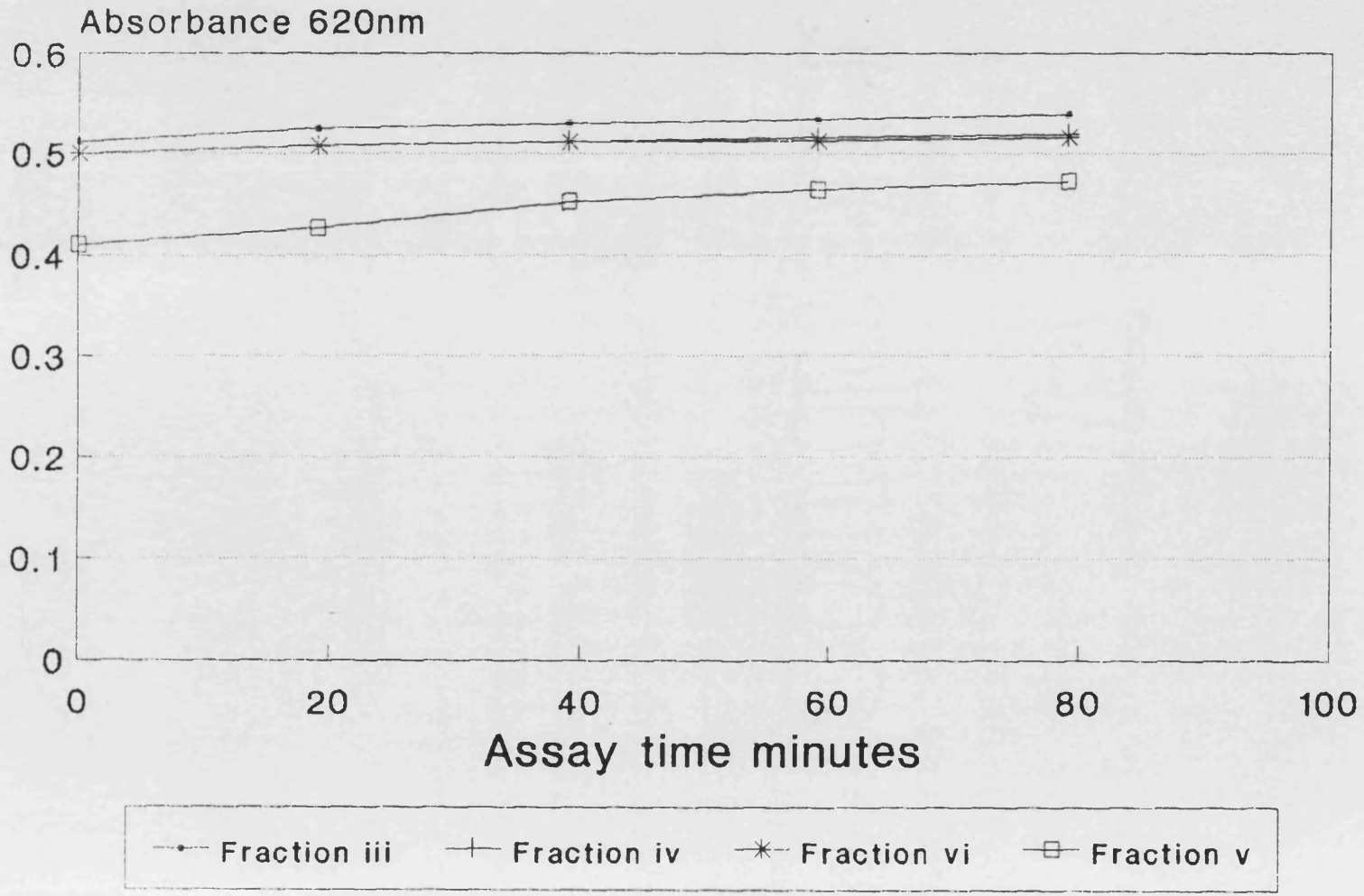
FRACTION iv the 35% sucrose gradient portion.

FRACTION v the sucrose gradient pellet.

FRACTION vi intact mitochondria.

The mitochondrial specific enzyme succinate dehydrogenase (SDH) (Chapter 2.7.2) was used to determine whether fractions other than fraction 6 contained mitochondrial membrane components. SDH activity of individual fractions shown as changes in absorbance at 620 nm are shown in Fig (4.1). Fraction v gave the highest SDH activity while fractions iii, iv, and vi had low activity. Subsequent mitochondrial purifications gave similar profiles. The low SDH activity of fraction vi was believed to be due to enzyme substrates having difficulty entering intact mitochondria. Homogenisation of fractions vi samples in later purifications produced activity levels similar to those in fraction v indicating that substrate penetration into the mitochondria was the problem and that fraction v by its position in the gradient and SDH assay probably contained damaged mitochondria.

FIG. 4.1 SUCCINATE DEHYDROGENASE ACTIVITY OF DIFFERENT CELLULAR FRACTIONS

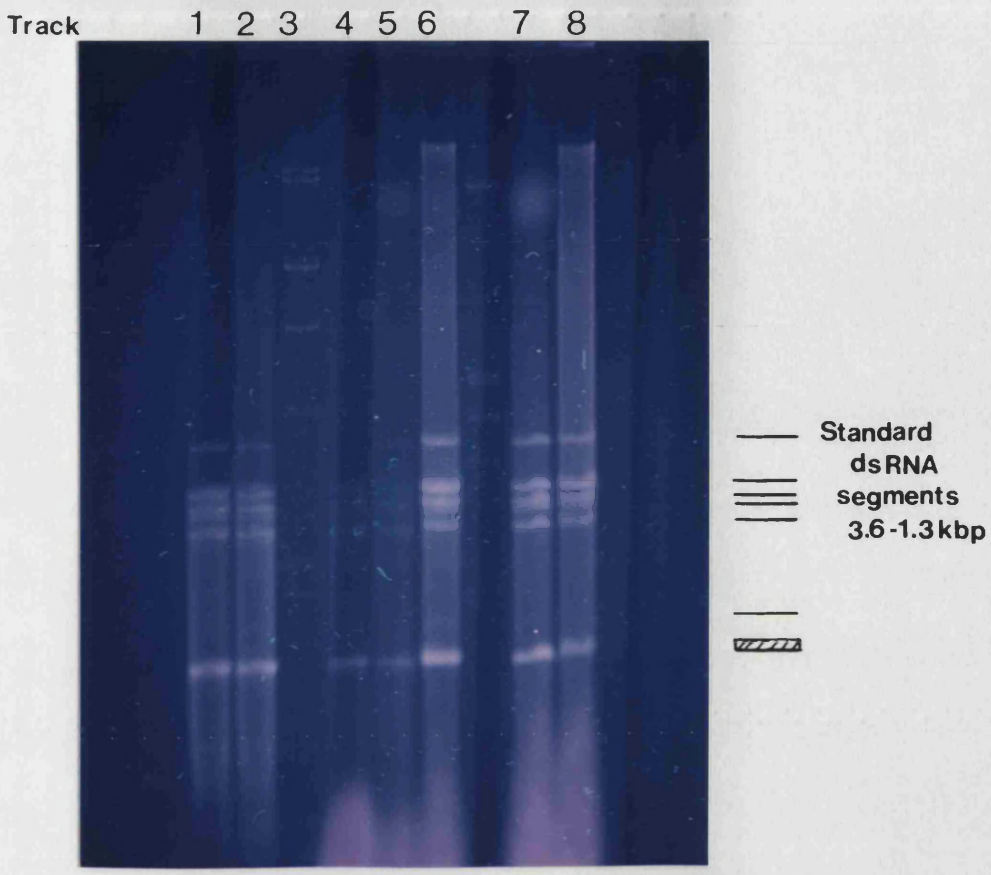


(i) Localisation of dsRNA in diseased sporophores (strain U3)

Difficulties in obtaining fresh diseased sporophore material meant that only sporophores used to produce cultured lines were available for mitochondrial purification. Unfortunately mitochondria were only purified from diseased sporophores H6F3. Total dsRNA extractions from H6F3 sporophores showed them to contain high molecular weight dsRNA segments 15, 12, 11.2 and 8.8 kbp and the "standard" dsRNA pattern 3.6 - 0.78 kbp (Chapter 3.2.1). Crude particle preparations showed a range of spherical and bacilliform particles to be present.

The dsRNA content of different cellular fractions of H6F3 sporophores are shown in (Plate 4.1). Fractions were not purified by CF11 chromatography prior to electrophoresis, hence the 0.8 and 0.78 kbp dsRNA segments which separated with fractionated DNA and ribosomal RNA were difficult to resolve. All fractions appeared to contain dsRNA segments 3.6, 3.0, 2.8, 2.7. 2.6, 1.5 and 1.3 kbp although concentrations differ. The two mitochondrial fractions vi and v contained the highest concentration of these segments, fractions iii and iv contained much lower levels and possibly represent dsRNA liberated from damaged mitochondria. Fraction i also contains a high level of dsRNA and is probably indicative of a large number of hyphae not being broken up during homogenisation. The cytoplasmic fraction ii also contains a high level of dsRNA segments 3.6 - 1.3 kbp and may be due to the high level of particles or unencapsidated dsRNA within the cytoplasm. The high molecular weight dsRNA segments 15 - 8.8 kbp were not detected in any of the fractions.

PLATE 4.1 AGAROSE ELECTROPHORESIS OF dsRNA EXTRACTED FROM DIFFERENT CELLULAR FRACTIONS OF DISEASED H6F3 SPOROPHORES.



Track 1 DsRNA extracted from fraction i.

" 2 " " " " ii.

" 3 Lambda DNA digested with Hind III (300ng).

" 4 DsRNA extracted from fraction iii.

" 5 " " " " iv.

" 6 " " " " v.

" 7 " " " " vi.

" 8 DsRNA extracted from diseased sporophores.

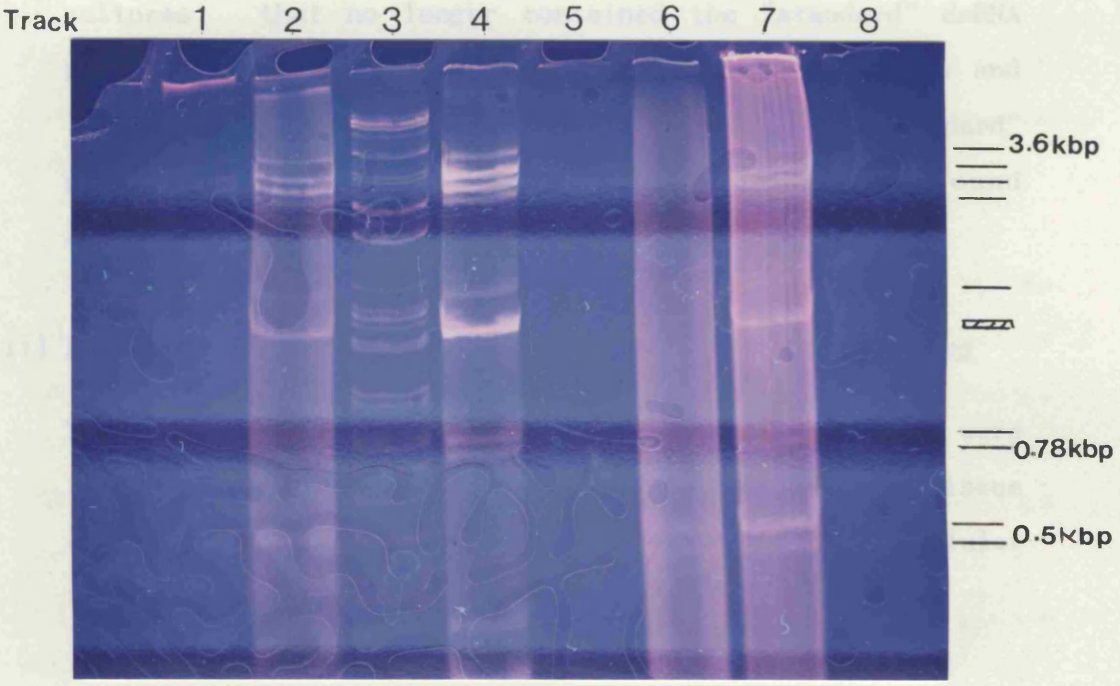
NB DsRNA prepared from each fraction was resuspended in the same volume of buffer, so that intensities of the dsRNA segments reflect their relative amounts in each fraction.

(ii) Localisation of dsRNA in diseased cultures (Strain U3)

Diseased culture material from cultures D and F was grown in liquid culture (chapter 2.1.1). At the time of mitochondrial isolation both diseased cultures contained the high molecular weight dsRNA segments 8.6, 8.0 and 6.6 kbp and still retained the "standard" pattern dsRNA segments 3.6 - 0.78 kbp. In general diseased culture preparations contained apparently low levels of virus particles in the electron microscope even when dsRNA levels were moderately high.

In diseased culture D fractions i, vi and v all contained dsRNA segments of the "standard" pattern (Plate 4.2). Fraction i contained the "standard" dsRNA segments plus two minor segments approximately 0.5 kbp. These two 0.5 kbp segments were also present in the fraction ii where dsRNA's of the "standard" pattern appeared absent (Plate 4.2). DsRNA was not found in fractions iii and iv. The two mitochondrial fractions vi and v both contained dsRNA segments 3.6, 3.0, 2.8, 2.7, 2.6, 1.3 kbp but the 1.5, 0.8 and 0.78 kbp dsRNA segments were either absent or not in a high enough concentration to be detected. Mitochondrial DNA was only in high enough concentration to be detected in fraction vi. High molecular weight dsRNAs 8.6 - 6.0 kbp did not appear in sufficient concentration in any fraction to be detected by ethidium bromide. Subsequent mitochondrial purification from La France diseased D and F cultures containing the "standard" dsRNA segments produced similar fraction profiles, with cellular fractions again not showing the high molecular weight dsRNA segments 8.6, 8.0 and 6.0 kbp.

PLATE 4.2 POLYACRYLAMIDE GEL ELECTROPHORESIS OF dsRNA EXTRACTED FROM DIFFERENT CELLULAR FRACTIONS OF DISEASED CULTURE D.



Track 1 DsRNA extracted from fraction v.
" 2 " " " " vi.
" 3 Lambda DNA digested with Hind III/ECORI (300ng).
" 4 DsRNA extracted from diseased culture D (total).
" 5 DsRNA extracted from fraction iv.
" 6 " " " " ii.
" 7 " " " " i.
" 8 " " " " iii.

NB DsRNA prepared from each fraction was suspended in the same volume of buffer so that intensities of the dsRNA segments reflect their relative amounts in each fraction.

On one occasion a diseased culture D preparation showed the high molecular weight 8.6 - 6.6 kbp dsRNA segments to be associated with mitochondrial fractions v and vi. Diseased cultures that no longer contained the "standard" dsRNA pattern but contained the 8.6, 8.0 and 6.0 kbp dsRNAs and low levels of the 3.6 and 1.5 kbp segments of the "standard" pattern were also examined, but dsRNA was not found associated with the mitochondria.

(iii) Localisation of dsRNA in "apparently" healthy sporophores

The mitochondria of "apparently healthy" sporophores were isolated from tissue that contained no dsRNA in total tissue extractions. DsRNA was not found in any of the cellular fractions following mitochondrial isolation.

Attempts were made to look for physical differences in the structure of mitochondria from diseased and "apparently healthy" sporophores by transmission electron microscopy (Chapter 2.7.3 - 2.7.4) but none were detected.

4.3 RESTRICTION PROFILES OF DISEASED AND APPARENTLY HEALTHY MITOCHONDRIAL DNA

A large number of mitochondrial related phenomenon including "stopper" mutants of N. crassa, senescence in P.ansernia and male sterility in maize all show changes in mitochondrial DNA (de-Vries et al, 1981; Esser et al, 1980; Leaver et al, 1982).

Mitochondrial DNA was isolated from purified mitochondria (Chapter 2.7.1). Mitochondrial DNA from diseased and apparently

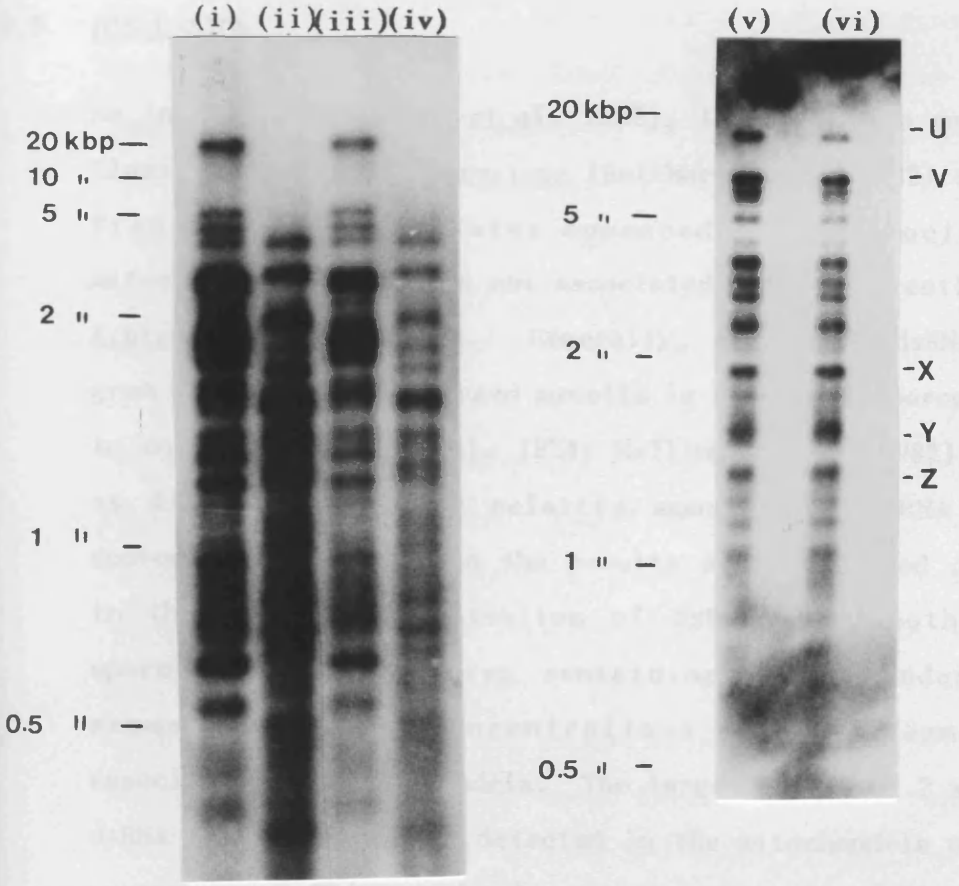
healthy mycelia were compared using Taq I, Sau3a and Hae III restriction digests (Chapter 2.3.3 (i)). Approximately equal amounts of mtDNA from healthy and diseased mycelia were digested, 100 ng in the case of Hae III and Sau3a and 10 ng for the Taq I digest. Southern blots of these digests probed with oligolabelled healthy mitochondrial DNA (Chapter 2.4.2) showed no apparent differences in sizes of bands present (Plate 4.3).

Hae III and Sau3a (Plate 4.3 (i)-(iv)) showed differences in the relative intensities of particular bands in diseased and healthy digests. As these differences related to all bands they were probably due to differences in the concentration of the initial DNA rather than any significant differences in the amounts of particular sequences in the two mtDNA genomes. However, in the Taq I digest (Plate 4.3 (v) & (vi)) some bands appear to give a similar signal in the two digests (bands X, Y, Z) while other bands (U, V) appeared less intense in the diseased than the healthy digest.

4.4 HOMOLOGY BETWEEN TOTAL dsRNA AND MITOCHONDRIAL DNA FROM DISEASED AND APPARENTLY HEALTHY ISOLATES

Mitochondrial DNA (5 ng) from diseased isolates H6F3 (containing "standard" dsRNA segments), culture F (has lost dsRNA of the "standard" pattern) and "apparently healthy" isolates was left intact and cut with restriction enzymes (Chapter 2.3.3). Samples were Southern blotted and probed with a c-DNA probe (Chapter 2.4.1 (ii), 2.4.4 (i) to 100 ng of total dsRNA (containing the "standard" dsRNA segments 3.6-0.78 kbp plus minor segments. The above experiment repeated three times indicated no evidence for homology between dsRNA and mitochondrial DNA from healthy or

PLATE 4.3 *DISEASED AND "APPARENTLY HEALTHY" MITOCHONDRIAL DNA CUT WITH RESTRICTION ENZYMES AND PROBED WITH OLIGOLABELLED HEALTHY MITOCHONDRIAL DNA*



(i) Healthy mtDNA digested with Hae III.

(ii) " " " " Sau 3a.

(iii) Diseased mtDNA digested with Hae III.

(iv) " " " " Sau 3a.

(v) Healthy mtDNA digested with Taq I.

(vi) Diseased mtDNA digested with Taq I.

diseased isolates.

4.5 DISCUSSION

As in O. Ulmi (Rodgers et al, 1987), leaf and stem rusts (Kim & Classen, 1989) and S.cereviase (Beitharz et al, 1982) dsRNA in La France diseased isolates appeared to be associated with mitochondria. DsRNA was not associated with "apparently healthy" A.bisporus mitochondria. Generally, levels of dsRNA/VLPs per gram dry weight of diseased mycelia is higher in sporophores than in cultures (Atkey et al, 1974; Hollings et al, 1982). As well as differences in the relative amounts of dsRNA found in sporophores and cultures the results also indicated differences in the cellular localisation of dsRNA. In both diseased sporophores and cultures containing the "standard" dsRNA segments, highest concentrations of these segments were associated with mitochondria. The large 15, 12, 11.2 and 8.8 kbp dsRNA segments were not detected in the mitochondria of diseased sporophores. This may be due to a concentration effect as these segments were usually present in low concentrations (<5ng/g tissue). However, with levels of the "standard" dsRNA segments at 600ng in the mitochondrial fraction high molecular weight dsRNA segments should be detectable if present. The 8.8, 8.0 and 6.0 kbp dsRNAs in cultured material were also generally not found associated with mitochondria However, in one diseased mitochondrial preparation segments were isolated that could represent the 8.8, 8.0 and 6.0 kbp dsRNAs. Diseased cultures showing a loss of the majority of "standard" dsRNA segments did not appear to have dsRNA associated with mitochondria.

Fraction ii from diseased sporophores contained high levels (300 ng) of the "standard" dsRNA segments while in diseased cultures they were undetectable in this fraction. As sporophores were stored at 4° prior to mitochondrial extraction these differences could be due to the break down of mitochondria in diseased sporophores during storage, or the result of a reduced concentration of dsRNA in diseased cultures. However, it is possible that differences in cellular localisation of dsRNA in diseased sporophores and culture were linked with the apparent lack of particles seen in cultured material (Atkey et al, 1974; Hollings 1982). Concentrations of particles were shown by Atkey et al (1974) to be reduced almost beyond the level of detection once diseased sporophores were established in culture. However, in other work (Hicks & Haughton, 1986) dsRNA was shown to be present in easily detectable levels in cultures from La France affected sporopores. Studies by Last et al, (1967) indicated that in La France diseased crops symptom severity increased with time. However, symptoms in cultured material appeared relatively stable, (Last et al, 1967). The above observations, together with dsRNA segment changes induced during culturing (Chapter 3), indicate possible differences between sporophore and culture maintenance of dsRNA/VLPs. Differences in the cellular localisation of dsRNA may provide further evidence for fundamental differences existing in the molecular biology of La France disease in the crop and in culture.

It is possible that La France disease symptoms could be linked to some direct or indirect effect of dsRNA in the mitochondria. Early studies on La France diseased mitochondrial structure showed them to appear deformed and associated with VLPs (Albouy, 1972). DsRNA has been shown by Penman & Summers (1965), Levin

et al., (1980) and Itmar et al (1984) to have an inhibitory effect on protein synthesis. In O.ulmi cytochrome ratios in mitochondria containing dsRNA were different from those without dsRNA (Rodgers et al, 1987). As levels of cytochromes increased as well as decreased it is unlikely that a simple inhibition of protein synthesis was responsible. Abnormalities in cytochrome ratios of dsRNA containing O.ulmi isolates are probably linked to growth rate reductions, as ATP production would undoubtedly be affected by such changes. Cytochrome levels were not investigated in diseased A.bisporus mitochondria although this area is worthy of further study.

In N.crassa (Collins et al., 1981 & Atkins et al., 1984) and A. amleostami (de Vries et al., 1981) abberant growth, similar to that seen in La France disease cultures was associated with re-arrangements of mtDNA. DsRNA was not associated with the above mutants and only in O.ulmi (Rodgers et al., 1987) and CMS-S maize plants (Leaver & Gray, 1982) has dsRNA associated with mitochondria been linked with any abberant growth. In O.ulmi mitochondrial DNA, re-arrangements have not been detected in d-infected isolates (Buck, 1990).

The possibility of rearrangements in mtDNA and integration of sequences related to dsRNA segments was investigated in La France diseased A.bisporus. A number of restriction enzymes failed to detect RFLPs between "apparently healthy" and diseased mitochondrial DNA. The presence of a large deletion or amplified region would have been detected with all the restriction enzymes used. However, it is possible that a point mutation in mt DNA may go undetected unless the enzymes cut at

the particular site of the mutation. In future work on the presence of mt DNA rearrangements in diseased isolates, enzymes must be selected for the detection of point mutations. As in O.ulmi (Rodgers et al, 1987) no evidence was found for homology between viral dsRNA and mt DNA in A.bisporus.

5. HYBRIDISATIONS

5.1 INTRODUCTION

An understanding of the molecular basis of transmissible La France disease in A. bisporus requires detailed knowledge of the structural and functional properties of the disease associated dsRNAs. A preliminary investigation of sequence homology between the various segments is required so that their degree of relatedness to each other can be determined. Similar studies have been carried out on other fungal dsRNA related phenomenon; hypovirulence in E. parasitica (L'Hostis et al., 1985, Hiremath et al 1986) and G. gramminis (Jamil et al., 1984) and killer factor production in U. maydis (Field et al., 1983).

In E. parasitica no homology was found between dsRNA segments of European and American hypovirulent strains (L'Hostis et al, 1985). Within isolates sequence homology was indicated between a number of the dsRNA segments (Hiremath et al., 1986). In G. graminis virus particles and the segments they contain have been placed in different virus groups based on their serological and biophysical properties (Buck et al 1981., McFadden et al., 1983; Buck 1984; Jamil et al., 1984). Hybridisation experiments showed no homology between dsRNA segments in different virus groups, also few relationships were detected between segments within a particular virus group (Jamil et al., 1984). Several relationships were found between dsRNAs of a particular virus group in strains that were not vegetatively compatible (Jamil et al., 1984). Killer toxin production in U. maydis is associated with heavy, light and

medium dsRNA segments (Bozarth, 1981). Sequence homology was not shown between heavy segments and the medium or light segments, only the smallest light segment and the medium segments had any homology (Field et al., 1983).

5.2 NORTHERN TRANSFER PROCEDURES

The two most frequently used methods of Northern transfer are: (i) to run the denatured dsRNA sample on a denaturing gel or (ii) run glyoxylated dsRNA on a non-denaturing gel and then blot onto an inert support. It is important to be able to visualize denatured dsRNA on the gel prior to blotting so that migration rates of particular bands (which may differ from those on a non-denaturing gel) can be determined. A third more primitive method is to cut and extract individual dsRNA segments from non-denaturing gels and spot denatured samples of individual segments onto an inert support.

5.2.1 DsRNA run under denaturing conditions and Northern Transfer.

DsRNA (400ng) was treated with glyoxal/DMSO or formaldehyde/formamide as indicated in Chapter 2.2.5 (i) and 2.2.5 (ii) respectively. In both cases after repeated experiments denatured dsRNA could not be detected with ethidium bromide (0.5 µg/ml) or acridine orange 30 µg/ml after electrophoresis. Non-denatured dsRNA (400ng) was readily detectable. Extensive treatment of reagents and electrophoresis tanks with 0.01% DEPC did not result in the denatured dsRNA segments being detected. Because of problems in detection and the similar sizes of the 5 major

dsRNA segments 3.6 - 2.6 kbp it was decided not to continue with this method as difficulty was envisaged in interpreting the results.

5.2.2 Electroblotting

Electroblotting (Chapter 2.2 (iii)) of dsRNA was attempted with a certain amount of success. Segments visualised by Etbr staining appeared to transfer efficiently on to Hybond strips. By altering the distance of slits from individual tracks and varying the time of electrophoresis, approximately equal concentrations of dsRNA could be transferred.

Denaturation of Hybond strips following transfer showed that when identical strips were probed with cDNA prepared from 200ng of total dsRNA (Chapter 2.4.1 (ii)) those denatured with 10 mM NaOH for 20 minutes gave the optimum signal (Plate 5.1). Formamide treated and non-denatured dsRNA segments also gave good signals following autoradiography. Attempts were made to mark the position of individual dsRNA segments on strips by small nicks in the Hybond. Preliminary experiments with individual dsRNA segments as probes resulted in problems identifying particular segments on the autoradiograph. Because of this potential ambiguity in interpreting results the method was not used in subsequent experiments. However, the above method clearly has potential as a quick and simple method for detecting homology between well separated dsRNA segments.

PLATE 5.1 THE EFFECT OF DIFFERENT NaOH DENATURING CONDITIONS ON THE HYBRIDISATION OF SLOT BLOTTED dsRNA PROBED WITH RADIOLABELLED cDNA TO TOTAL dsRNA

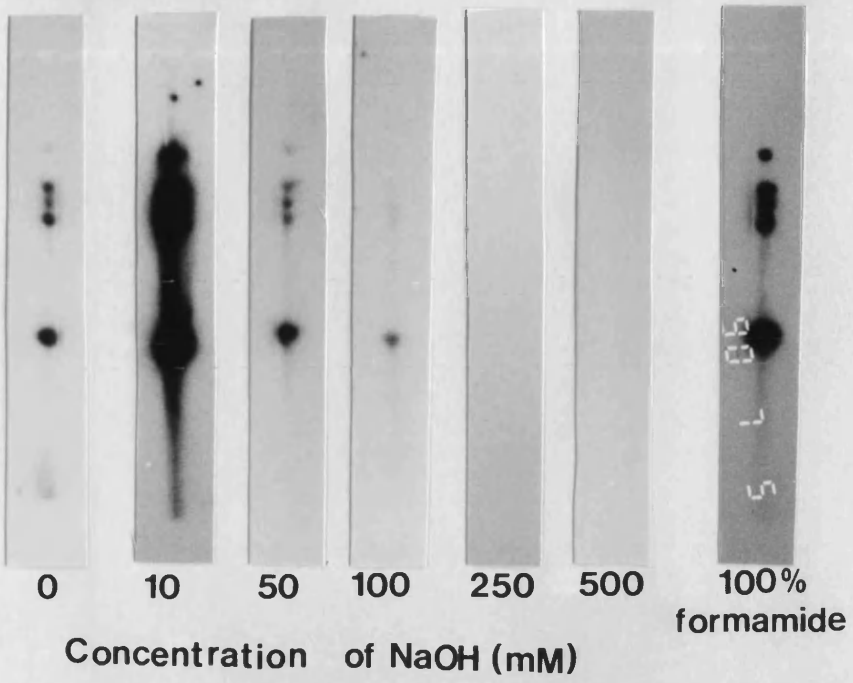
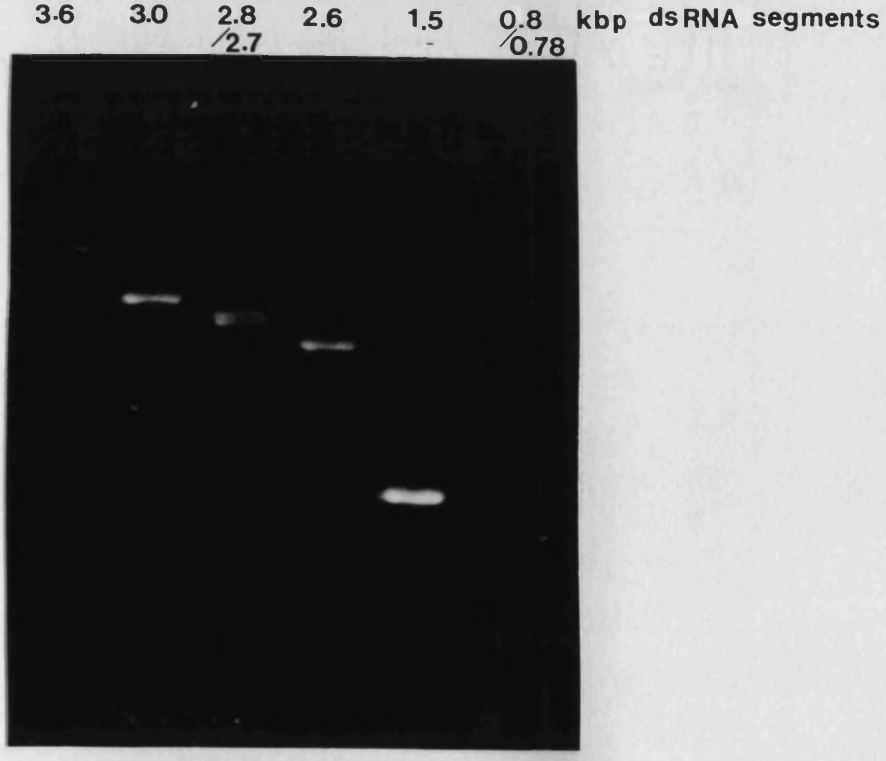


PLATE 5.2 PROBE AND DOT BLOT PURITY ASSESSED BY AGAROSE ELECTROPHORESIS AND ETHIDIUM BROMIDE STAINING.



5.2.3 Dot blots.

Individual dsRNA segments were extracted from polyacrylamide and agarose gels by a number of methods (Chapter 2.2.4 (i) (ii) (iii)). Chapter 2.2.4 (i) (polyacrylamide tube gels) and 2.2.4 (ii) (low melting point agarose gels) were the methods utilized as dsRNA bound to Whatman DE81, (Chapter 2.2.4 (iii) proved difficult to remove. Low melting point agarose electrophoresis enabled large amounts of individual dsRNA segments (400ng) to be extracted. Extraction from polyacrylamide tube gels only yielded between 20-40ng of each segment. However, dsRNA segments extracted from low melting point agarose gels appeared to inhibit the cDNA synthesis reaction (Chapter 2.4.1 (ii)), presumably due to the presence of inhibitory substances in the agarose. Using dsRNA segments extracted from polyacrylamide tube gels no problems were encountered in the synthesis reaction. DsRNA was therefore extracted from low melting point agarose gels for preparing dot blots and polyacrylamide tube gels for cDNA probe production. All segments were assessed for purity by agarose electrophoresis prior to their use as dot blots or probes (Plate 5.2).

5.3 NORTHERN HYBRIDISATIONS

In the literature when probing for well matched hybrids Northern hybridisation is generally carried out at 42^{o}C with 50% formamide, 5 X SSPE and 0.5% (w/v) SDS (Arrand, 1987, Anderson &,Young 1987, Thomas, 1980, 1983, Koenig et al.,1988; Powel & Van Alfen, 1987). The washing stage after hybridisation is the point at which stringencies are

altered by varying the temperature and salt concentrations. By using a range of washes and exposing blots to X-ray film after each wash, information can be obtained on the level of relatedness of different sequences.

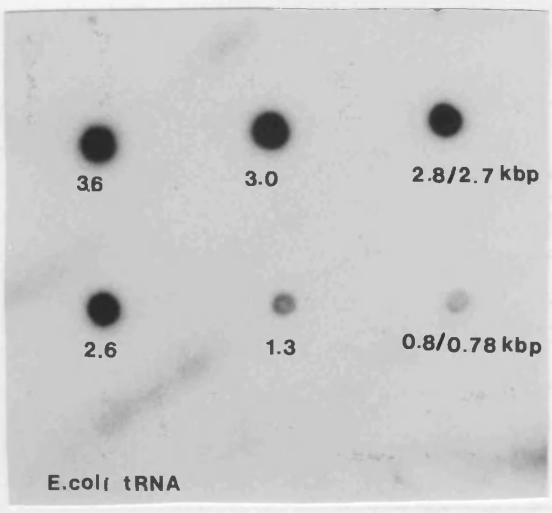
5.3.1 Homology between dsRNA segments of the "standard" pattern

Northern hybridisations were carried out using dot blots of 20ng of denatured dsRNA segments 3.6, 3.0, 2.8/2.7 and 1.3 kbp and <5ng of the denatured 0.8/0.7 kbp dsRNA segment. A negative control of 20ng *E. coli* tRNA was used to allow the problems of non-specific binding to be addressed. cDNA probes were prepared from 20ng of dsRNA segments 3.6, 3.0, 2.8/2.7 and 1.3 kbp as described in Chapter 2.4 (ii) and northern hybridisations carried out as in Chapter 2.4.4 (i).

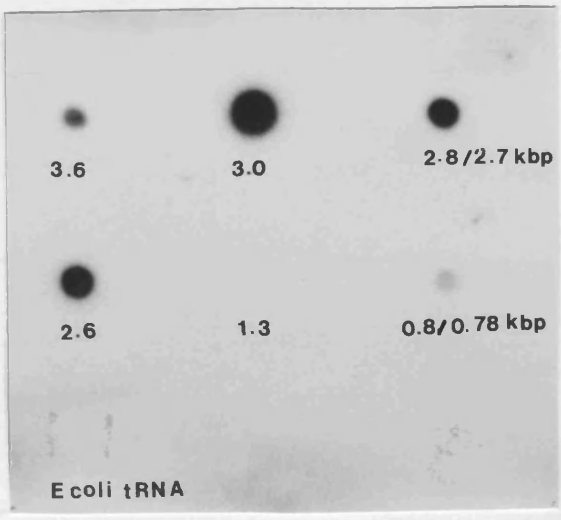
Levels of homology are shown in (Plate 5.3) and summarised in Table 5.1. All final washes were carried out in 0.1 x SSPE, 0.1% (w/v) SDS for 2 x 15 minutes at RT. Washes in 2 x SSPE, 0.1% (w/v) SDS for 30 minutes at 65°C resulted in no further removal of the signal. A 75°C wash with 2 x SSPE. 0.1% (SDS) resulted in the removal of cDNA from the positive control blots to the level of non-detectability.

PLATE 5.3 DOT BLOT HYBRIDISATION ANALYSIS OF HOMOLOGY BETWEEN dsRNA SEGMENTS OF THE "STANDARD" PATTERN.

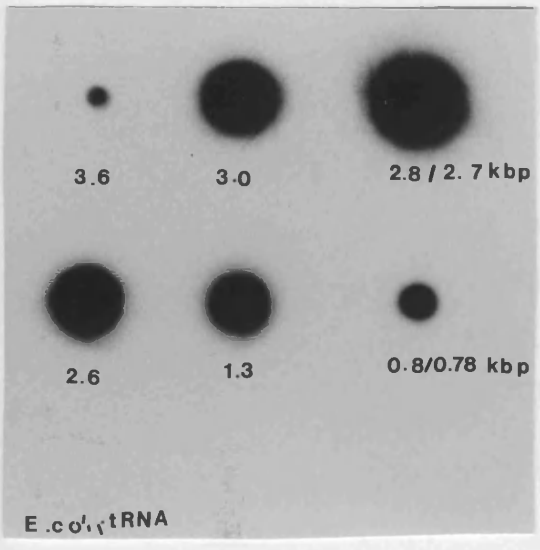
(i) 3.6 kbp dsRNA probe



(ii) 3.0 kbp dsRNA probe



(iii) 2.8/2.7 kbp dsRNA probe



(iv) 1.3 kbp dsRNA probe

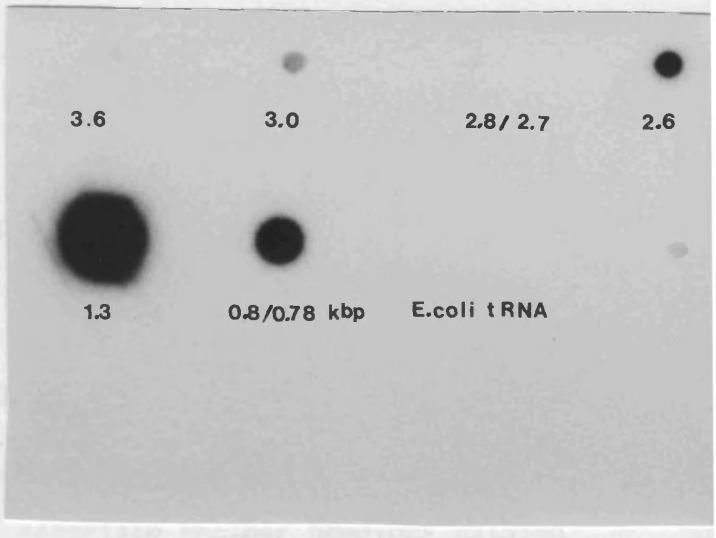


TABLE 5.1

DOT BLOT HYBRIDISATIONS BETWEEN dsRNA SEGMENTS OF THE 'STANDARD' PATTERN

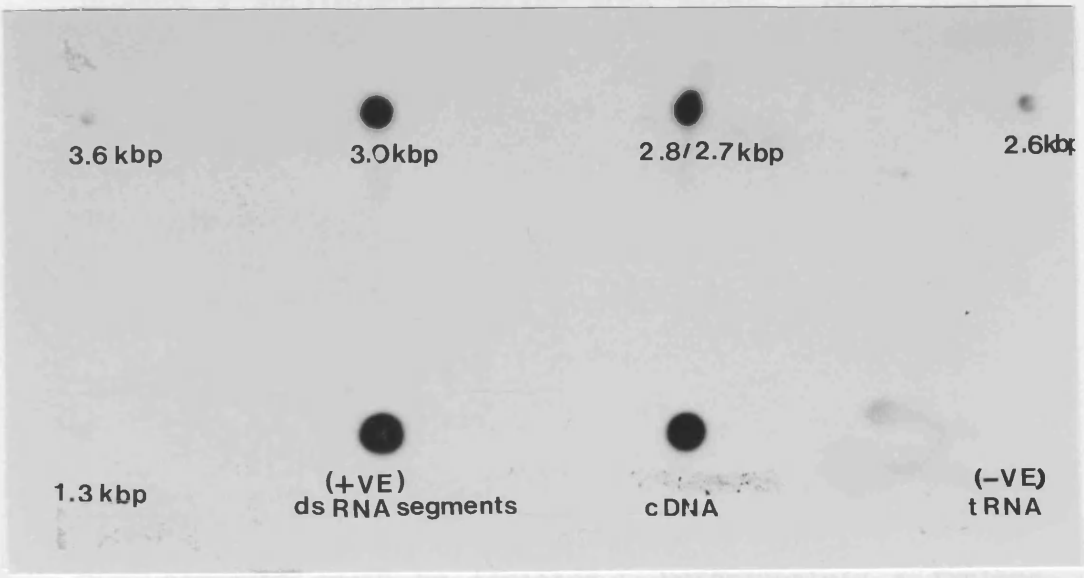
	DsRNA segments kbps			
Probe Dot Blots	3.6	3.0	2.8/2.7	1.3
3.6	+++*	++	+	+
3.0	+++	+++	+++	+
2.8/2.7	+++	+++	+++	-
2.6	+++	+++	+++	++
1.3	+		++	+++
0.8/0.78	+	+	++	++
E.coli t.RNA	-	-	-	-

* Relative hybridisation : no hybridisation -; weak +; moderate ++; strong +++.

5.3.2 Homology between the very high molecular weight dsRNA segments (VHMS) (15, 12, 11.2 and 8.8 Kbp), high molecular weight segments (HMS) (8.8, 8.0, 6.6 Kbp) and dsRNA segments of the "standard" pattern.

Because of the dsRNA segment changes reported in Chapter 3, it was very important to ascertain whether homology existed between the higher molecular weight dsRNA segments and those of the "standard" pattern. Low concentrations of both types

PLATE 5.4 DOT BLOT HYBRIDISATION BETWEEN THE VHMW dsRNA SEGMENTS 15, 12, 11.2 AND 8.8 kbp AND dsRNA SEGMENTS OF THE "STANDARD" PATTERNS.



of high molecular weight segments in diseased material (chapter 3) caused problems in extracting enough dsRNA to produce a sufficiently active cDNA probe. DsRNA segment levels below 1 ng used in the production of cDNA generally did not even detect the positive control on the blot.

(a) cDNA to 15, 12, 11.2 and8.8 kbp dsRNA segments

Concentrations of the VHMW dsRNA segments (15, 12, 11.2 and 8.8 kbp) were increased by running overloaded dsRNA from between 50-100g of severely diseased sporophores on low melting point agarose gels. Using this method between 5-10ng of the 15, 12, 11.2 and 8.8 kbp dsRNA segments could be isolated. Unfortunately a further purification step on polyacrylamide tube gels to remove agarose impurities was required. Hence the total dsRNA segment yields after polyacrylamide tube gels were further reduced to <5ng.

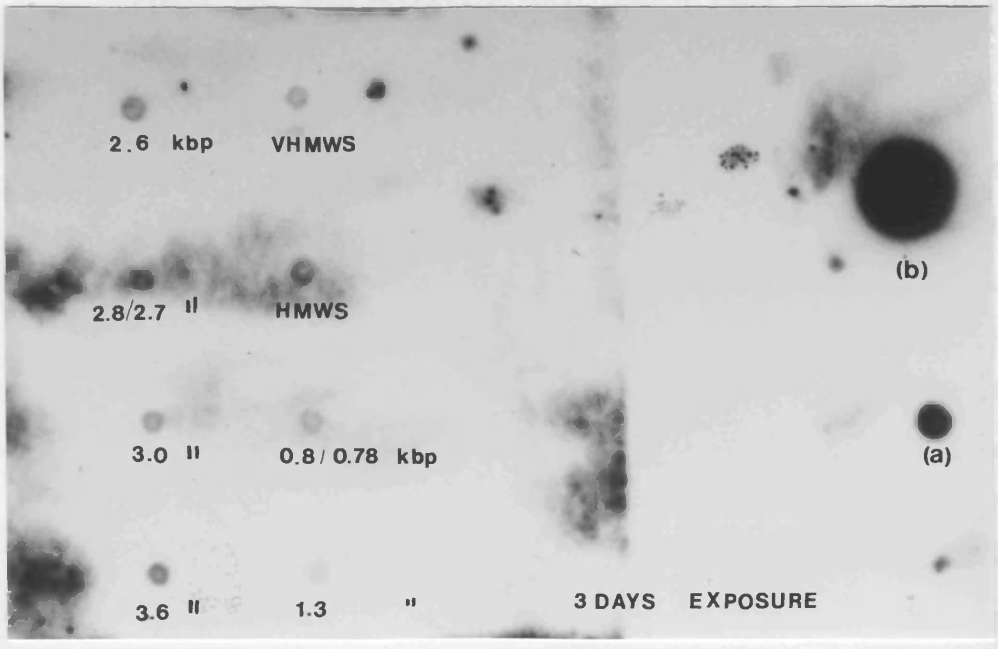
Northern hybridisations were carried out using dot blots as in Chapter 5.3 (i). cDNA produced from the VHMW dsRNA segments showed fairly strong homology with the 3.0 and 2.8/2.7 kbp dsRNA after a high stringency wash (0.1 X SSPE, 0.1% SDS, RT 30 minutes). The 2.6 and 3.6 kbp segments showed slight homology as did E. coli t-RNA (Plate 5.4).

(b) cDNA to 8.8, 8.0, 6.6 kbp Segments.

High enough concentrations of 8.1, 8.6 and 6.6 kbp dsRNAs could not be extracted to produce an adequate concentration of radiolabelled probe. An attempt was

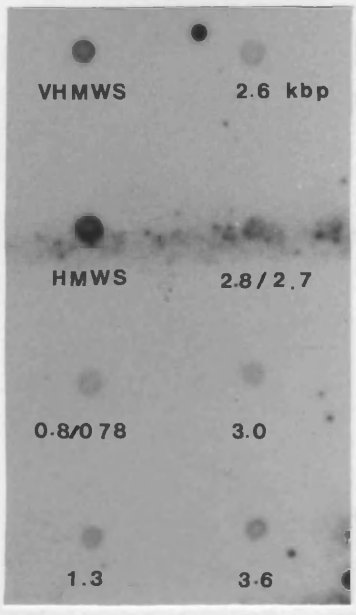
PLATE 5.5 DOT BLOT HYBRIDISATION BETWEEN THE HIGH MOLECULAR WEIGHT dsRNA SEGMENTS 8.6, 8.1 AND 6.6 kbp AND dsRNA SEGMENTS OF THE "STANDARD" PATTERN.

(i) Following a high stringency wash at 0.1 x SSPE, 0.1% SDS at RT.



(a) 50ng of 3.6, 3.0, 2.8/2.7, 2.6 kbp dsRNAs.
(b) (+ve) control containing 5-10ng of 8.6, 8.1 & 6.6 kbp dsRNA.

(ii) Following a very high stringency wash at 0.1 x SSPE, 0.1% SDS at 65°C.



14 DAYS EXPOSURE

NB All dot blots contained 20ng of each segment except the HMS and VHMS which contained 2-5ng.

made to increase the amount of radiolabelled cDNA produced (Chapter 2.4.1(iii). The adaptation appeared successful and a probe was produced at approximately 30 times the concentration that would have been produced from a comparable single labelling reaction.

Northern hybridisations were carried out using dot blots as in Chapter 5.2 (i). Following a high stringency wash at 0.1 X SSPE, 0.1% SDS at RT, the 3.6, 3.0, 2.8/2.7, 2.6, 1.3 and 0.78/0.8 kbp and the VHMW dsRNA segments all appear to show homology (Plate 5.5 (i)). However, a high degree of shadowing was also shown on the autoradiograph. A second very high stringency wash at 65^{o}C, 0.1 X SSPE, 0.1% SDS was used in an attempt to remove this non-specific binding. The majority of the hybridisation signal was removed from all dots including those containing the 8.8, 8.6 and 6.6 kbp segments (Plate 5.5 (ii)). Weak homology was still shown with dsRNA segment of the "standard" pattern and the VHMW dsRNA segments found in sporophores and early diseased cultures.

5.4 POSSIBLE INTEGRATION OF dsRNA SEGMENTS INTO THE GENOME OF DISEASED CULTURES.

The changes in dsRNA segment profiles observed in cultures (Chapter 3), together with the maintenance of the diseased phenotype was a completely unexpected observation. If the dsRNA associated with the disease symptoms was able to act like certain retroviruses and integrated into fungal DNA

while still maintaining the diseased state, this may explain the maintenance of the disease although not the dsRNA segments changes. As dsRNA segments are fairly large such an integration into nuclear or mitochondrial DNA would be readily detectable by comparing restriction digest profiles of diseased and healthy DNA.

Two approaches were followed in the "search" for integrated viral dsRNA.

(a) A comparison of total and mitochondrial DNA restriction profiles (Chapter 4) of diseased and healthy cultures.

(b) Probing restriction digested diseased total DNA with cDNA from dsRNA segments.

Although all isolates were of the U3 strain isogenic isolates were not available. Previous work had shown that RFLPs do not appear to exist within particular hybrid strains (Loftus et al., 1988). Also Hintz et al., (1985) examined restriction patterns of mitochondrial DNA from four strains of A. bisporus and found them to be identical. Hence, if variation was seen between diseased and healthy DNA profiles it is more likely due to viral integration and not strain differences.

5.4.1 Total DNA digests.

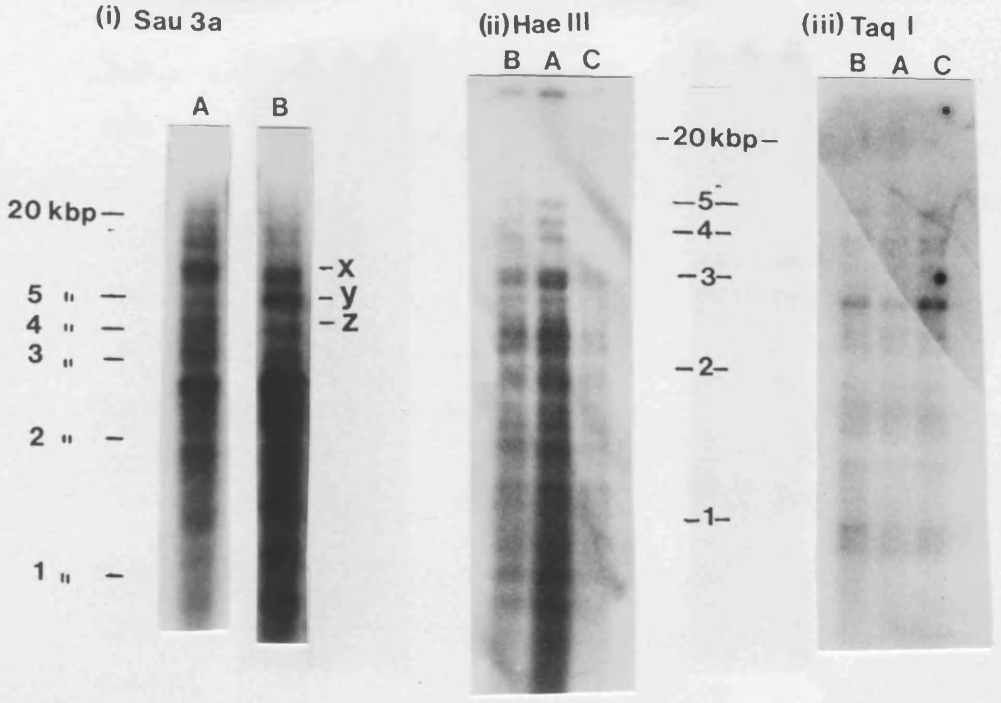
Total DNA was extracted from diseased culture F, H6F3 and "apparently healthy" cultures (Chapter 2.3.1). Diseased and "apparently healthy" DNA (10ng) were digested and Southern blotted as in Chapter 2.3.3. Mitochondrial DNA (20ng) and

the 20 kbp DNA fragment (20ng) isolated with the dsRNA segments following CF11 chromatography were oligolabelled (Chapter 2.4.2) and used as probes in a southern hybridisation (Chapter 2.4.4 (ii)).

"Apparently healthy" and H6F3 Sau 3A total DNA digests appear to show a number of differences when probed with healthy mitochondrial DNA (Plate 5.6 (i)). However, these differences appear to be due to concentration differences of bands X, Y & Z, and not the presence of new bands within the isolates (Plate 5.6(i)). Hae III and Taq I digests of apparently healthy and diseased H6F3 DNA probed with healthy mitochondrial DNA appeared to show no apparent differences (Plate 5.6 (ii) & (iii)). A comparison of Taq I digests of culture F (which no longer showed the complete "standard" dsRNA pattern) showed it to be no different from the "apparently healthy" or the diseased H6F3 digests (Plate 5.5 (iii)).

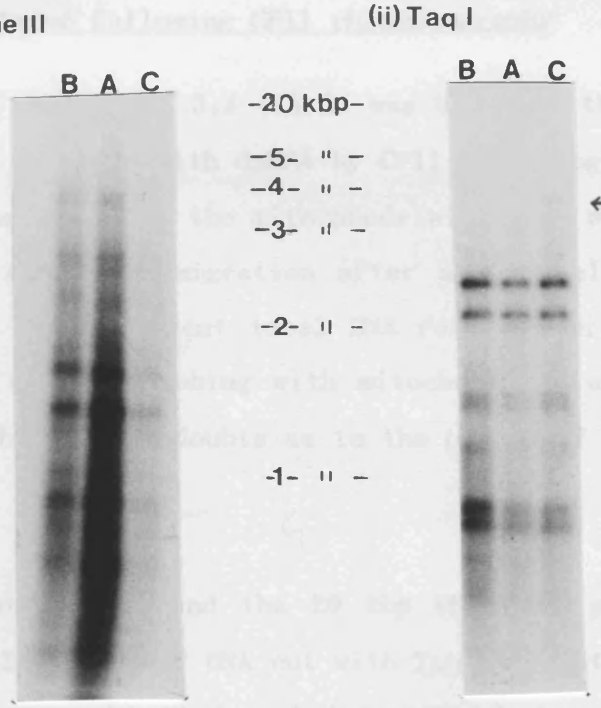
Diseased H6F3 (standard dsRNA pattern), culture F and "apparently healthy" restriction digests, probed with the oligolabelled 20 Kbp DNA fragment showed no obvious differences following restriction digests (Plate 5.7). Hae III digests though a little unclear in culture F also appeared to show no apparent differences (Plate 5.7 (ii)). Taq I digests showed no apparent differences between "apparently healthy" U3X and diseased H6F3 ("standard" pattern) (Plate 5.7 (ii)). However, a faint extra band 3.5 kbp (Plate 5.7 (ii)) was indicated in the culture F profile that was not present in the "apparently healthy" or H6F3.

PLATE 5.6 SOUTHERN HYBRIDISATIONS OF TOTAL DNA CUT WITH DIFFERENT RESTRICTION ENZYMES AND PROBED WITH OLIGOLABELLED "APPARENTLY HEALTHY" MITOCHONDRIAL DNA



(A) "Apparently Healthy" DNA.
(B) DNA from a diseased isolate containing the "standard" dsRNA segments (H6F3).
(C) DNA from a diseased isolate that has lost the majority of the "standard" dsRNA segments (Culture F).

PLATE 5.7 SOUTHERN HYBRIDISATIONS OF TOTAL DNA CUT WITH DIFFERENT RESTRICTION ENZYMES AND PROBED WITH OLIGOLABELLED 20 kbp DNA



(A) "Apparently Healthy" DNA.
(B) DNA from a diseased isolate containing the "standard" dsRNA segments (H6F3).
(C) DNA from a diseased isolate that has lost the majority of the "standard" dsRNA segments (Culture F).

5.4.2 Comparison of mitochondrial DNA from "apparently healthy" U3(X) and diseased isolates, with the 20 Kbp DNA band isolated following CF11 chromotography

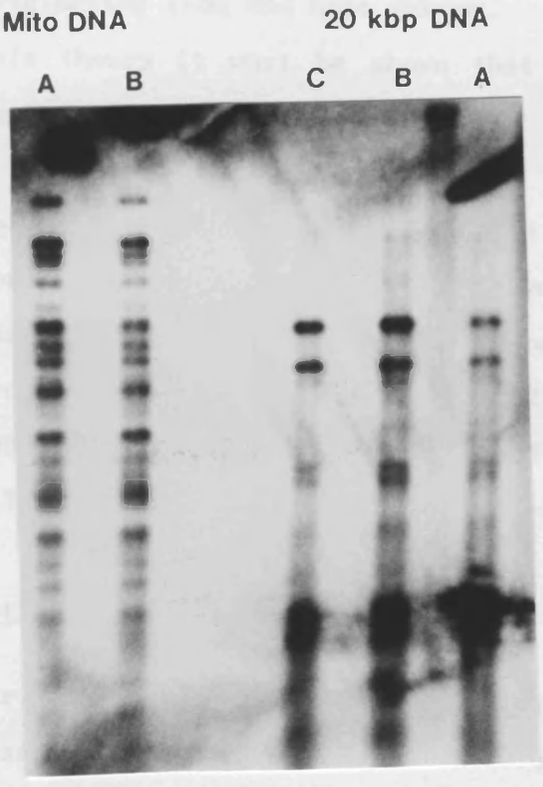
Prior to experiment 5.3 (i) it was believed that the 20 kbp DNA band isolated with dsRNA by CF11 chromatography (Chapter 2.2.1) was possibly the mitochondrial genome which was shown to have a similar migration after agarose electrophoresis. However, the different total DNA restriction profiles seen in 5.3 (i) when probing with mitochondrial and 20 kbp DNA band probes led to doubts as to the origin of the 20 kbp DNA band.

Mitochondrial DNA and the 20 kbp DNA were prepared as in Chapter 2 and 5ng of DNA cut with Taq I (Chapter 2.3.3 (i)). Samples were blotted and hybridised to oligolabelled mitochondrial DNA (Chapter 2.3.3, 2.4.2, 2.4.4 (i)). As can be seen from (Plate 5.8) mitochondrial DNA from healthy and diseased digests appear identical but completely different from 20 kbp DNA band digests from diseased and healthy cultures. Taq I 20 kbp DNA digests from "apparently healthy" and diseased cultures also appear identical (Plate 5.8).

5.4.3 Homology between total dsRNA and total and mitochondrial DNA from diseased and healthy isolates.

The results described in Chapter 5.3(i) and Chapter 4 suggested that loss of dsRNA segments and maintenance of the diseased phenotype in diseased cultures cannot be explained by the dsRNA integrating into the host genome. However, "apparently healthy" cultures (Chapter 3) after prolonged

PLATE 5.8 TAQ I DIGESTED MITOCHONDRIAL AND 20 kbp DNA PROBED WITH OLIGOLABELLED MITOCONDRIAL DNA



(A) "Apparently Healthy" DNA.
(B) DNA from a diseased isolate containing the "standard" dsRNA segments (H6F3).
(C) DNA from a diseased isolate that has lost the majority of the standard dsRNA segments (Culture F).

culturing, were occasionally found to contain high molecular weight dsRNAs. It is possible that the dsRNA could be linked with an integral component of the host genome (i.e. originating from the host genome. To provide evidence for this theory it must be shown that dsRNA is homologous to regions of the host genome.

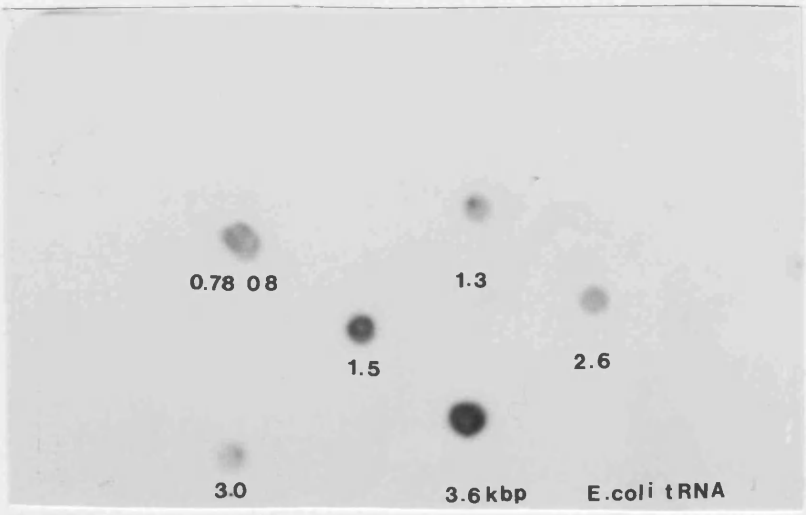
Total DNA (10ng) from H6F3, culture F and "apparently healthy" isolates was left intact and cut with restriction enzymes (Chapter 2.3.3). Samples were Southern blotted and probed with a c-DNA probe (Chapter 2.4.1 (ii), 2.4.4 (i)) to 100 ng of total dsRNA (containing 3.6, 3.0, 2.8, 2.7, 2.6, 1.3, 1.5, 0.78, 0.8 kbp dsRNA s plus minor segments).

Total DNA

Early work with uncut DNA from "apparently healthy" and diseased isolates (H6F3 and culture F) indicated possible homology. However, specific DNA bands were not picked out from restriction digested DNA. Using total dsRNA resulted in a problem with probe purity as the 20 kbp DNA band which may be present in very small amounts was impossible to remove by DNase treatment (RNase contamination cannot be easily removed from DNase). As reverse transcriptase can make copies of DNA when random primers are present the homology indicated may be between the 20 kbp DNA (in the probe itself) and the uncut samples.

This problem was removed by using Southern dot blots of individual dsRNA segments (20ng per segment) and probing with 10ng of oligolabelled 20 kbp DNA. It was shown that

PLATE 5.9 HOMOLOGY BETWEEN THE 20 kbp DNA SEGMENT AND dsRNA SEGMENTS OF THE "STANDARD" PATTERN



homology existed between the 20 kbp DNA and most of the "standard" dsRNA segments (Plate 5.9).

5.5 DISCUSSION

Recent hybridisation studies on the "standard" pattern of dsRNA segments associated with La France disease indicated no homology existed between the individual dsRNA s (Harmsen *et al*, 1989). Conditions were such as to allow hybridisation of only perfectly matched hybrids. However, the results presented here indicated that dsRNA segments of the standard pattern show extensive homology with each other (Plate 5.3, Table 5.1). Individual dsRNA segments used for probes and dot blots were shown to be free of other contaminating dsRNA segments by electrophoresis. Isolated segment purity was only addressed using ethidium bromide staining (sensitive to < 1ng). Hence it is possible that very low levels of similar sized dsRNA segments could have been present. It is unlikely that the presence of subnanogram levels of contaminants would give hybridisation signals comparable to those obtained with the positive controls (Plate 5.3). Furthermore dsRNA segments including the 3.6 and 2.6 kbp segments are separated by 2-3 cm during electrophoresis. Hence, it is unlikely that low level contamination between these segments could occur.

Possible explanations accounting for discrepancies between results shown here and those of Harmsen *et al*., (1989) include the fact that dsRNA segments used in the two studies could be completely different or from different viral strains. However, this is unlikely as the "standard" dsRNA

segment pattern associated with La France disease appears fairly consistent throughout the world (Marino et al, 1974, Hicks & Haughton, 1986; Wach & Romaine, 1987; Romaine et al., 1989; Harmsen et al., 1989). Differences between results are probably due to differences in the hybridisation stringencies used. Harmsen (1989) carried out RNA-RNA hybridisation at 65°C, 1M NaCl and 50% formamide. During experiments described here comparable RNA-DNA hybridisations were carried out at 42°C, 5 X SSPE, 0.5% (w/v) SDS, 5 X Denhardts. DNA-DNA, RNA-DNA and RNA-RNA hybrids have different thermal stabilities, the melting temperature (Tm) for RNA-RNA duplexes is about 10°C higher than that of comparable DNA-DNA hybrids, RNA-DNA hybrids are of intermediate stability (Wahl et al., 1987). Generally, optimal temperatures for nucleic acid reassociations in aqueous salt solution lie in the range 60-75°C (Young and Anderson, 1987).

The incorporation of formamide into the hybridisation solution allows reactions to be carried out at lower temperatures avoiding problems of high temperature probe degradation. Generally the temperature of hybridisation with 50% formamide can be lowered from 65°C to 42°C without any significant effect on stringency (Young & Anderson, 1987) Most workers when assessing homology of RNA-RNA and RNA-DNA hybrids carry out hybridisation at 42°C, 50% formamide, 5 X SSC (Finnegan & Brown, 1986; Paul et al., 1988; Shelbourne et al., 1988; Hunst et al., 1986; Thomas, 1980; Jamil et al., 1984; Koenig, 1988). RNA-RNA hybrids are substantially more stable than DNA-RNA or DNA-DNA hybrids, stringent hybridisation and washing conditions must

be employed when RNA probes are used to analyse Northern, slot or dot blots. Some protocols (Zinn et al., 1985 & Wahl et al., 1987) use temperatures up to 60°C with 50% formamide to cicumvent the problem of false positives produced from very small homologous regions.

Harmsen et al's., (1989), high stringency 65°C and 50% formamide RNA/RNA hybridisations showed dsRNA segments of the standard pattern to have no obvious sequence homologies. RNA/DNA hybridisation of comparable segments in this chapter under fairly stringent conditions indicated extensive homology. It is unlikely that segment homology shown here was the result of false positives. Ecoli tRNA failed to hybridise with all dsRNA segments used to produce the probe. Washes at 75°C, 2 X SSPE removed all the probe from all the blots Unfortunately as viral dsRNA was not used as a negative control it cannot be certain that homology was not due to some common viral sequence. Only by cloning and sequencing the dsRNA segments can we be sure of the level of homology that exists within dsRNA segments of the standard pattern.

Homology is also indicated between the VHMW and HMW dsRNA segments and dsRNA segments of the "standard pattern" (Plate 5.4 & 5.5). As levels of these high molecular weight segments were low it is difficult to determine whether the faint signals detected are a true indication of levels of homology. Future experiments should involve cloning these high molecular weight segments so that probes and blots contain sufficient levels of DNA or RNA.

Homology between dsRNA segments associated with hypovirulence, degenerative diseases and killer factor production has been studied in other fungal systems (L'Hostis et al., 1985; Hiremath et al., 1986; Jamil et al., 1984; Field et al., 1983; Bruenn and Kane, 1978). Mixed results were obtained, some systems showing extensive sequence homology and others little homology between dsRNA segments. In E. parasitica significant dsRNA sequence homology was shown within European isolates expressing a variety of dsRNA segment profiles (Hiremath et al, 1986, Tartagalia et al., 1986). Homology was shown in the terminal regions of large (4.5 - 6.0 X 10^6 Da) and small (0.33 - 0.45 X 10^6 Da) dsRNA s (Hiremath et al., 1986). However, a lack of cross hybridisation was shown between dsRNA of European and American isolates (L'Hostis et al., 1985). DsRNA segment variability and hybridisation studies led Rae et al., (1989) to propose that in E. parasitica most of the <9kbp dsRNAs are derived from the largest dsRNA by internal deletion events. Current investigations by Hillman et al., (1989) to clone and sequence the dsRNA segments of E. parasitica will provide more conclusive evidence for this theory.

The possible production of smaller dsRNAs from larger dsRNAs has been shown in S. cerevisae and U. maydis (Field et al , 1978; Bruenn & Kane, 1978; Field 1983; Shelbourne et al., 1988). Suppressive sensitive strains of S.cerevisae no longer have M dsRNAs (1.9 Kbp) but contain smaller (S) dsRNAs (Tzen et al., 1974; Vodkin et al., 1974). Hybridisation studies demonstrated that S dsRNAs are derived from their parental M dsRNA by internal deletion (Fried &

Fink 1978; Bruenn & Kane, 1978). In U. maydis, heteroduplex analysis (Field et al., 1983) and hybridisation studies (Shelbourne et al., 1988) suggested that the light dsRNA segment found in U. maydis was homologous to one of the medium sized segments. However, unlike the situation in E. parasitica homology does not exist between any of the other dsRNA segments associated with killer toxin production in U. maydis or S. cerevisae (Field et al., 1983; Shelbourne et al., 1988; Fried & Fink, 1978; Bruenn & Kane, 1978). The only other fungal system where dsRNA segments have been compared is G. graminis (Jamil et al, 1984). In G.graminis dsRNA segments within particular virus groups showed little homology with each other.

Loss of the standard dsRNA segments during culturing with the maintenance of the La France phenotype and high molecular weight segments could indicate that the larger segments contain all necessary information for the maintenance of the disease. The results of hybridisation studies indicate that the smaller segments could have come from the larger segments. Low molecular weight RNAs may be analogous to the sub-genomic RNAs associated with plant viruses representing translatable repeated copies of individual dsRNA segments (Mathews, 1981). Alternatively, high molecular weight dsRNA segments could represent replicative structures involved in the replication of the "standard" dsRNA segments. Some sequence homology is also suggested between dsRNA segments of the "standard" pattern. This was not supported by Harmsen et al., 1989) although conditions used by Harmsen would probably only detect hybrids with 90-100% homology. As has already been

suggested cloning and sequencing of the dsRNA segments is required to determine levels of sequence homology.

The integration of dsRNA into the host genome would provide a possible explanation for the loss of dsRNA segments and maintenance of the disease phenotype. Diseased and healthy mitochondrial DNA (Chapter 4) was shown to contain no obvious RFLP s and have no homology with dsRNA segments. RFLP analysis of diseased hyphae (with and without the "standard" dsRNA segments) and "apparently healthy" hyphae revealed no obvious points of dsRNA integration. However, homology was shown between the oligolabelled 20 kbp DNA segment (probe) and some of the dsRNA segments.

Generally, VLPs and dsRNA segments found in fungi show little homology with host DNA. In S. cerevisae (Wickner & Leibowitz, 1977 Wesolawski 1984), O. ulmi (Rodgers et al., 1987), U. maydis (Ben-Zvi, 1984) and stem and leaf rusts of cereals (Kim & Klassen, 1989) dsRNA shows no homology with mitochondrial or total DNA. However, other probably non-viral dsRNAs do show homology with cellular DNA. DsRNA found in S. cerevisae mitochondria not related to the ScVs does show homology with mtDNA (Beilharz et al., 1982). This dsRNA molecule is believed to be produced as a result of symmetrical DNA translation. DsRNA associated with male sterility in V. faba showed homology with nuclear genomes of sterile and fertile lines (Turpen, 1988). Sequence homology has also been found between some plant viroid RNAs and host derived nuclear RNA molecules (Kiss et al., 1983).

The origin of the 20 kbp DNA segment is unknown, although presumably due to its structure it is isolated along with dsRNA during CF11 chromatography. It was initially thought to represent mtDNA as it had a similar migration to mtDNA after 1.0% agarose gel electrophoresis However, restriction analysis showed them to be completely different Also the fact that mtDNA showed no homology with dsRNA and the 20 kbp DNA band apparently did, is further evidence that the latter is not mitochondrial in origin. The 20 kbp DNA isolated in diseased cultures with and without the standard pattern and "apparently healthy" cultures appeared identical. Homology of this DNA segment with dsRNA and the fact that "apparently healthy" cultures can be induced to produce dsRNA during culturing, possibly implicates this 20 kbp fragment in dsRNA production. Morris & Dodds (1979) and Koltin, (1986) have also reported co-purification of DNA segments with dsRNA after CF11 chromatography but dismissed these as preparatory artifacts. The fact that DNA plasmids are extremely common in fungi (Esser et al., 1986 Bockelmann et al., 1987) coupled with our findings indicate that these segments should not be dismissed so lightly. In a number of cases O. ulmi (Takai et al., 1984; Buck, 1990), R. solani (Hashiba et al., 1984) and G. graminis (Honeyman et al., 1983) DNA plasmids 22-2 Kbp have been isolated along with dsRNA segments. The aquatic fungus Rhizidiomyces has also been found to contain DNA mycoviruses containing 25 kbp DNA segments (Dawe & Kuhn, 1983).

The majority of linear DNA plasmids in fungi, including those in S.cerevisae (Gunge et al., 1981), Ascobolus immersus (Meinhardt et al., 1986) and

Claviceps purpurea (Tudzynski & Esser, 1986) have terminal repeats and proteins bound to their 5' termini. Adenoviruses are the only group of extrachromosomal genetic elements that show a comparable structure and replication, to these linear DNA plasmids (Kempston et al., 1989). It is possible that these linear DNA species may be vestiges of viral ancestors. One interesting point which may have analogies amongst dsRNA viruses is that the majority of DNA plasmids reside inside mitochondria (Bockelmann et al., 1987). DsRNA in O.ulmi (Rodgers et al., 1987) and A. bisporus (Chapter 4) also appears to reside in mitochondria. As indicated earlier, the existence of viruses as intracellular elements results in them becoming more plasmid than virus like. It is possible that dsRNA viruses and linear DNA plasmids were once linked and have evolved to form two completely different molecules. If we consider the current range of dsRNA segments in fungal systems, ScV in S. cerevisae (Wickner, 1980) may still resemble the original virus particles that infected S. cerevisae. In E. parasitica (Van-Alfen, 1986) and O. ulmi capsid proteins are no longer present and dsRNA elements resembling plasmids reside in other cellular locations. The current situation in A. bisporus appears intermediate between ScV s in S. cerevisae and dsRNA segments in O. ulmi, dsRNA segments are still encapsidated but the majority appear to be found in the mitochondria or in club shaped particles (Lesseman et al, 1977).

6. EFFECT OF PHYSICAL AND CHEMICAL TREATMENTS ON LA FRANCE DISEASED A.BISPORUS CULTURES

6.1 INTRODUCTION

The inability to unequivocally infect "apparently healthy" isolates of A.bisporus with virus particles or dsRNA has led to a limited understanding of the molecular aspects of the disease. An alternative approach to elucidate the aetiology of La France disease is to attempt to eliminate dsRNA segments or virus particles either partially or completely from La France diseased isolates.

Heat therapy is well established as a technique for the treatment of certain plant virus disorders (Nyland, et al., 1969). Heat treatment of La France diseased A.bisporus cultures has, however, produced inconclusive results. Early work by Hollings (1962) showed that normal growth could be obtained by growing diseased cultures at 33°C for two weeks before returning hyphal tip cultures to 23°C. Later work by van Zaayan (1979), showed that cultures heat treated at 30-34°C for two weeks were not free from virus particles despite increased growth rates. Last et al, (1974) and Nair (1973) showed that the time at which heat treatment commenced after culturing affected the outcome. Diseased isolates cultured at 25°C, prior to heat treatment usually showed lower or negligible growth rate increases, compared to isolates which were not pre-incubated.

Chemical treatments have also been used to eliminate viruses and virus-like particles from eukaryotes. Cycloheximide treated killer strains of S.cerevisae lost the ability to inhibit other sensitive yeast strains (Fink & Styles, 1972). This loss has been attributed to the selective removal of a dsRNA segment associated with the ScV particle M in the cytoplasm of the killer strain of yeast (Young & Yagua 1978). Fulbright (1984) showed that certain dsRNA containing hypovirulent strains of E.parasitica appeared morphologically normal and contained no dsRNA after cycloheximide treatment. However, certain hypovirulent genotypes were not cured of dsRNA by cycloheximide (Fulbright, 1984). Ribavirin (B-D-ribofuranosyl -1, 2, 4-triazole 3-carboxamide) is a nucleoside analogue with a broad spectrum of anti-viral activity (Sidwell et al, 1980). Ribavirin has been shown to inhibit the replication of influenza and other viruses both in cell culture and in-vivo (Sim & McCullagh, 1985).

Ethidium bromide was also used as a chemical treatment due to its ability to intercalate DNA, especially mitochondrial DNA. Studies on the cellular localisation of dsRNA in La France disease (Chapter 4) showed a high proportion of total cellular dsRNA to be associated with the mitochondria of infected cultures. It is possible that the dsRNA may be acting on the mitochondrial genome and hence it was worth investigating whether ethidium bromide had any effect on disease symptoms. In the following experiments different physical or chemical treatments were carried out on La France diseased material in an attempt to interupt particular

aspects of viral life cycles. The effects of treatments were studied by looking at the morphology and dsRNA content of cultures.

6.2 HEAT TREATMENT

(i) La France diseased H6F3

H6F3 (strain U3) cultures containing the "standard" dsRNA segment pattern and "apparently healthy" strain U3 cultures were grown on MA at 15°, 23°, 30° and 37°C for between 4-6 weeks. Sub-cultures of "apparently healthy" isolates were taken before colonies reached the edge of the plate. The growth rates of peripheral and mid plate sub cultures were assessed on MA at 23°C, and liquid cultures established for the determination of dsRNA content (Chapter 2.2.1).

Culture H6F3 treated at 30°C showed a significant increase in growth rate (Fig. 6.1) ($p<0.05$, anovar, Bonferroni's inequality test, Appendix 1). and generally looked healthier than cultures grown at lower temperatures (Plate 6.1).

Mid and peripheral regions from cultures grown at 30°C both showed growth rate increases, although the growth rate of hyphae taken from mid regions had a slower final than initial growth rate. Growth rates of diseased H6F3 cultures grown at 15°C were not significantly different

FIG. 6.1 MEAN GROWTH RATES OF ISOLATES ESTABLISHED FROM MID AND PERIPHERAL REGIONS OF DISEASED H6F3 FOLLOWING HEAT TREATMENT

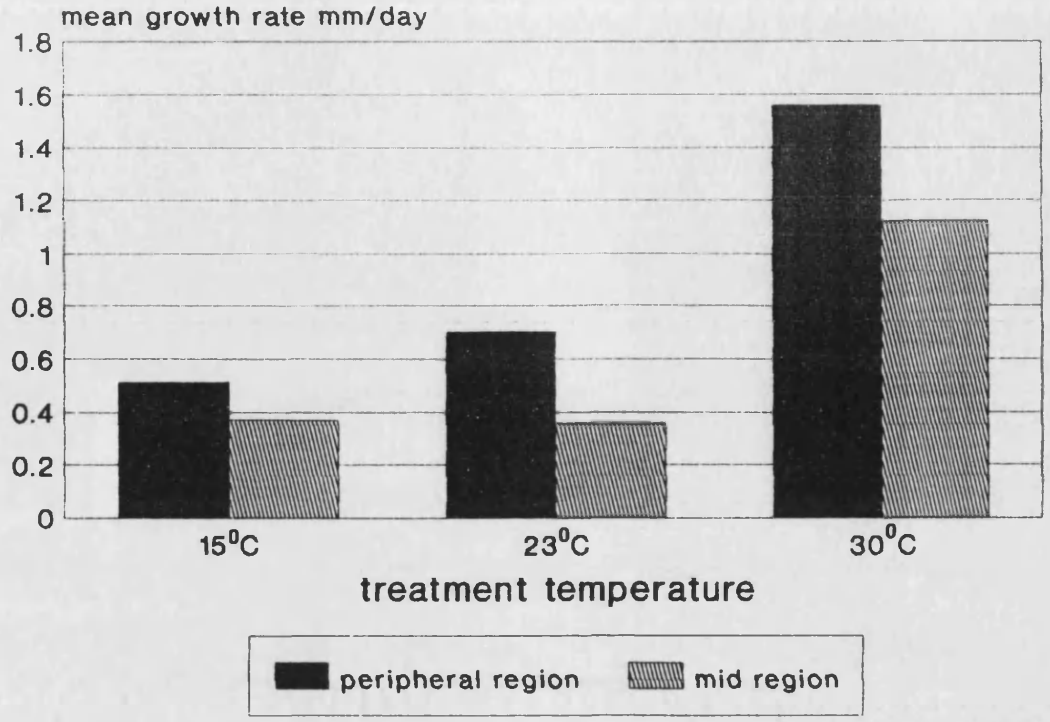


FIG. 6.2 MEAN GROWTH RATES OF CULTURES ESTABLISHED FROM MID AND PERIPHERAL REGIONS OF CULTURE 616 FOLLOWING HEAT TREATMENT

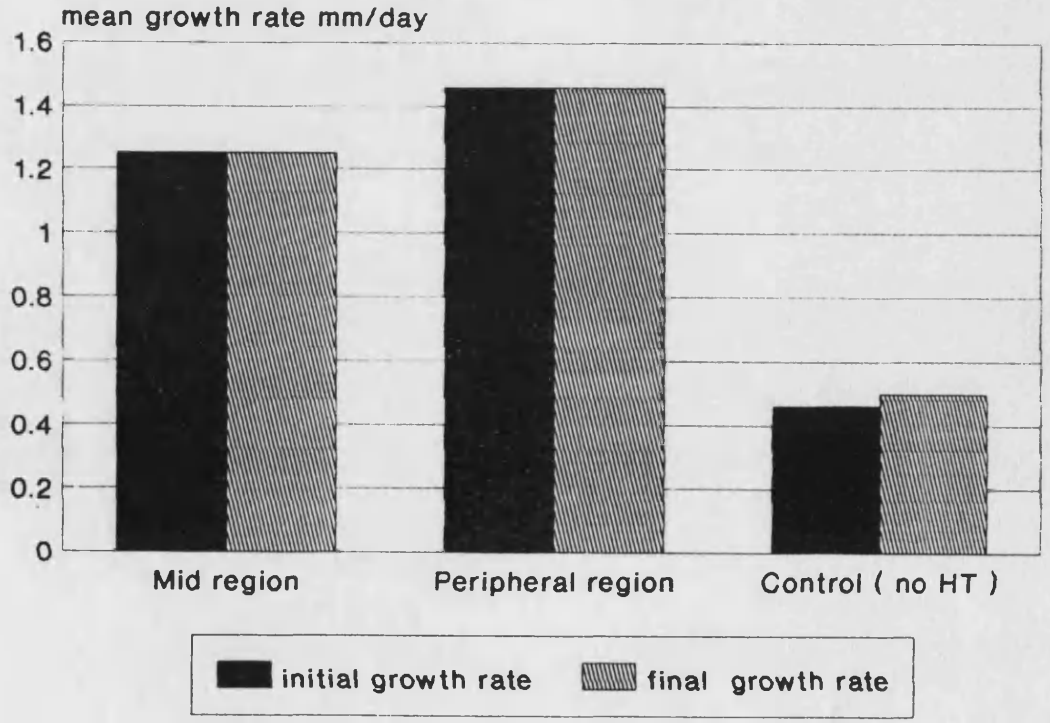
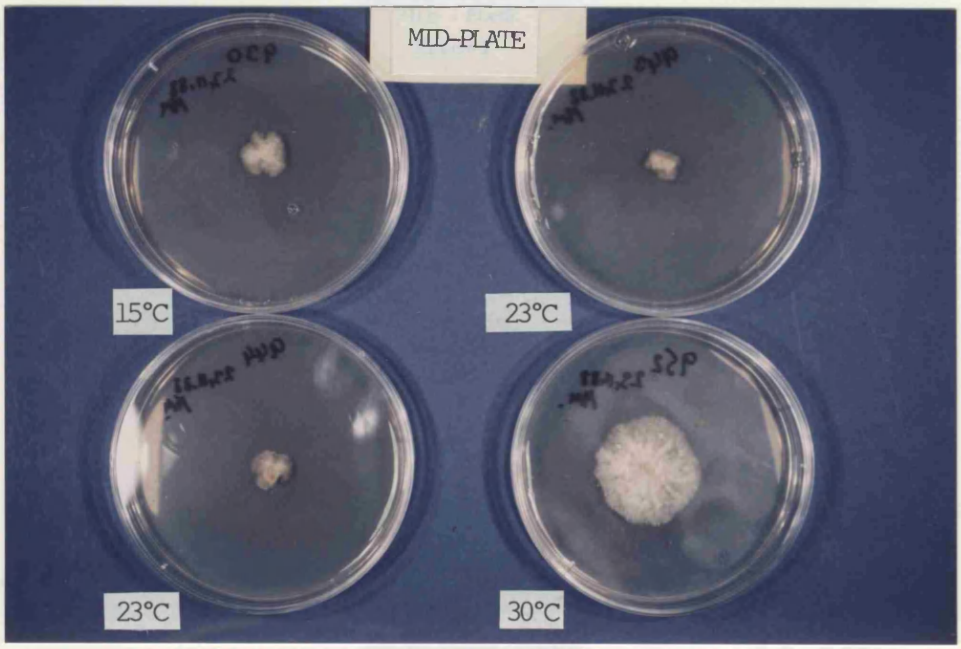


PLATE 6.1 H6F3 CULTURES ESTABLISHED ON MA FOLLOWING HEAT TREATMENT.

(i) Cultures established from mid-plate regions.



(ii) Cultures established from peripheral regions.

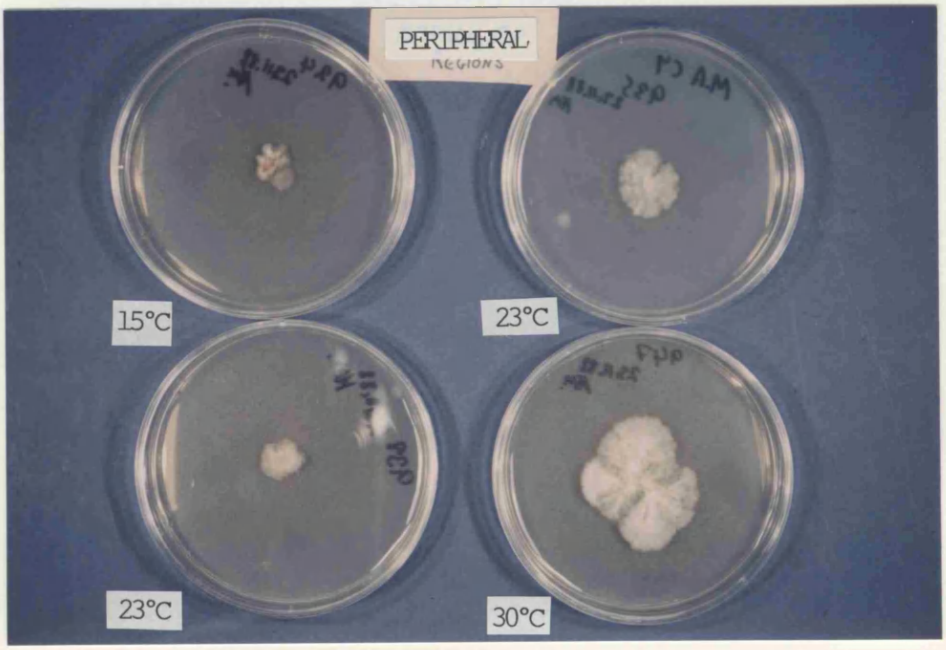


PLATE 6.2 DsRNA EXTRACTED FROM PERIPHERAL H6F3 CULTURES FOLLOWING HEAT TREATMENT

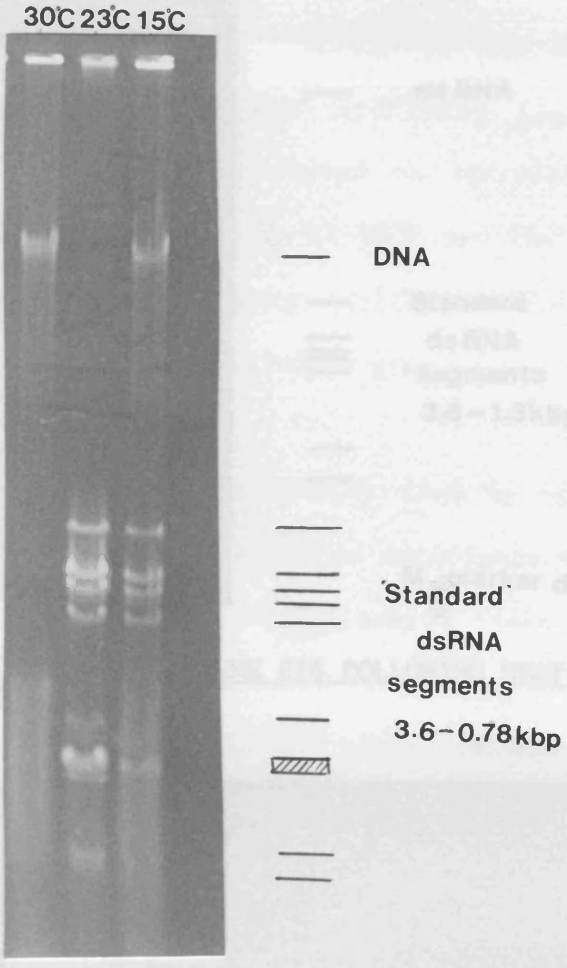


PLATE 6.3 DsRNA EXTRACTED FROM THE MITOCHONDRIA OF PERIPHERAL H6F3 CULTURES FOLLOWING HEAT TREATMENT

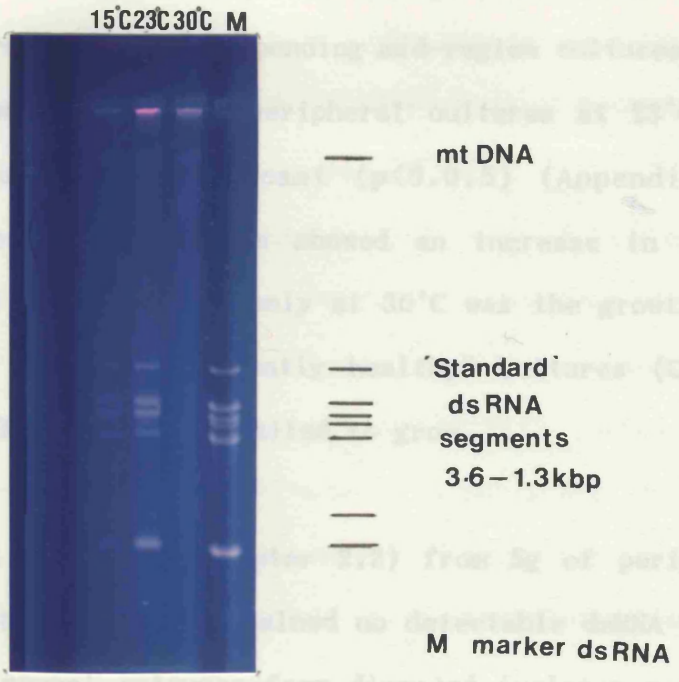
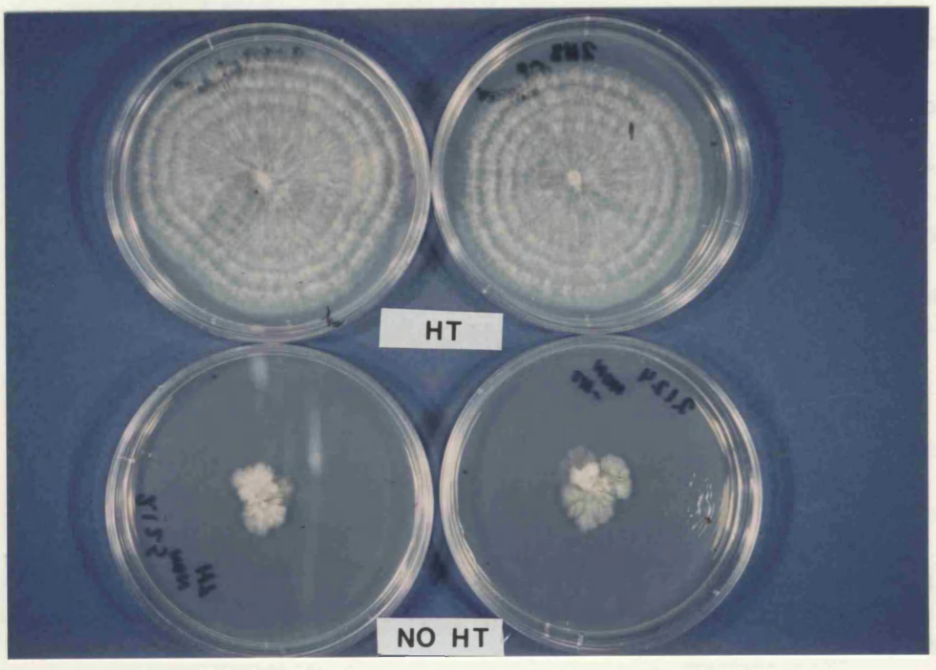


PLATE 6.4 PERIPHERAL ISOLATES OF CULTURE 616 FOLLOWING HEAT TREATMENT AT 30°C



($p>0.05$) from cultures grown at 23°C (Appendix 1). Cultures taken from peripheral regions at 15°C and 23°C showed increases over their corresponding mid-region cultures (Fig. 6.1). However, only in peripheral cultures at 23°C were these differences significant ($p<0.0.5$) (Appendix 1). Although peripheral regions showed an increase in growth rate at all temperatures, only at 30°C was the growth rate similar to that of "apparently healthy" cultures (Chapter 3). All cultures at 37°C failed to grow.

Total dsRNA extracted (Chapter 2.2) from 5g of peripheral hyphae treated at 30°C contained no detectable dsRNA (Plate 6.2). Peripheral cultures from diseased isolates grown at 23°C showed a significant increase in growth rate above that of the mid region (Fig. 6.1) but showed no apparent change in dsRNA segment concentration. DsRNA extracted from diseased mitochondria extracted from 10g of hyphae following treatment at 15°C, 23°C and 30°C showed little difference in the relative concentrations of dsRNA in 15°C and 23°C cultures (Plate 6.3). Mitochondrial cultures treated at 30°C no longer contained dsRNA (Plate 6.3).

(ii) La France diseased culture (616)

The diseased culture 616 produced by anastomosis of an "apparently healthy" U3 strain and H6F3 (Chapter 7) was heat

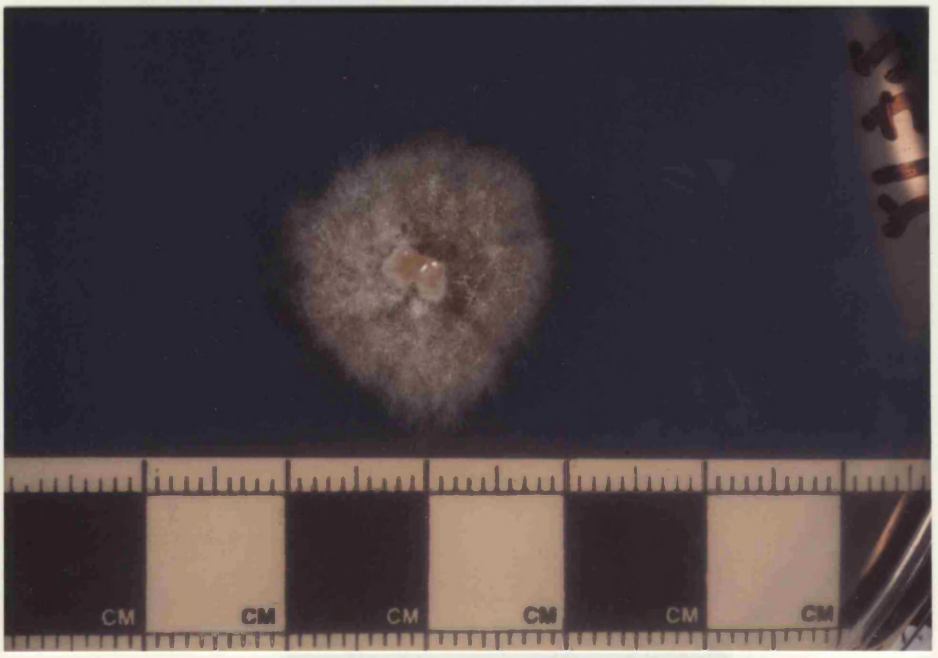
treated at 30°C. After 6 weeks at 30°C isolates from mid and peripheral regions of culture 616 had significantly higher growth rates on MA ($p<0.05$, Appendix 2) than similar isolates grown at 23°C (Fig. 6.2, Plate 6.4). Initial and final growth rates of cultures established from mid and peripheral regions showed no significant differences ($p>0.05$, Appendix) (Fig. 6.2). DsRNA extracted from mid and peripheral heat treated 616 showed neither to contain detectable dsRNA. Assessment of growth rates and morphologies of cultures established from peripheral regions ten weeks after heat treatment indicated that the majority of cultures had growth rates and morphologies similar to "apparently healthy" isolates.

(iii) La France diseased Culture F

Diseased culture F which has lost all the standard dsRNA segments except the 3.6 and 1.5 kbp dsRNAs was also subjected to heat treatment. After a 6 week heat treatment at 30°C, both mid and peripheral regions showed a significant increase in growth rate ($p<0.05$, anovar Bonferroni's inequality test), when compared to the non-heat treated disease control (Fig. 6.3 Plate 6.5 Appendix 3). However, the growth rate was still below that found for "apparently healthy cultures. DsRNA segments of the stardard pattern were not detected from mid and peripheral regions of heat treated culture F (Plate 6.6). High

PLATE 6.5 PERIPHERAL ISOLATES OF CULTURE F GROWN AT 23°C BEFORE AND AFTER HEAT TREATMENT AT 30°C

(i) Before



(ii) After

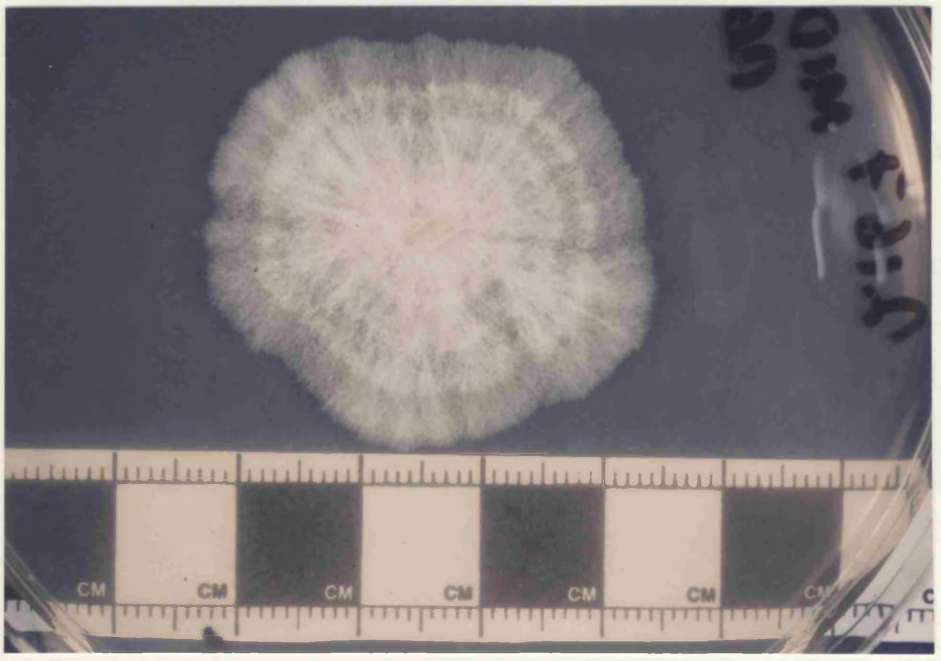
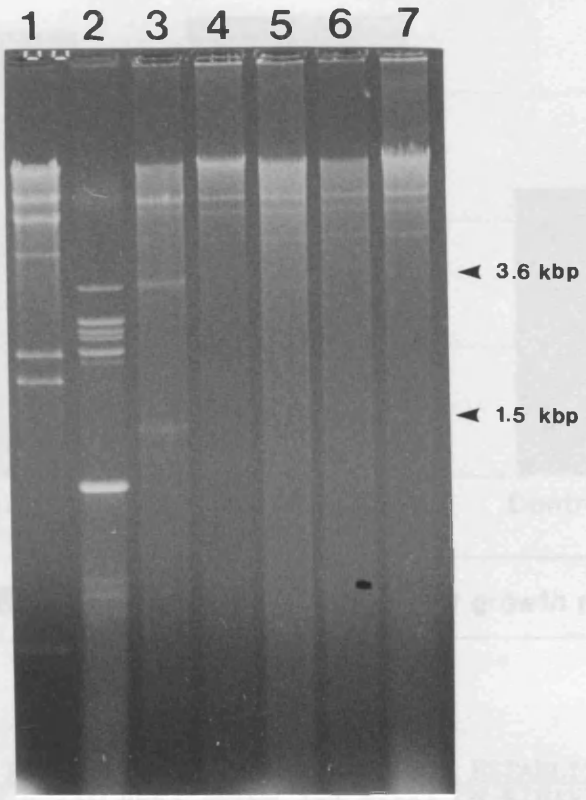


PLATE 6.6 AGAROSE GEL ELECTROPHORESIS OF dsRNA EXTRACTED FROM PERIPHERAL AND MID ISOLATES OF HEAT TREATED CULTURE F



(1) λ DNA digested with Hind III (300 ng).

(2) "Standard" dsRNA segments.

(3) No heat treatment (23°C).

(4) Cultures established from mid regions of heat treated colonies.

(5) Cultures established from mid regions of heat treated colonies.

(6) Cultures established from peripheral regions of heat treated colonies.

(7) Cultures established from peripheral regions of heat treated colonies.

NB dsRNA was extracted from 5g of mycelia in each case.

FIG. 6.3 MEAN GROWTH RATES OF CULTURES ESTABLISHED FROM MID AND PERIPHERAL REGIONS OF HEAT TREATED CULTURE F

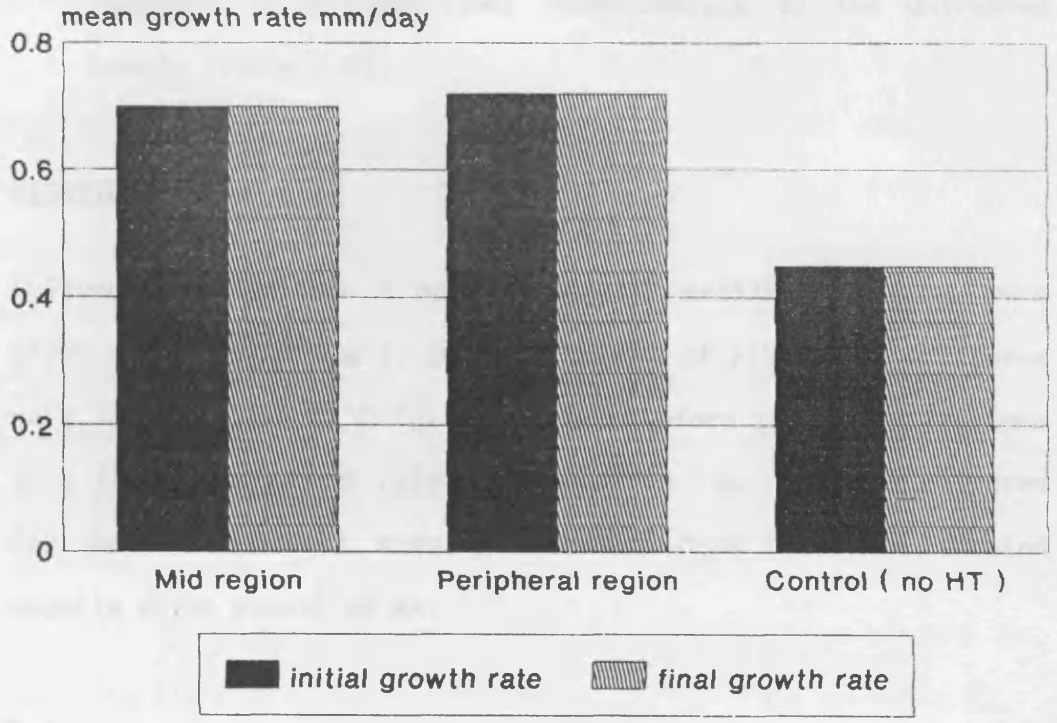
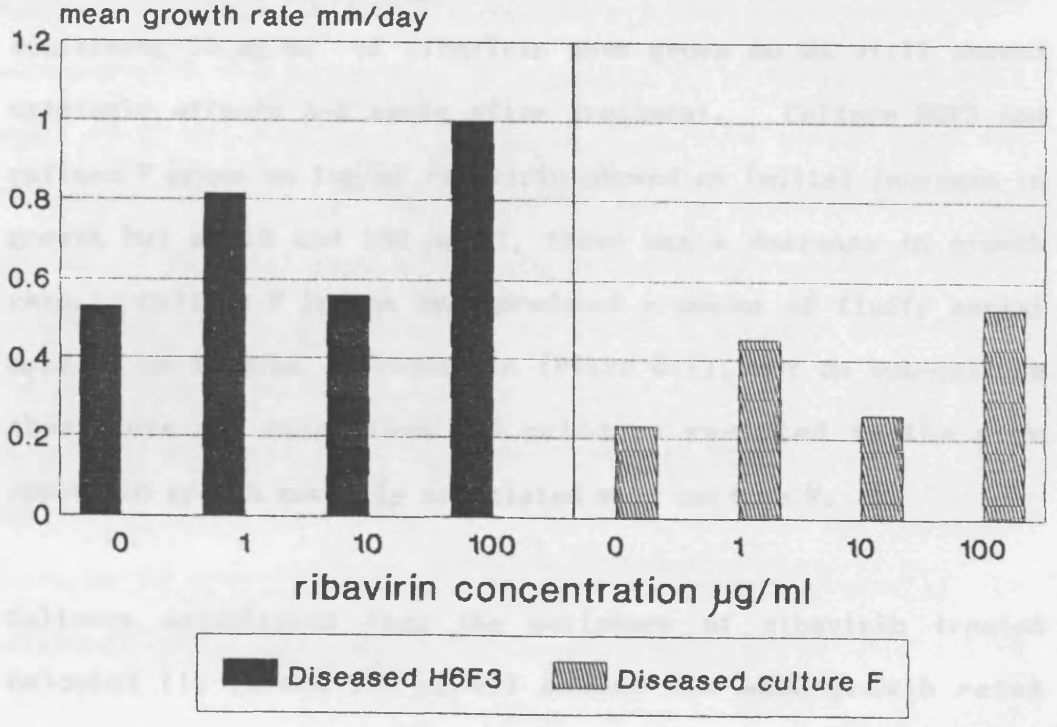


FIG. 6.4 MEAN GROWTH RATES OF PERIPHERAL ISOLATES ESTABLISHED FROM CULTURE F AND H6F3 AFTER 3-4 WEEKS ON RIBAVIRIN



molecular weight 8.6, 8.1 and 6.6 kbp dsRNA segments remained at an equivalent concentration to the untreated sample (Plate 6.6).

6.3 RIBAVIRIN

Culture H6F3, Culture F and "apparently healthy" isolates were grown on MA containing 1, 10 and 100μg/ml of ribavirin. Cultures were incubated at 23°C for 20-25 days before peripheral regions were taken for growth rate assessment on MA. Liquid cultures for dsRNA assessment were established from ribavirin treated mycelia after growth on MA.

Ribavirin had a cytotoxic effect on "apparently healthy" cultures at concentrations of 10 and 100 μg/ml A concentration of 1 μg/ml did not appear to affect growth rates when compared to those on MA. "Apparently healthy" cultures taken from plates containing 10 μg/ml of ribavirin when grown on MA still showed cytotoxic effects 3-4 weeks after treatment. Culture H6F3 and culture F grown on 1ug/ml ribavirin showed an initial increase in growth but at 10 and 100 μg/ml, there was a decrease in growth rate. Culture F in one case produced a sector of fluffy aerial mycelia on 1 μg/ml of ribavirin (Plate 6.7), but on sub-culture these were not maintained and cultures reverted to the slow appressed growth normally associated with culture F.

Cultures established from the periphery of ribavirin treated colonies (1, 10 and 100 μg/ml) showed the mean growth rates

indicated in Fig. 6.4. Treatment of H6F3 with 1µg/ml and 100 µg/ml of ribavirin resulted in a significant increase in growth rate when compared to MA (Fig. 6.4, Appendix 4(i)). A greater abundance of cord like structures were evident and an increase in the amount of aerial hyphae was observed (Plate 6.8). The increase in growth rate of culture F after treatment with 1µg/ml and 100 µg/ml of ribavirin was not significantly different from non-treated cultures (Fig. 6.4 ($p>0.05$) Appendix 4(ii)). However, on 100 µg/ml ribavirin culture F also produced more aerial hyphae and a rhythmic type of growth commonly associated with "apparently healthy" mycelia (Plate 6.9). Growth of peripheral cultures (H6F3 and F) grown on 10 µg/ml ribavirin showed no significant difference from growth on MA (Fig. 6.4, Appendix 4).

Ribavirin treated cultures with the healthiest morphology and fastest growth rate were placed into liquid culture for dsRNA analysis. DsRNA extraction was carried out for diseased H6F3 cultures with the following growth rates, MA (0.53 mm per day),1 µg/ml (0.92 and 0.77 mm per day), 10 µg/ml 0.47 mm per day) and 100 µg/ml (1.0 mm per day). DsRNA extracted from equal wet weights of diseased mycelia was electrophoresed overnight. Lower concentrations of the "standard" dsRNA segments were observed in the more rapidly growing 1 ug/ml treated cultures (Plate 6.10 (i)) when compared with the non-treated H6F3 culture. The culture treated with 10 µl/ml ribavirin showed no apparent increase in growth rate and had similar concentrations of the "standard" dsRNA segments to the non-treated control (Plate 6.10

PLATE 6.7 AERIAL SECTORS PRODUCED FROM CULTURE F ON 1µg/ml OF RIBAVIRIN

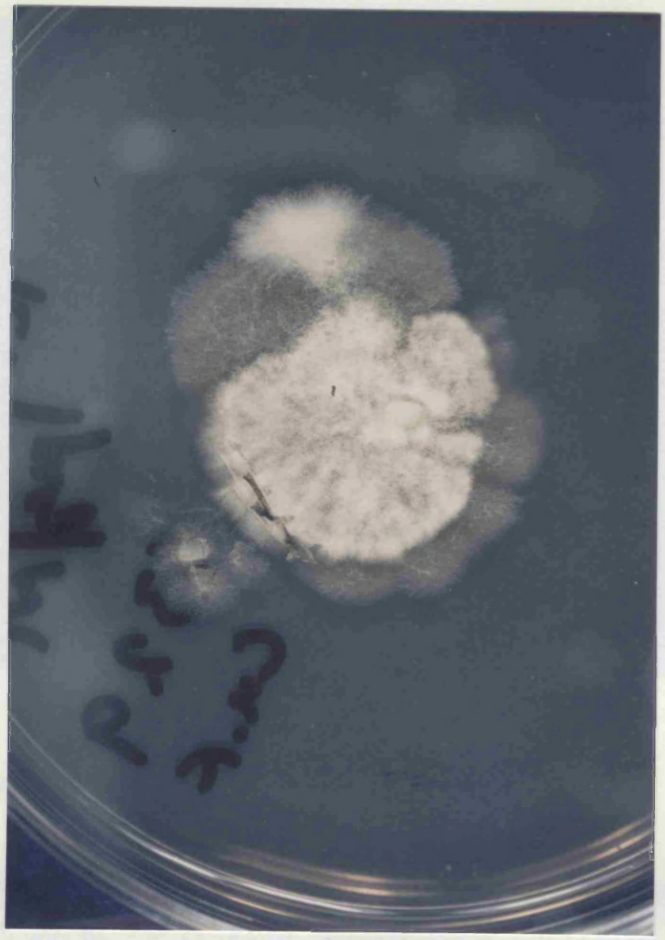
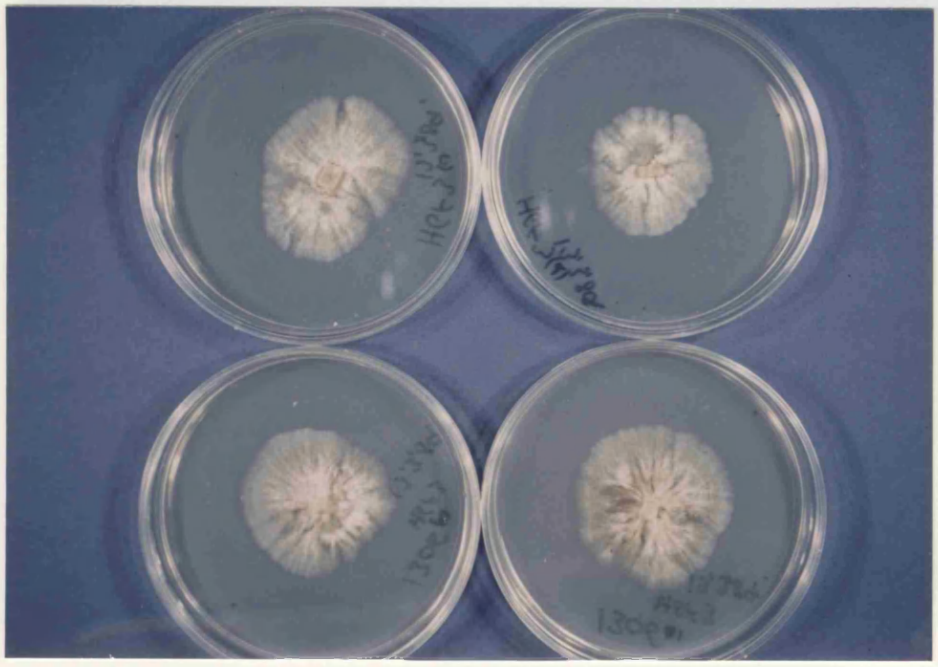


PLATE 6.8 PERIPHRAL ISOLATES OF H6F3 ON MA AFTER 3-4 WEEKS TREATMENT ON RIBAVIRIN

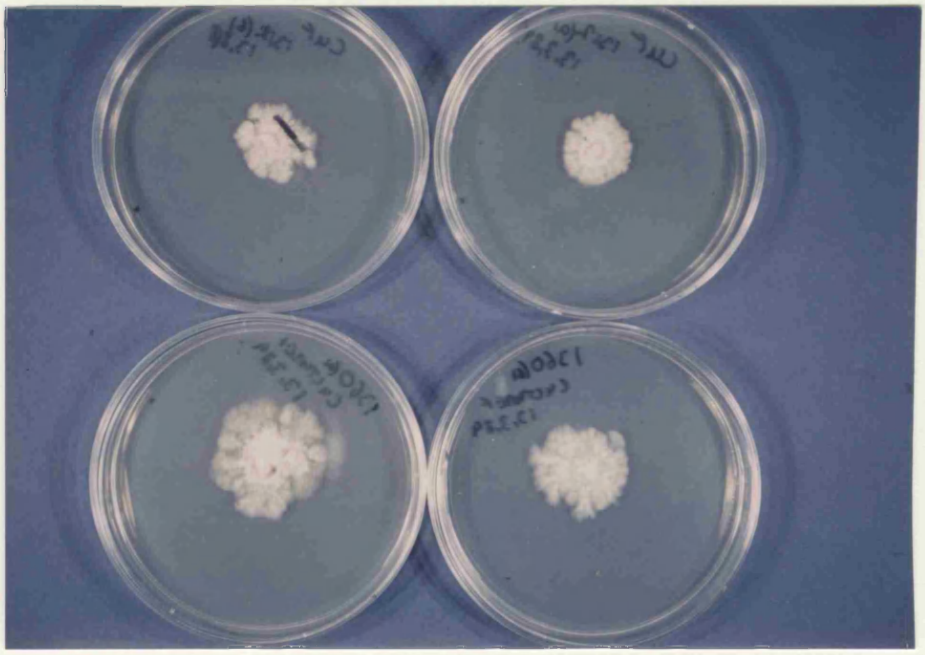
(i) Control



(ii) 100µg/ml ribavirin.

PLATE 6.9 PERIPHERAL ISOLATES OF CULTURE F ON MA AFTER 3-4 WEEKS TREATMENT WITH RIBAVIRIN

(i) Control



(ii) 100µg/ml ribavirin.

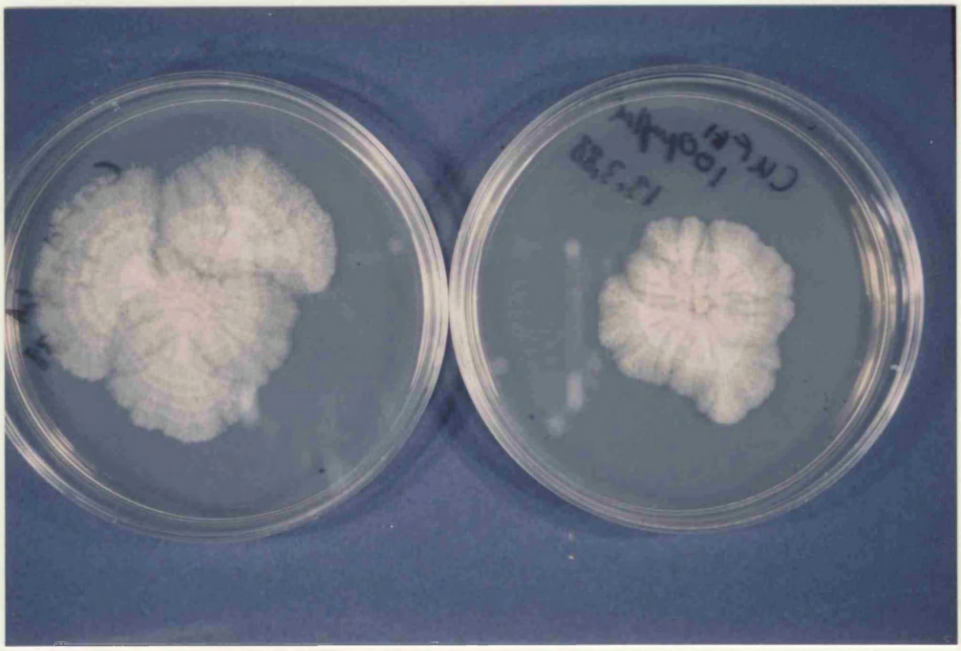


PLATE 6.10 AGAROSE GEL ELECTROPHORESIS OF dsRNA EXTRACTED FROM PERIPHERAL ISOLATES OF RIBAVIRIN TREATED CULTURE F AND H6F3

(1) λ DNA Hind III digest.

(2) "Standard" dsRNA segments (100ug).

(3) " " " (300ng).

(4) No treatment (control).

(5) 1µg/ml ribavirin.

(6) " "

(7) 10µg/ml ribavirin.

(8) 100µg/ml "

(9) "Standard" dsRNA segments.

(10) "Apparently healthy".

(11) λ DNA Hind III digest.

(12) "Standard" dsRNA segments (300ng).

(13) No treatment (control).

(14) 1µg/ml ribavirin.

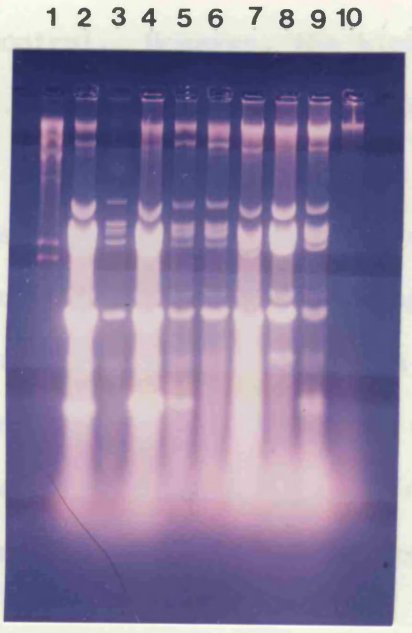
(15) 10µg/ml ribavirin.

(16) 100µg/ml ribavirin.

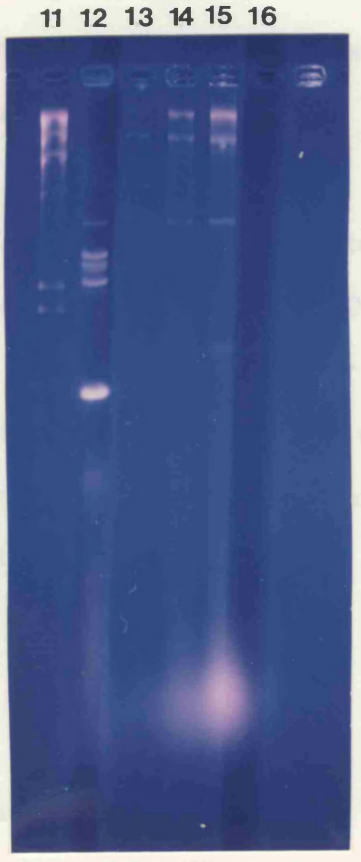
* DsRNA was extracted from equal wet weights of mycelium.

PLATE 6.10

(i) H6F3



(ii) Culture F



(i)). Diseased H6F3 treated with 100 µg/ml ribavirin showed an increase in growth rate but appeared to contain approximately equal amounts of the "standard" dsRNA segments as the non-treated control. However, the high molecular weight dsRNA segments 8.6, 8.1 and 6.6 kbp (Plate 6.10 (i)) appeared extremely faint.

DsRNA extraction was carried out on ribavirin treated culture F cultures with the following growth rates, no treatment, (0.23mm per day), 1 µg/ml (0.57 mm per day), 10 µg/ml (0.25 mm per day) and 100 µg/ml (0.66 mm per day). No obvious differences in dsRNA levels were shown between ribavirin cultures showing growth rate increases and non-treated cultures (Plate 6.10 (ii)).

6.4 CYCLOHEXIMIDE

Culture H6F3, Culture F and "apparently healthy" isolates were grown on MA containing 5, 15, 25 and 50 µg/ml of cycloheximide. Growth rates were monitored on cycloheximide and after 5-6 weeks cultures were established on MA from the peripheral regions of different treatments. Growth rates were monitored and liquid cultures established from the more rapidly growing isolates of each treatment.

The cytotoxic effects of cycloheximide were evident as a reduction in the growth rate of the "apparently healthy" isolate when the cycloheximide concentration was gradually increased. Diseased cultures H6F3 and F showed little difference in growth

FIG. 6.5 MEAN GROWTH RATES OF PERIPHERAL ISOLATES ESTABLISHED FROM CULTURE F AND H6F3 AFTER 5-6 WEEKS ON CYCLOHEXIMIDE

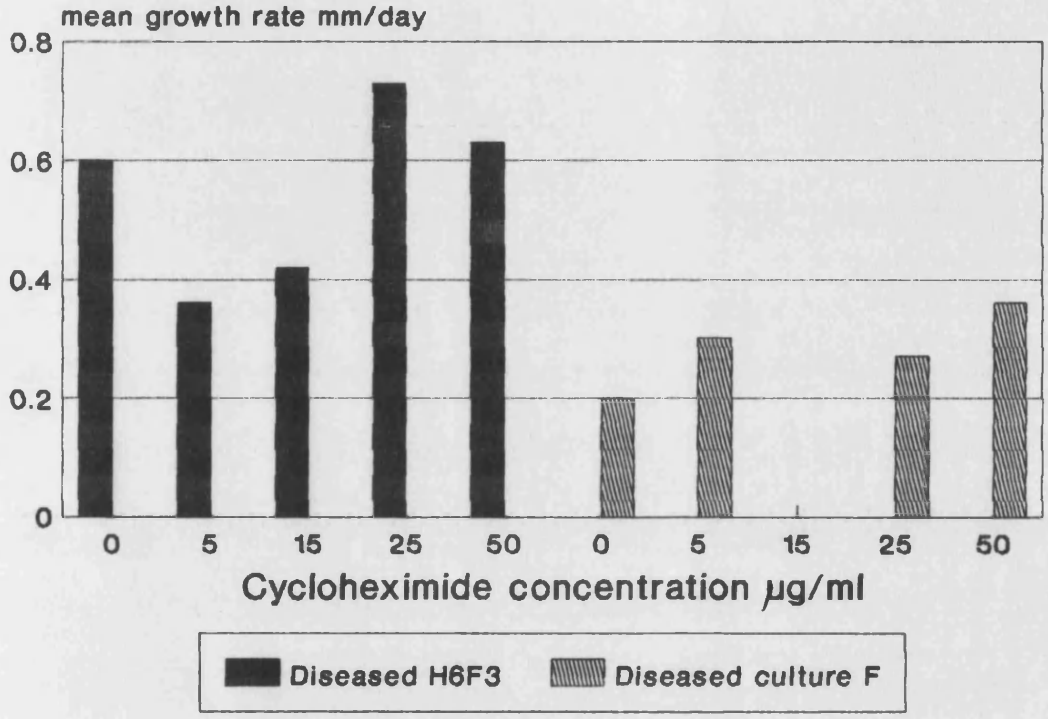
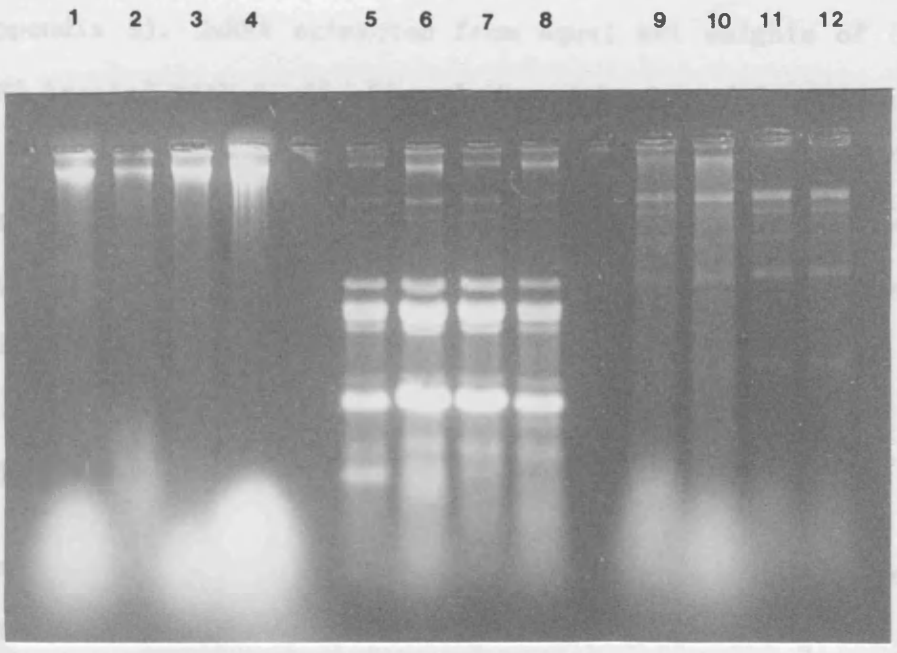


PLATE 6.11 AGAROSE GEL ELECTROPHORESIS OF dsRNA EXTRACTED FROM PERIPHERAL ISOLATES OF CYCLOHEXIMIDE TREATED CULTURE F, H6F3 AND "APPARENTLY HEALTHY" CULTURES



(1) "Apparently Heathy" 5µg/ml cycloheximide
(2) " " 10µg/ml "
(3) " " 25µg/ml "
(4) " " 50µg/ml "
(5) H6F3 5µg/ml cycloheximide
(6) " 10µg/ml "
(7) " 25µg/ml "
(8) " 50µg/ml "
(9) culture F MA
(10) " 5µg/ml cycloheximide
(11) " 25µg/ml "
(12) " 50µg/ml "

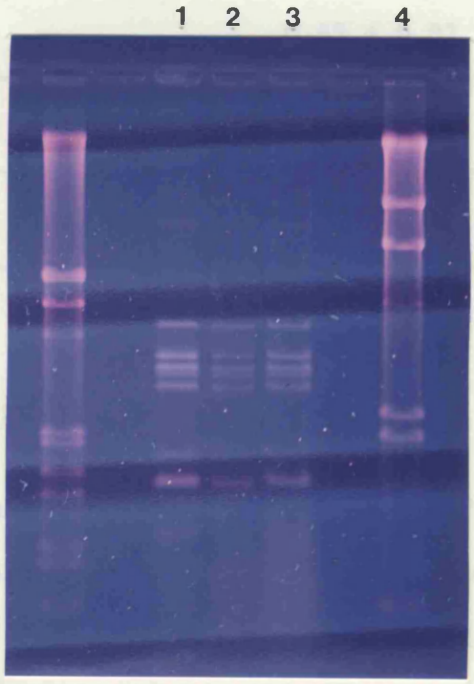
rate even when the cycloheximide concentration was raised to 50 µg/ml. Growth rates of diseased cultures established on MA after cycloheximide treatment showed no significant differences from the growth rates of non-treated diseased mycelia (Fig. 6.5) (Appendix 5). DsRNA extracted from equal wet weights of culture H6F3 treated with 5, 15, 25 and 50 µg/ml of cycloheximide showed no obvious differences in the intensities of the "standard" dsRNA segments (Plate 6.11). DsRNA from culture F also showed no apparent difference between the 5, 50 µg/ml treated samples (Plate 6.11).

6.5 ETHIDIUM BROMIDE

Diseased culture H6F3 containing the standard dsRNA segment pattern and "apparently healthy" cultures were grown on MA containing 10^{-6}, 10^{-5}, 10^{-4} M ethidium bromide (EtBr). After 4 weeks, cultures from peripheral regions were established on MA. Growth rates were monitored throughout the experiment and liquid cultures established for dsRNA extraction.

At concentrations of 10^{-4}M, EtBr had an extremely toxic effect on diseased H6F3 and "apparently" healthy cultures. At lower concentrations, (10^{-5}M and 10^{-6}M), EtBr had no discernable effect on the growth rates of healthy or diseased cultures (Table 6.1). No difference in growth rates was observed in diseased cultures grown in 10^{-5} and 10^{-6}M EtBr. Similar amounts of dsRNA were present in cultures grown on MA containing 10^{-5} and 10^{-6}M Etbr and control cultures not containing Etbr (Plate 6.12).

PLATE 6.12 AGAROSE GEL ELECTROPHORESIS OF dsRNA EXTRACTED FROM PERIPHERAL ISOLATES OF ETHIDIUM BROMIDE TREATED H6F3



1 10^{-5}M ethidium bromide

2 10^{-6}M

3 MA

4 DNA Hind III digest

TABLE 6.1 MEAN GROWTH RATES OF PERIPHERAL CULTURES ESTABLISHED AFTER 4 WEEKS ON ETHIDIUM BROMIDE

Ethidium Bromide Concentration	H6F3	U3(X)
MA	0.53 ± 0.07	2.4 ± 0.0
10^{-6} M	0.37 ± 0.04	2.4 ± 0.04
10^{-5} M	0.54 ± 0.19	2.46 ± 0.06

6.6 CHLORAMPHENICOL

Diseased cultures H6F3 containing the "standard" dsRNA segments and culture F which has lost the standard dsRNA segments, except the 3.6 and 1.3 kbp dsRNAs and "apparently healthy" isolates were grown on MA containing 0, 50, 75, 100 and 200 µg/ml of chloramphenicol. After 5-6 weeks cultures were established on MA from peripheral regions of the different treatments. Growth rates were monitored and liquid cultures established from the more rapidly growing isolates for dsRNA extraction.

Apparently healthy cultures seemed fairly tolerant to the cytotoxic effects of chloramphenicol (Table 6.2). However, growth rates of diseased cultures, particularly H6F3, were strongly inhibited by chloramphenicol. Little change was seen in growth rates when diseased and healthy cultures were returned to

MA following treatment (Table 6.3). Unfortunately, culture contamination prevented dsRNA extraction being carried out so data on dsRNA levels is unavailable.

TABLE 6.2 EFFECT OF CHLORAMPHENICOL ON THE GROWTH RATES OF DISEASED AND HEALTHY CULTURES

	Mean growth rates mm/day		
Chloramphenicol concentration µg/ml	U3(X) "Apparently Healthy"	H6F3	Culture F
MA	3.0 ± 0.57	0.51 ± 0.08	0.28 ± 0.05
50	2.13 ± 0.24	0.18 ± 0.03	0.162 ± 0.03
75	2.5 ± 0.08	0.25 ± 0.03	0.13 ± 0.0
100	2.4 ± 0.22	0.17 ± 0.05	0.1 ± 0.0
200	1.78 ± 0.19	0.1 ± 0.0	0.1 ± 0.0

TABLE.

TABLE. 6.3 MEAN GROWTH RATES OF DISEASED AND APPARENTLY HEALTHY CULTURES FOLLOWING CHLORAMPHENICOL TREATMENT

Chloramphenicol concentration µg/ml	U3(X) "Apparently Healthy"	H6F3	Culture F
MA	/	0.35 ± 0.07	0.2 ± 0.01
50	/	0.32 ± 0.05	0.16 ± 0.01
75	2.6 ± 0.05	/	0.25 ± 0.03
100	2.0 ± 0.12	0.38 ± 0.03	0.21 ± 0.00
200	1.9 ± 0.07	0.25 ± 0.02	0.17 ± 0.0

6.7 DISCUSSION

The results in Chapter 6 indicate heat treatment (HT) to be the most effective method for eliminating the La France phenotype and dsRNA. In the present study, HT at 30°C for 4-6 weeks significantly increased ($p<0.05$) the growth rates of H6F3, 616 and to a lesser extent culture F. DsRNA was not detected in HT cultures established from mid or peripheral regions of culture H6F3 or 616. However, HT mid regions from H6F3 although showing a significant increase in initial growth rate showed a decrease in final growth rate. This may indicate that although at undetectable levels dsRNA can still exert a deleterious effect on the growth rate of A.bisporus. Mitochondria isolated from HT

H6F3 also did not contain any dsRNA. Unlike the report of Koons et al., (1989) there was no effect of lower temperatures (<23°C) on the dsRNA content of diseased cultures. Differences in the severity of La France disease in culture H6F3 and 616 was reflected in the final growth rates and morphologies following HT.

Although a significant increase was shown in the growth rate of culture F following HT, it appeared harder to cure than culture H6F3 and 616. Loss of the very faint 3.6 and 1.5 kbp dsRNA segments coincided with an increase in vigour. However, the high molecular weight 8.6, 8.1 and 6.6 kbp dsRNA segments remained. Segments with similar molecular weight were not detectable in culture H6F3 or 616 after HT. The presence of the 8.6, 8.1 and 6.6 kbp dsRNA segments and the incomplete curing of the La France disease phenotype appears to implicate these dsRNA segments in disease expression. However, when detected in "apparently healthy" isolates (Chapter 3) similar sized dsRNA segments were not associated with symptoms. Whether or not the 8.6, 8.1 and 6.6 kbp dsRNAs isolated from diseased cultures were the same as those found in the "apparently healthy" has yet to be established.

A number of suggestions have been put forward for the success of HT in viral elimination. Peripheral mycelia may be outgrowing viral infection since elevated temperatures result in increased mycelial growth rates. This would have the effect of diluting

out the virus; but does not explain the observation that dsRNA can be reduced to undetectable levels in mid-regions as well as peripheral regions of HT cultures. Another hypothesis is that HT is affecting a key enzyme involved in dsRNA/virus replication, possibly the RNA polymerase or an enzyme of host origin involved in dsRNA replication. This was concluded by Koons et al., (1989) who believed that the secondary effect of HT on an enzyme involved in dsRNA or virus particle synthesis was at least as important as the mycelia growing away from the virus due to high temperature. Koons et al., (1989) also inferred that a reduction in temperature could affect the above process as much as high temperatures. No evidence was found for low temperature effects in this study.

Unlike the experiments of Last et al (1974) and Nair (1973) incubating diseased cultures at 23°C prior to HT did not appear to inhibit its effects on dsRNA elimination. Culture F showing dsRNA segment changes induced during culturing was less susceptible to HT than diseased cultures H6F3 and 616 not showing these segment changes. It is possible that in experiments by Last, et al (1974) and Nair (1973) cultures could have undergone comparable dsRNA segment changes during culturing making them less susceptible to curing by HT.

Chemical treatment of La France diseased cultures with ribavirin resulted in significant growth rate increases in culture H6F3 but not in culture F. Increase in growth of culture H6F3 after

1µg/ml of ribavirin was reflected by a reduction in the concentration of the "standard" dsRNA segments. However, with 100 µg/ml ribavirin a significant increase in growth rate was not reflected by a decrease in concentration of the "standard" dsRNA segments. A reduction in the concentration of the 8.6, 8.1 and 6.6 kbp dsRNA segments was, however, correlated with the increase in growth rate. DsRNA levels in ribavirin treated culture F appeared no different from the controls. Together with data from HT and hybridisation studies (Chapter 5) the above results appear to implicate the 8.6, 8.1 and 6.6 kbp dsRNA segments in the aetiology of La France disease.

Cycloheximide has been shown to inhibit viral dsRNA synthesis in fungal (Fink & Styles, 1972 and Fulbright, 1984) and mammalian hosts (Stryer, 1981). In eukaryotes cycloheximide inhibits protein synthesis on cytoplasmic ribosomes (80s) but does not affect the mitochondrial protein synthesising system. (Novikoff & Holtzmann, 1976). Chloramphenicol, however, affects mitochondrial not cytoplasmic protein synthesis. Variability in sensitivity to cycloheximide has been shown with S.cerevisae 80s ribosomes which are strongly inhibited whereas closely related S.fragilis 80s ribosomes are resistant (Franklin & Snow, 1981). No significant effect was observed after cycloheximide treatment on the growth rates or dsRNA content of culture H6F3 or culture F. Initially the above results were surprising as Fink & Styles (1972) and Fulbright (1984) had shown cycloheximide to eliminate dsRNA from S.cerevisae and E.parasitica. In S.cerevisae the

killer phenotype was removed in all cases (Fink & Styles, 1972). Fulbright (1984) using 10-20 μg/ml cycloheximide was able to cure most hypovirulent E.parasitica strains of dsRNA, producing the healthy phenotype. However, in certain genetic backgrounds dsRNA proved impossible to eliminate; only when segments were transferred by anastomosis into a susceptible background could they be eliminated (Fulbright, 1984).

The non-susceptibility of certain E.parasitica strains to curing, as in S.fragilis (Franklin & Snow, 1981) may be due to resistance to cycloheximide. A similar explanation may be applicable to La France diseased A.bisporus. However, in all the A.bisporus strains used cycloheximide was extremely cytotoxic. A more feasible explanation revolves around differences in the cellular localisation of dsRNA in S.cerevisiae,E.parasitica and La France diseased A.bisporus. DsRNA resides in cytoplasmic virus particles of S.cerevisae (Wickner, 1980) and membranous vesicles in E.parasitica (Dodds, 1980). In both cases the dsRNA requires the 80s cytoplasmic ribosomes for translations. DsRNA in La France diseases A.bisporus appears to be mainly localised within the mitochondria (Chapter 4), so its translation is likely to be initiated by 70s ribosomes not affected by cycloheximide.

Treatment of La France diseased cultures with chloramphenicol was used to specifically inhibit mitochondrial protein synthesis. However, growth rates of diseased cultures following treatment appear no different from those prior to treatment . DsRNA

extraction from treated cultures was not carried out due to contamination problems, hence it cannot be assumed that chloramphenicol has had no effect on dsRNA levels. It has previously been shown in culture F that most of the "standard" dsRNA segments can be reduced to undetectable levels and severe La France symptoms still be expressed. The chloramphenicol experiment, is therefore worth repeating so that dsRNA levels following treatment can be ascertained.

Ethidium bromide was used to investigate the possibility that DNA plasmid release from diseased mitochondria may be responsible for La France symptoms. Previous studies with mtDNA (Chapter 4) had indicated that mtDNA rearrangements did not occur in diseased myclelia and this was supported by the finding that ethidium bromide had no effect on La France disease.

7. TRANSFER OF LA FRANCE DISEASE VIA HYPHAL ANASTOMOSIS AND CELL FREE METHODS

7.1 INTRODUCTION

DsRNA mycoviruses are unusual in that the majority do not lyse their hosts and are only transmitted by intracellular routes. This occurs either within an individual or between individuals by hyphal anastomosis. Basidiomycetes such as A.bisporus are septate and grow only by apical tip extension. Growth is a result of the fusion of vesicles carrying cell wall precursors and enzymes within the apical plasmalemma (Bartnicki-Garcia et al., 1978). A.bisporus hyphal tips appear to be either virus-free or contain very low levels of virus particles (Hollings, 1978). It would seem, therefore, that virus replication takes place in the distal part of the peripheral growth zone and particles are carried forward towards the tip in the net flow of protoplasm. Septal pores which allow the transfer of organelles as large as nuclei (Trinci et al., 1971) will not be a barrier to the movement of particles.

(i) Transfer by hyphal anastomosis

Hyphal anastomosis as a means of transmission of dsRNA mycoviruses has been demonstrated for several fungi including Penicillium chrysogenum (Lhoas, 1971), E.parasitica (Anagnostakis, 1979), R.solani (Castanho & Butler, 1978) and A.bisporus (van-Zaayan 1979). Hyphal anastomosis is generally limited to individuals within species (Delhotal, 1976) though even within a species transmission is likely to be restricted by vegetative incompatibility (Caten, 1972). Fusion incompatibility

occurs in different anastomosis groups of R.solani (Finkler et al., 1985) where the inability of hyphae to fuse restricts viral transfer. Anagnostakis (1982) concluded that the efficiency of transmission of dsRNA in E.parasitica was inversely related to both the number of vegetative compatability (V-C) gene differences and the "strength" of individual V-C genes. Brasier (1984) came to similar conclusions with d^2-factor transfer in C.ulmi, transfer was only 4% successful when all the V-C genes were different, 50% when only one V-C gene was different and 100% when all V-C genes were the same.

La France disease is spread as a result of anastomosis between hyphae of a healthy colony and either germinating infected spores or hyphae remaining in the growing trays (Gandy, 1960a, 1960b). The use of strains of A.bisporus from different V-C groups for successive crops has been employed as a method of reducing the spread of La France disease. A.bitorquis has been regarded as immune to La France disease because cultures did not become diseased when inoculated with infected mycelial fragments of A.bisporus (van-Zaayan 1976). Vegetative incompatibility between the two strains could also have prevented virus transmission.

(ii) Cell free transmission

Numerous attempts to infect fungal mycelium with purified virus preparations have been unsuccessful and it is generally considered that the cell wall is a barrier to virus penetration (Hollings, 1962). Hollings et al., (1968) and van-Zaayan (1968) reported that A.bisporus could be

infected by injection of partially-purified virus preparations from diseased mushrooms into developing sporophores. However, the process was very inefficient and difficult to reproduce and it is hard to be certain that chance contamination from air-borne spores or spores in the virus preparation did not occur. Apparently as few as one to ten spores are sufficient to initiate infection (Schisler & Sinden, 1967). The finding that mushroom spawns can contain at least low levels of particles (Nair, 1972, Passmore & Frost 1979), introduces the possibility that a latent infection may have been reactiviated in the very few cases when infection was detected.

Most current work on the infection of fungi with cell free virus preparations involves the transformation of "apparently" uninfected fungal protoplasts. Early studies proved relatively unsuccessful. However, Lhoas (1971) reported that when protoplasts of P.stoloniferum were incubated with a mixture of viruses PsV-S and PsV-F, 12 out of 20 colonies regenerated from single protoplasts were infected with PsV-S. Levels of infection were low, 10% of that of the donor strain from which the virus inoculum was obtained. This level was apparently maintained after two further single conidial subcultures. Similar results were obtained by Pallet (1976) who also reported infections of P.chrysogenum protoplasts with viruses from P.chrysogenium and P.stoloniferum. However, the level of virus in the newly infected cultures was low (1% or less of that of the donor strains) and the infection unstable, the virus becoming undetectable after 3 years of subculture.

The use of polyethylene glycol (PEG) in transformation media has generally resulted in higher transformation efficiencies. Stanway and Buck (1984) achieved infection of 10% of protoplasts of G.graminis. In this case the recipient was known to be free from virus particles and susceptible to virus infection. Virus preparations were filter sterilised and completely free from fungal propagules. Virus particles transferred to protoplasts were isolated and thoroughly characterised, showing levels of particles to be similar to those in the initial infected cultures. Levels of particles remained stable over three successive subcultures (Buck, 1986). Other fungi, H.victoriae (Ghabrial, 1986) and E.parasitica (Van-Alfen, 1984; 1987) have not proved so amenable to protoplast transformation. Ghabrial (1986) used 190S and 145S particles to transform healthy protoplasts of H.victoriae producing stunted and abnormal colonies. Thin sections of hyphae from newly diseased but not control colonies revealed the presence of aggregates of VLPs. The frequency of infection was low and isolation and characterisation of virus particles from the newly diseased tissue was not achieved.

Cell free infection of E.parasitica protoplasts with vesicles containing dsRNA was carried out by Van-Alfen (1984; 1986), dsRNA introduced into virulent strains transformed them to the hypovirulent morphology. However, dsRNA was not maintained beyond two colony transfers. Hillman (1989) has recently developed a DNA mediated transformation system for E.parasitica and is currently attempting to introduce cDNA clones specifying dsRNA encoded

gene products into virulent strains of the fungus. In S.cerevisiae viral particles extracted from K_1 and K_2 killer strains have been shown to be infectious using cell free preparations, infected cells showing the killer phenotype, (El-Sherbeini & Bostain, 1987).

7.2 ANASTOMOSIS

Attempts to transfer dsRNA/virus particles by anastomosis were carried out in order to show that the "apparently healthy" U3 isolate was susceptible to La France disease. This was essential if it was to be used for future cell free transmission experiments. Anastomosis was also used to investigate whether "disease" factors from diseased isolates no longer showing the "standard" dsRNA segments could be transferred to a compatible "apparently healthy" strain.

(i) Methods

Diseased isolates were allowed to grow on one side of a YMDA/MA plate for 2-3 weeks. A 4 mm plug from an "apparently healthy" isolate was placed approximately 30 mm from the leading edge of the diseased colony. If isolates were vegetatively compatible they would grow together and fusion would occur. Two or three weeks after fusion plugs were taken from the "apparently healthy" isolate at varying distances from the point of fusion. Growth rates of cultures infected by anastomosis were monitored prior to seeding liquid cultures for dsRNA extraction (section 2.2.1.)

(ii) Anastomosis between diseased culture F (U3 strain) and an "apparently healthy" Culture (U3 strain)

Preliminary anastomosis experiments were carried out on La France diseased culture F which had lost much of the "standard" dsRNA segment pattern but retained the high molecular weight 8.6, 8.1 and 6.6 dsRNA segments (Chapter 3). Anastomosis was carried out on YMDA as the possible implications of potential stresses caused by this media on dsRNA segment changes had not yet been realised.

Diseased culture F (strain U3) was prepared for anastomosis with the "apparently healthy" strain U3 as indicated in section 7.2 (i). Mean growth rates of plugs taken from the "apparently healthy" side of the interaction zone are shown in Fig. 7.1.

A slight reduction in growth rate (<0.5mm per day) was shown in "apparently healthy" cultures after fusion had occurred. Growth rate decreases were minimal and still indicative of an "apparently healthy" phenotype. The initial "apparently healthy" culture used as the recipient during anastamosis did not contain any dsRNA segments (Fig. 7.2). Culture F the La France diseased donnor isolate contained high molecular weight dsRNA segments 8.6, 8.1 and 6.6 kbp and the 3.6 and 1.5 kbp dsRNA segments of the standard pattern (Fig. 7.2). DsRNA extracted from the anastomosed "apparently healthy" cultures was of two distinct types (Fig. 7.2). "Apparently healthy" cultures taken 5, 24 and 45 mm from the point of interaction contained dsRNA segments 8.6, 8.1 and 6.6 kbp. Cultures 17.5 and 29 mm from the

FIG. 7.1 MEAN GROWTH RATES OF ISOLATES FROM AN "APPARENTLY HEALTHY" CULTURE AFTER ANASTOMOSIS WITH CULTURE F.

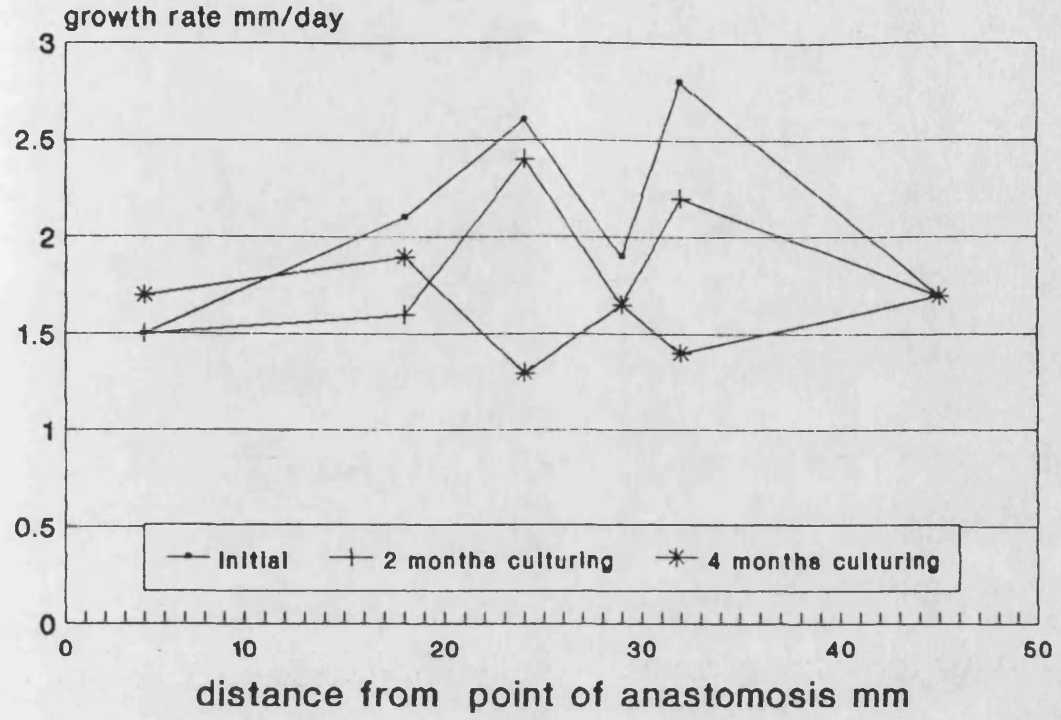
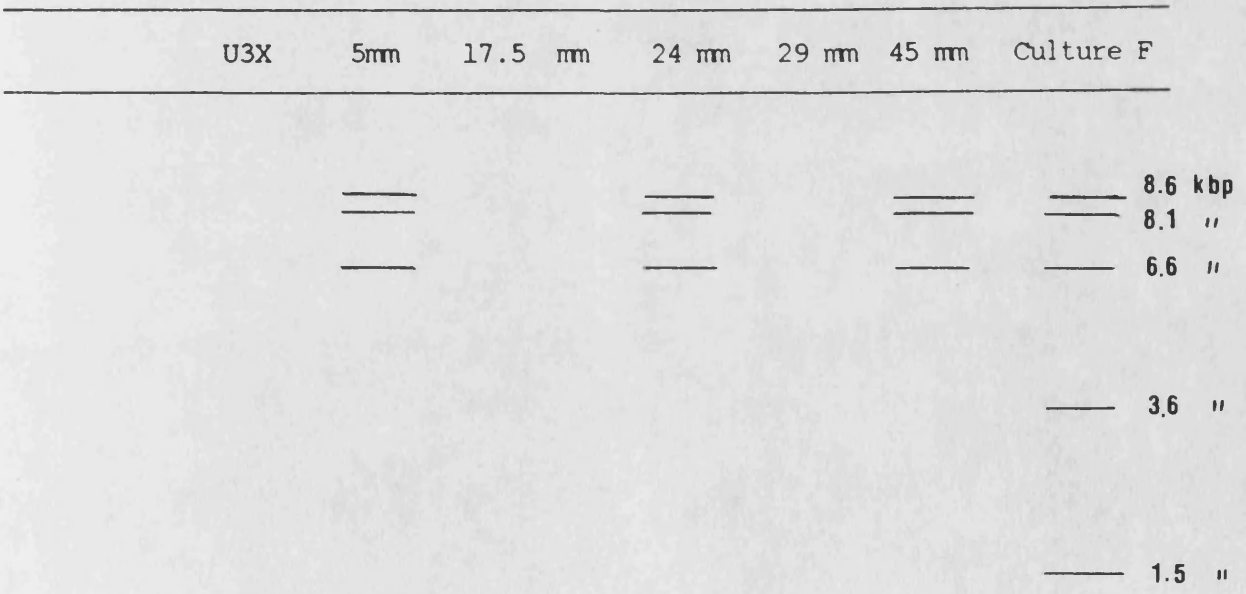


FIG. 7.2 DsRNA SEGMENTS TRANSFERRED FROM CULTURE F (STRAIN U3) TO AN "APPARENTLY HEALTHY" ISOLATE (STRAIN U3) DURING ANASTOMOSIS



point of interaction did not contain any dsRNA segments. Acquisition of dsRNA segments by the "apparently healthy" cultures did not correlate with any change in growth rates.

(iii) Anastomosis between an "apparently healthy" U3 strain, a severely La France diseased U3 strain and a moderately diseased D strain

Anastomosis on MA was carried out as in section 7.2(i) between:

(a) Culture H6F3 a La France diseased U3 strain containing dsRNA segments of the "standard" pattern and an "apparently healthy" culture (strain U3).

(b) Culture H9F2 (D-strain) an isolate showing moderate disease symptoms and an "apparently healthy" strain U3.

(c) Severely diseased H6F3 (strain U3) and the moderately diseased H9F2 isolate (strain D).

Anastomosis of H6F3 (strain U3) with the "apparently healthy" culture (strain U3) produced cultures from the "apparently healthy" side of anastomosis that showed a variety of growth rates (table 7.1). Some of the colonies produced the characteristic morphological symptoms of severe La France disease (Plate 7.1). DsRNA extraction revealed that "apparently healthy" isolates taken from up to 39 mm from the point of fusion contained the "standard" dsRNA segments 3.6 - 0.78 kbp and expressed diseased growth rates

PLATE 7.1 "APPARENTLY HEALTHY" ISOLATES (STRAIN U3) AFTER ANASTOMOSIS WITH H6F3 (STRAIN U3)

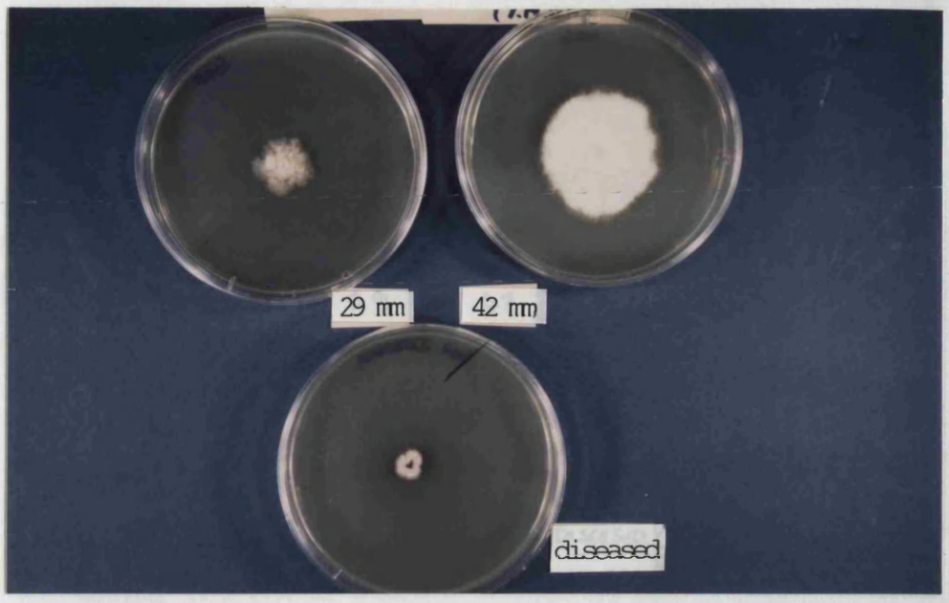
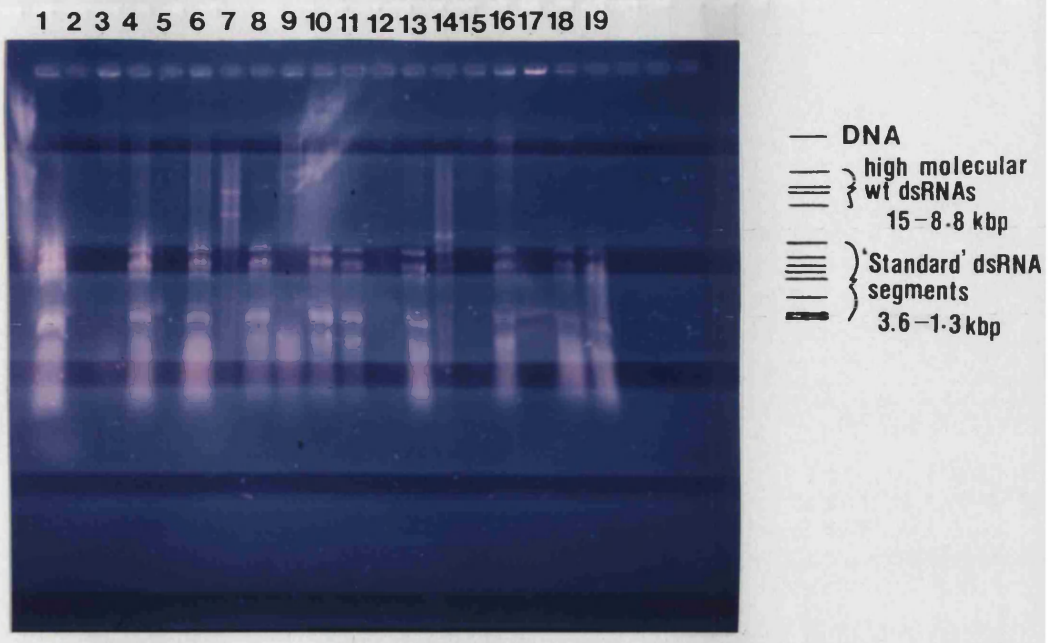


PLATE 7.2 AGAROSE GEL ELECTROPHORESIS OF dsRNA EXTRACTED FROM "APPARENTLY HEALTHY" AND DISEASED ISOLATES AFTER ANASTOMOSIS



DsRNA extracted from culture N°

Track 1	613
" 2	Empty
" 3	"Apparently healthy" control
" 4	607*
" 5	Empty
" 6	615*
" 7	λHind III digest
" 8	604
" 9	603*
" 10	605
" 11	607*
" 12	606
" 13	612
" 14	λHind III/EcoRI digest
" 15	611*
" 16	605
" 17	601
" 18	616
" 19	619*

* dsRNA extracted from the diseased side of anastomosis.

(Plate 7.2). Initial "apparently healthy" cultures did not contain any detectable dsRNA segments (Plate 7.2(3)) H6F3 cultures 607 and 615 also contained the 8.6, 8.1 and 6.6 high molecular weight dsRNAs. These segments appear to have been transferred to cultures 604, 605, 612 and 616 but were not detected in 606 (Plate 7.2).

TABLE 7.1

GROWTH RATES OF CULTURES FOLLOWING ANASTOMOSIS OF H6F3 AND H9F2 WITH THE APPARENTLY HEALTHY CULTURE

"Apparently Healthy" U3 isolate Anastomosed with	Culture No.	Distance from point of fusion (mm)	Mean Growth Rates mm/day (MA)
	600	5	2.3 mm
	601	13	1.6 mm
H9F2	602	30	1.67 mm
	603*	20	0.86 mm
	608	3	/
H9F2	609	12	2.0 mm
	610	35	1.8 mm
	611*	17	1.25 mm
	604	2	0.37
H6F3	605	29	0.62
	606	42	2.33
	607*	4	0.4
	612	3	1.22
H6F3	613	24	/
	614	46	0.8
	615*	6	0.5
	616	2	0.83
H6F3	617	23	2.3
	618	42	2.5
	619*	5	0.6

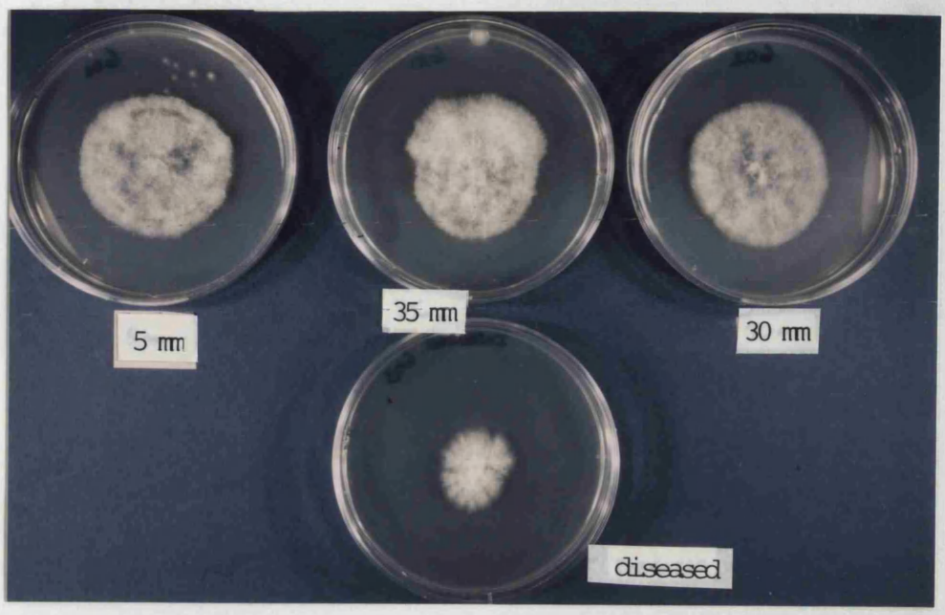
*Indicates culture established from the diseased side of anastamosis, all others from the apparently healthy side.

TABLE 7.2

GROWTH RATES OF CULTURES PRODUCED FOLLOWING ANASTOMOSIS OF H6F3 WITH H9F2

Side from which plug was taken	Culture No.	Distance from Point of Fusion (mm)	Mean Growth rates mm/day (MA)
H9F3	630	3.5	/
	631	13	0.9
	632	22	0.8
	631	33.5	1.0
H6F3	634	2.5	/
	635	10	0.53
	636	19	0.50
	637	31	0.36
H9F2	638	3	1.1
	639	12	/
	640	25.5	1.4
	641	38	/
H6F3	642	4	/
	643	8	0.5
	644	10.5	0.5
	645	21	0.7
H9F2	646	3.5	1.1
	647	11.0	1.1
	648	22.0	1.2
	649	31.0	1.2
H6F3	650	6.0	0.6
	651	13.0	0 5
	652	23.0	0.6
	653	31.0	0.5

PLATE 7.3 "APPARENTLY HEALTHY" ISOLATES (STRAIN U3) AFTER ANASTOMOSIS WITH H9F2 (STRAIN D)



Anastomosis between the moderately diseased H9F2 (D-strain) and the "apparently healthy" isolate (strain U3) produced colonies that maintained the "apparently healthy" phenotype (Plate 7.3) and growth rate (Table 7.1). DsRNA extracted from the "apparently healthy" recipient colonies produced no detectable dsRNA (Plate 7.2 (17)). Only the 8.6, 8.1, 6.6 kbp dsRNAs could be detected in the diseased H9F2 culture used in the anastomosis experiment (Plate 7.2(9,15)).

Anastomosis between the moderately diseased H9F2 (D strain) and the severely diseased U3 isolate, H6F3 appeared to result in no transfer of dsRNA. Sub cultures taken from the H9F2 side of the fusion zone produced colonies with growth rates (table 7.2) and morphologies typical of H9F2 cultures. Sub cultures taken from the H6F3 side produced colonies with growth rates (table 7.2) and morphologies typical of H6F3 cultures.

7.3 CELL FREE TRANSMISSION

(i) Protoplasts

The isolation of protoplasts from fungi using lytic enzymes is now a well established technique (Peberdy, 1989). Hyphae are treated with a mixture of cell wall degrading enzymes in an isotonic buffer solution. The type of enzymes and digestion conditions differ for different fungi (Hamlyn et al., 1981; Davis, 1985). Generally mycelia in the early phase of linear growth are preferred (Davis, 1985),

The slow growth of A.bisporus resulting in relatively low levels of cells in the early growth phase has been one of the major limiting factors in the regeneration of this species, (Lucas, 1989). A range of protoplast sizes are produced and viabilities and regeneration frequencies are low (Lucas, 1989). As a protocol for producing protoplasts from A.bisporus was not available it was decided to compare two protoplasting methods developed for other fungal species. The first, for the deuteromycete Aspergillus Niger (Buxton et al., 1985) and the second for the basidiomycete Schizophyllum commune (Munoz Rivaz, 1986). Both methods chapter 2.6.2 (i) and (ii) use $MgSO_4$ as the major component of the osmotica but differ in the way protoplasts are harvested. A third method essentially the same as Buxton et al., (1985), but using 0.6M NaCl instead of $MgSO_4$ was also attempted.

All three methods were fairly efficient in the liberation of protoplasts from "apparently healthy" cultures. (Table 7.3 (i) (ii) (iii)), and digestion times of 2-4 hours were sufficient to remove the majority of protoplasts from hyphae (Plate 7.4). Buxton et al., (1985) indicated that 10^8 protoplasts per ml were needed for the efficient transformation of Aspergillus niger with the arg B gene of A.nidulans. Total protoplast levels using all three methods were approximately 1×10^7, however, protoplast viability was poor, (5-7%). Percentage regeneration from agar overlays was very low and it was difficult to determine whether regeneration was due to individiual protoplast or contaminating hyphal fragments.

PLATE 7.4 PROTOPLAST PRODUCTION FROM A.BISPORUS HYPHAE

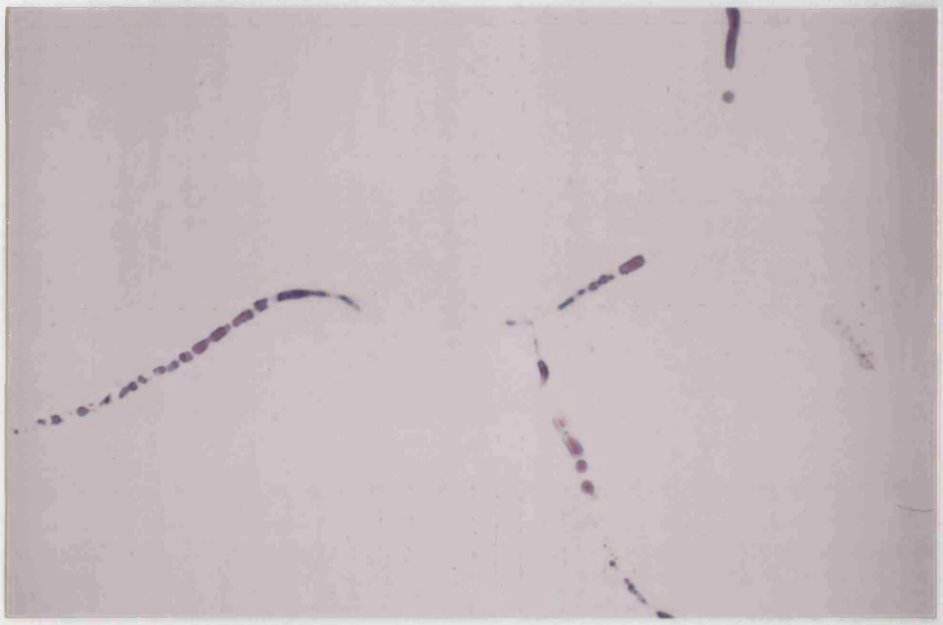


TABLE 7.3

PROTOPLAST YIELD AND PERCENTAGE VIABILITY OF THE THREE METHODS ATTEMPTED.

(i) PROTOPLASTS HARVESTED BY FLOTATION IN $MgSO_4$.

Sample No.	Mean Grid Counts *	Mean No. Protoplasts per ml.	Total Protoplasts	% Viability
1	30.13±5.1	8.0×10^6	3.2×10^7	6
2	25.26±3.4	6.7×10^6	2.7×10^7	7
3	28.4±4.7	7.6×10^6	3.0×10^7	4
4	31.0±3.0	8.26×10^6	3.3×10^7	6

* 10 replicates.

(ii) PROTOPLASTS HARVESTED BY CENTRIFUGATION, USING $MgSO_4$ AS THE OSMOTICA

Sample No.	Mean Grid Counts *	Mean No. Protoplasts	Total Protoplasts	% Viability
1	52.73±8.58	1.4×10^7	3.5×10^7	5
2	51.5±5.17	1.37×10^7	3.4×10^7	6
3	42.5±6.8	1.13×10^7	2.8×10^7	5
4	53.75±3.96	1.4×10^7	3.5×10^7	4

* 10 replicates of each.

(iii) PROTOPLASTS HARVESTED BY CENTRIFUGATION USING NaCl AS THE OSMOTICA

Sample No.	Mean Grid Counts *	Mean No. Protoplasts per ml.	Total Protoplasts	% Viability
1	45.2±6.15	1.2×10^7	3×10^7	5
2	39.1±4.2	1.0×10^7	2.5×10^7	6
3	40.0±3.5	1.1×10^7	2.8×10^7	5
4	46.4±2.0	1.2×10^7	3.0×10^7	7

* 10 replicates of each.

(ii) Whole Cell Transformation

Whole cell transformation is a relatively new method using whole fungal cells rather than protoplasts. Alkali salts coupled with PEG treatment and a brief heat shock induce competence of fungal tissue. The procedure was originally developed for yeast (Ito et al., 1982) and later extended to N.crassa (Dhawale et al., 1984). Recently Dickman (1988) transformed the alfalfa fungal pathogen Collelotrichum trifolii with genes for benomyl and hygromycin B resistance. Foreign DNA was stably integrated into the fungal chromosome.

Whole cell transformation has not yet been used for the infection of fungi with dsRNA or viruses. Due to problems encountered with protoplasting, whole cell transformation was seen as a potential method for infecting A.bisporus with virus particles/dsRNA segments.

Method

The protocol used for whole cell transformation was adapted from Dickmann (1988). "Apparently healthy" static cultures were grown at 23°C for four weeks and harvested by vacuum filtration. The fungal mat was washed with sterile TE pH 7.6 and resuspended in 0.1M lithium acetate (5ml/gm). The mixture was incubated with shaking at 30°C for 30 minutes and then centrifuged at 9,000g, 4°C for 5 minutes. The pellet was resuspended in 0.5 - 1ml of 0.1M Lithium acetate and divided into four eppendorfs containing: (i) 10-20µg melted total dsRNA in TE pH 7.6 (ii) virus particles dialysed post $CsSO_4$ gradient (iii) virus particles and 10-20µg melted total dsRNA and (iv) 20ul TE pH 7.6. All fractions were made up to a final volume of 50ul with TE pH 7.6, spermidine (Sigma) spermine (Sigma) were added at 5 mM and 1 mM respectively. Samples were incubated with shaking for 30 minutes at 30°C. Ten volumes of 40% PEG (4,000) in 0.1M lithium acetate were added and the incubation continued at 30°C for a further hour. The suspension was heated for 5 minutes at 37°C and tissue collected by centrifugation at 9,000g for 5 minutes and washed with sterile water. Centrifugation was repeated and the pellet resuspended in sterile water prior to plating on MA. After 1-2 weeks growth, colonies were randomly selected and grown on fresh MA. Mid regions of these colonies showing diseased growth rates and morphology were taken and grown on MA prior to seeding liquid cultures for dsRNA analysis.

Results

Growth rates of cultures established from a random selection of putative transformants are shown in Fig. 7.3. Colony growth rates were compared using a t-test. Mean growth rates of cultures incubated with dsRNA or virus particles, and dsRNA with virus particles were not significantly different (Appendix 6). The growth rate of control "apparently healthy" mycelia was however significantly higher than that of the three experimental treatments (Appendix 6). A comparison of the levels of sectoring between potentially transformed and "apparently healthy" control colonies were also investigated (Fig. 7.4). As previously discussed in Chapter 3, sectors are a normal feature of moderately diseased La France colonies distinguishable from the rest of the colony by their high growth rate. Conversely, "apparently healthy" colonies when grown on MA are characterised by an absence of sectors and a concentric growth pattern. Sectoring data were analysed using an STP non-parametric multiple comparison test based on the Mann-Whitney test (Appendix 7). In the analysis, all 3 experimental treatments had significantly higher sectoring levels than the control but were not significantly different from each other (Appendix 7).

Cultures generated from putative transformants produced from the 3 experimental treatments outlined above were maintained on MA for long term observation. After 6 weeks all cultures irrespective of treatment showed an increase in growth rate and apparent loss of the La France disease morphology. A paired t-test of growth rate data for each experimental treatment 3 and 6 weeks after "transformation" indicated a

FIG. 7.3 MEAN GROWTH RATES OF PUTATIVE TRANSFORMANTS PRODUCED FROM THE THREE TREATMENTS

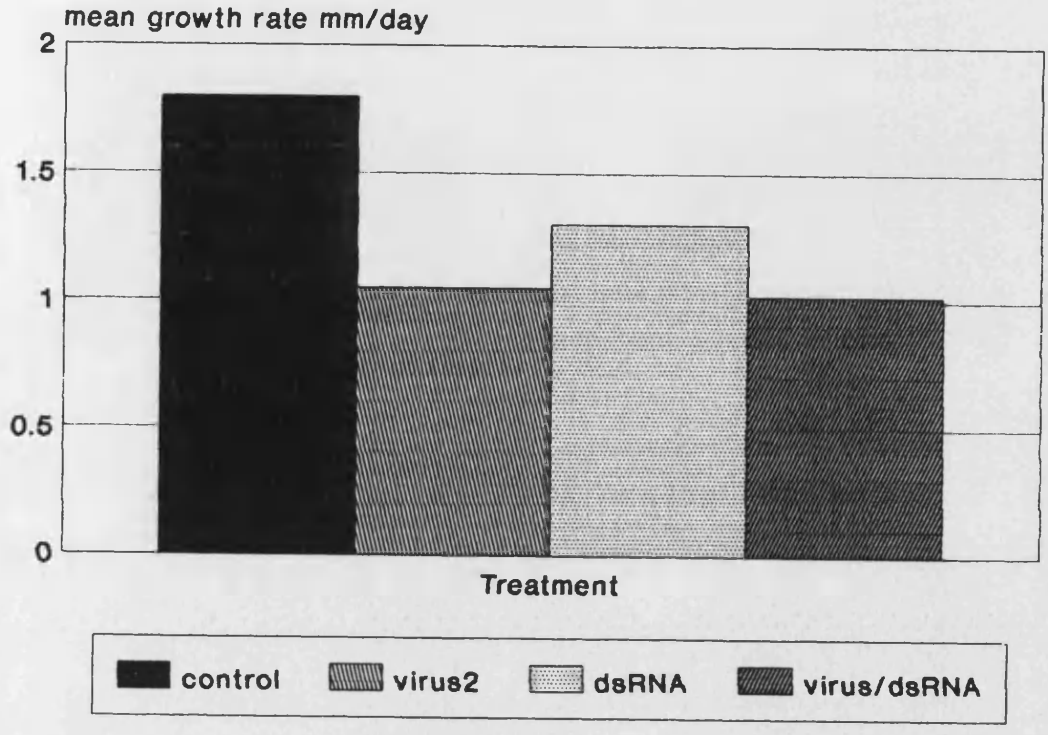
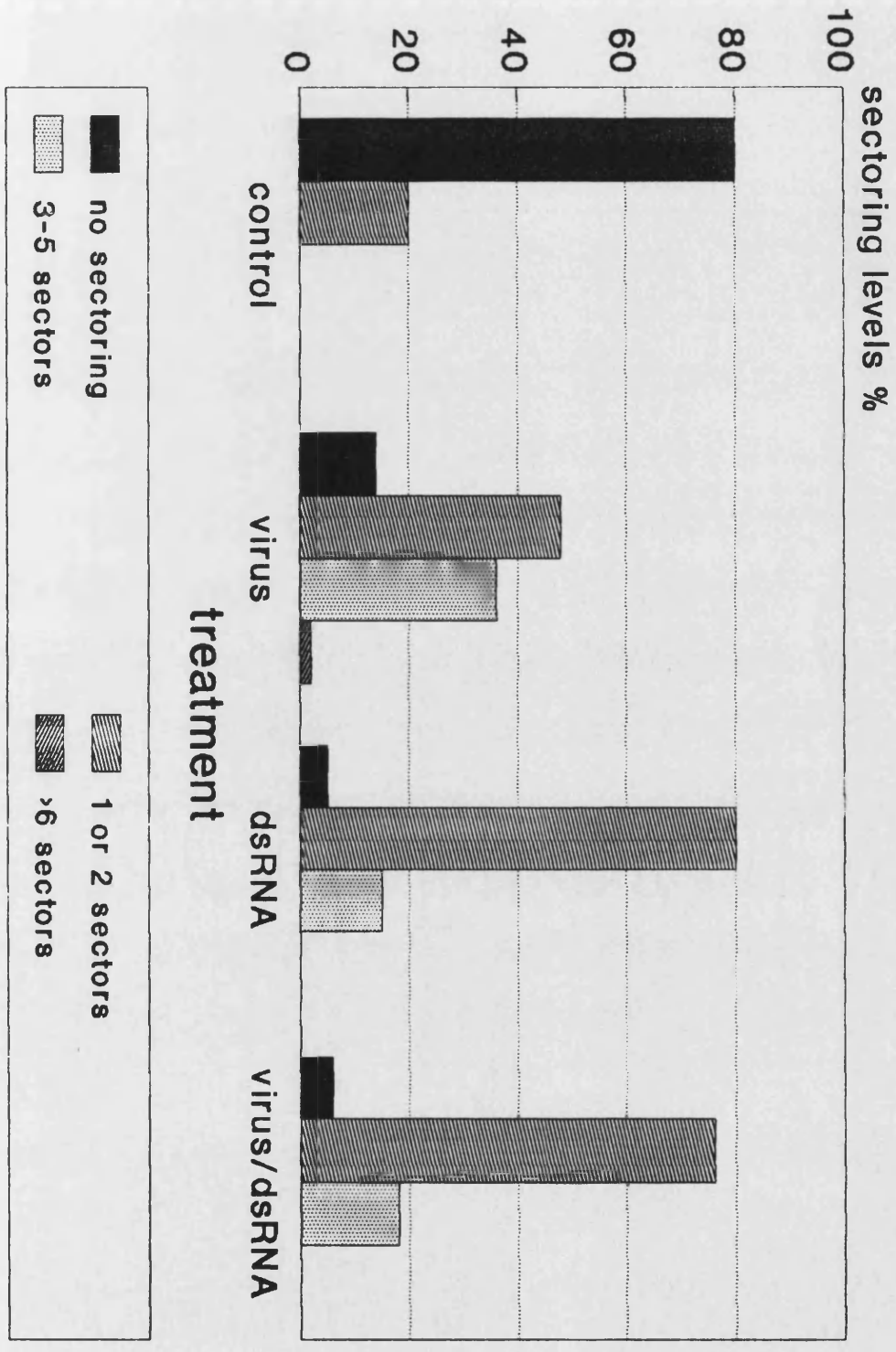


FIG. 7.4 EFFECT OF THE DIFFERENT TREATMENTS ON SECTORING LEVELS



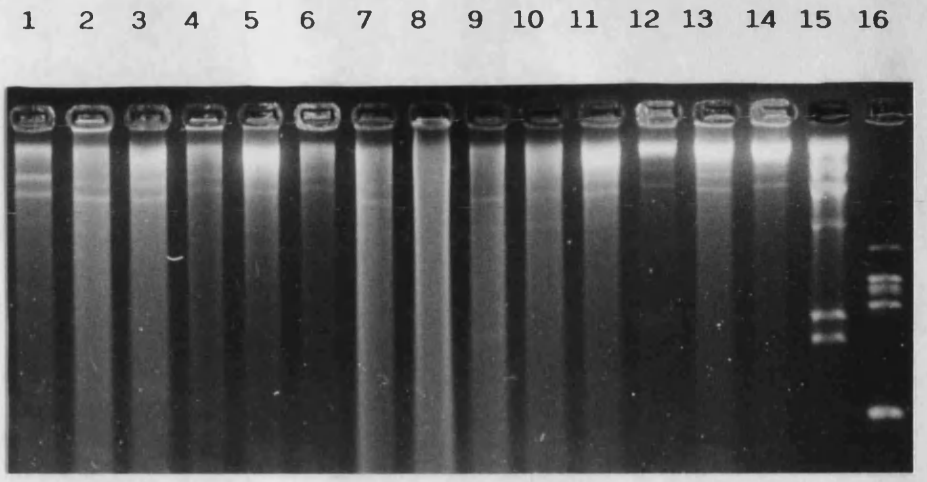
highly significant increase in growth rates of hyphae from all 3 treatments (Table 7.4).

Hyphae from colonies with the following growth rates: Controls 2.12 and 2.1mm per day; virus 0.88, 0.95, 0.92 and 1.33mm per day; dsRNA 0.71, 1.75, 0.33, 1.25mm per day; Virus/dsRNA 1.11, 1.15 and 1.4mm per day were used to seed liquid cultures for dsRNA extraction. After 6 weeks, dsRNA was purified from equal weights of mycelium; this was analysed by 1% agarose electrophoresis. The "standard" dsRNA pattern was absent from "apparently healthy" cultures as well as cultures inoculated with putative transformants from all 3 experimental treatments. High molecular weight dsRNA segments 8.6, 8.1 and 6.6 kbp were present in 3 of the 4 cultures examined for each experimental treatment (Plate 7.5). High molecular weight dsRNA segments were also present at high concentrations in all of the "apparently healthy" cultures examined.

TABLE 7.4 GROWTH RATES OF PUTATIVE TRANSFORMANTS, 3 AND 6 WEEKS AFTER TREATMENT

	3 weeks Growth Rate	6 weeks Growth Rate mm per day	Mean growth rate 3weeks	6 weeks
Control	///	///	///	1.84 ± 0.44
	0.5	1.56		
	0.56	1.36		
	0.22	0.88		
Virus	0.2	0.95	0.58 ± 0.15	1.17 ± 0.26
	0.66	1.5		
	0.77	1.2		
	0.58	0.92		
	0.57	0.88		
	0.72	1.33		
	0.66	0.71		
	0.88	1.25		
	0.8	1.13		
dsRNA	1.14	1.66	0.67 ± 0.28	1.17 ± 0.47
	0.25	1.4		
	0.44	1.75		
	0.5	0.33		
	0.2	1.11		
	0.5	1.26		
dsRNA/ virus	0.4	1.77		
	0.35	1.04	0.41 ± 0.20	1.26 ± 0.18
	0.33	1.15		
	0.86	1.6		
	0.25	1.4		

PLATE 7.5 AGAROSE GEL ELECTROPHORESIS OF dsRNA EXTRACTED FROM CONTROL AND TREATED CULTURES FOLLOWING ATTEMPTED WHOLE CELL TRANSFORMATION



(1-3) Controls
(4-6) DsRNA / virus treatment
(7-10) DsRNA "
(11-14) Virus "
(15) λ DNA Hind III digest
(16) DsRNA segments of the "standard" pattern

7.4 DISCUSSION

The invasive spread of extrachromosomal elements during hyphal fusion is a phenomenon which has been established for several decades. Dickson (1936) showed that as a consequence of hyphal contact between a fluffy variant and normal Coprinus macrorhysis the normal receptor mycelia acquired the fluffy characteristic regardless of mating type. Since these early studies, nuclei (Snider, 1963, 1965), mitochondria (Sanford & Skoropad, 1955, Scazzacchio, 1986), DNA plasmids (Gunge, 1983) and viruses (Buck, 1986) have been shown to be transferred during hyphal anastomosis. It is established in A.bisporus that transfer and spread of La France disease can occur via hyphal anastomosis (Hollings, 1962, Hollings et al., 1963 and van-Zaayan, 1979).

Results shown in chapter 7 indicate that La France disease is only transferred between vegetatively compatible isolates when the diseased culture contains the "standard" dsRNA segments. In all cases where the La France phenotype was observed following anastomosis the "standard" dsRNAs were detected. The distance sub-cultures are taken from the point of fusion is also an important factor in the spread of the disease. Cultures taken more than 42mm from the point of fusion did not show disease symptoms or contain the "standard" dsRNA segments. A similar phenomenon has been observed in the field where the degree of disease severity decreases as the distance from the point of infection increased. (Hicks & Haughton, 1986; Last et al., 1974). High molecular weight dsRNA segments 8.6, 8.1 and 6.6 Kbp were also occasionally transmitted, although there was no obvious correlation between their presence and the severity of the

disease. Generally, high molecular weight dsRNA segments were only found in cultures established from regions at or near the point of anastomosis, It is therefore possible that although these segments are transferable they are only able to move short distances. Alternatively it is possible that anastomosis between "apparently healthy" and diseased strains is acting as an inducer of these segments in "apparently healthy" isolates. Cell free infectivity studies (Chapter 7.3) and cultural effects (Chapter 3) indicate that high molecular weight dsRNA segments may be inducible.

Diseased cultures which had lost the standard dsRNA segments, (except low concentrations of the 3.6 and 1.5 kbp dsRNAs) were not able to transfer the disease-causing factors to "apparently healthy" cultures of the same vegetative compatability group. Occasionally, high molecular weight dsRNAs appeared to pass across with no effect on phenotype. As indicated earlier doubts exist as to whether these high molecular weight dsRNA segments are transferred or are induced. Induction of extrachromosomal elements during laboratory culture has been shown in vegetative death of Aspergillus glaucus (Jinks, 1959), senescence in P.asserina (Marcou & Schecroun, 1959) and the production of the stopper phenotype in N.crassa (Bertrand, 1968). All the above arose spontaneously during prolonged vegetative culturing. Disease transfer between non-vegetative compatible A.bisporus strains did not occur and incompatibility reactions were not observed.

Initial results from cell-free infectivity studies appeared promising, with significant effects seen on initial growth rates and sectoring levels of treated cultures. However, over a period

of several weeks growth rates had increased to levels approaching those of "apparently healthy" isolates. Even though growth rates were still lower than the control, dsRNAs of the "standard" pattern were not detectable in these cultures. The 8.6 and 8.1 and 6.6 kbp high molecular weight dsRNA segments were found in most treatments but also in the control cultures. Previous evidence indicated that these dsRNA segments may be associated with the "standard dsRNA segments (Chapter 5) but do not appear to cause disease symptoms alone. Previous results indicate that high molecular weight dsRNA segments may be inducible from a very low steady state to detectable levels by culturing changes. It is possible that during cell-free transmission experiments these high molecular weight dsRNA segments may have been induced by the transformation procedure and not by infection with dsRNA/virus particles.

Initial effects of the dsRNA/virus particles on growth may have been due to the inhibitory effects of a high dsRNA concentration on protein synthesis (Penman & Summers, 1965, Levin et al., 1980; Itmar et al., 1984). Although symptoms were very similar to those exhibited by La France disease cultures it is likely that the initial effect seen is a result of a direct action on protein synthesis rather than the establishment of La France disease. It is likely that factor(s) essential for the maintenance of dsRNA were not transferred along with dsRNA/VLPs during the treatment. In other fungi results of attempted transformations systems using dsRNA/virus particles have been mixed. Transformation of P.stoloniferum (Lhoas, 1971; Pallet, 1976), G.graminis (Stanway & Buck, 1984) and S.cerevisae (Bostain, 1983) with VLPs has resulted in detectable levels of VLPs surviving for several subcultures. Attempts at transformation in H.victorae

(Ghabrial, 1986) and E.parasitica (Van-Alfen, 1984; 1986) were unsuccessful. In H.victorae VLPs are detected by electron microscopy but an insufficient number were present after liquid culture for characterisation (Ghabrial, 1986). Transformation in E.parasitica resulted in the dsRNA not being maintained beyond two colony transfers (Van-Alfen, 1986). One surprising observation is that in both successful and unsuccessful transformations the disease or dsRNA associated phenotype was initially produced.

The situation in E.parasitica parallels that in A.bisporus, in both cases the majority of dsRNA appears to be associated with organells (Dodds, 1980, Chapter 4). It is possible that VLPs which have evolved for a solely intracellular existence have dispensed with viral genes involved in a previous extracellular phase. Genes associated with infectivity may have become redundant and the role of genes coding for key enzymes may have been taken over by those from the host. Indirect evidence for this comes from the fact that all factors required for transmission of hypovirulence in E.parasitica and La France disease in A.bisporus can be transmitted during anastomosis (Anagnostakis & Day, 1979; van-Zaayan, 1979). While transformation in both E.parasitica (Ghabrial, 1980) and A.bisporus proved unsuccessful. However, it is also feasible that key enzymes coded for by dsRNA are not purified with the dsRNA used in infectivity studies. Alternatively a virion associated enzyme may be damaged during purification. Also, if an alternative site was available for dsRNA localisation free from the effects of degradative enzymes then genes for capsid components could also be redundant. This is possibly the case in hypovirulent isolates of E.parasitica and d^2-infected isolates of

O.ulmi where dsRNA associated with the above phenomenon is localised in membranous vesicles and mitochondria (Dodds, 1980 & Rodgers et al., 1987), In La France diseased A.bisporus the majority of dsRNA appears localised within mitochondria although the situation may differ between diseased sporophores and cultures (Chapters 4 & 5).

Hillman et al., (1989) have developed a system for the stable integration of DNA sequences into E.parasitica chromosomal DNA Attempts are currently being made to transform apparently healthy E.parasitica protoplasts with DNA plasmids containing complementary sequences to dsRNA segments. This approach will hopefully circumvent the problem of maintaining the dsRNA in the fungus by integrating a DNA copy into the host genome. However, problems may be encountered in obtaining expression of the cloned c-DNA, other genes (some of host origin) required for expressing dsRNA segments may not be switched on or present in the new host. Although a number of problems will have to be overcome, this approach if successful will allow a more comprehensive understanding of how individual segments contribute to the dsRNA associated phenomenon seen in fungi. A similar system would be applicable for A.bisporus as the lithium acetate whole cell transformation system does appear capable of getting dsRNA into A.bisporus.

8. GENERAL DISCUSSION

Results from this study along with those of Harmsen et al., (1989); and Romaine et al., (1989) strongly suggest that the mere presence of dsRNA in sporophores and cultures of A.bisporus is insufficient for the expression of La France disease. The disease, however, is strongly associated with a "standard" pattern of 9-10 dsRNA segments 3.6-0.78 kbp (Hicks & Haughton, 1986; Wach et al., 1987; Harmsen et al., 1989; Romaine et al., 1989). In the present study, whenever disease symptoms were observed in sporophores these segments have been present. Furthermore, reduction of disease symptoms by heat or ribavirin was associated with a complete loss or reduction of the "standard" dsRNA segments. Anastamosis between vegetative compatible "apparently healthy" and diseased strains resulted in the simultaneous transfer of disease causing factor(s) and the "standard" dsRNA segments. Cell free transmission of dsRNA/VLPs, however, has proven inefficient and difficult to reproduce (Hollings, 1962; van-Zaayan, 1969; 1979).

DsRNA segments of the "standard" pattern have been isolated from "apparently healthy" sporophores showing no obvious symptoms (Deahl et al., 1986; Harmsen et al, 1989; Romaine et al., 1989). Studies by Harmsen et al., (1989) indicated that high concentrations of the "standard" dsRNA segments were present in symptomless sporophores around severely diseased areas indicating that essential factors required for symptom expression were

either absent or at too low a concentration for disease expression. No evidence for the association of the "standard" dsRNA segments with "apparently healthy" sporophores or cultures were found in this study. The loss of the majority of the "standard" dsRNA segments during culturing with the maintenance of the disease phenotype caused further confusion as to the exact role of the "standard" dsRNA segments. Loss of the remaining 3.6 and 1.5 Kbp dsRNA segments during heat treatment correlated with a significant increase in growth rate and vigour, although heat treated cultures did not appear as healthy as the "apparently healthy" control. The maintenance of disease like symptoms following the loss of the majority of the "standard" dsRNA segments could be a direct result of mutations in host genes involved in dsRNA maintenance. Mutations in host genes could result in the loss of the "standard" dsRNA segments but maintain disease like symptoms via debilitating effects on host metabolism. Studies have already shown mycoviruses in other fungi to be under host control (Koltin, 1986; Bruenn, 1986). The incomplete curing of the disease phenotype by HT in cultures where loss of "standard" dsRNA segments has occurred may be due to host mutations preventing cultures returning to normal even when all the standard dsRNA segments have been eliminated. Alternatively dynamic drifting of dsRNA segments as described by Ben Zvi et al., (1978) (Chapter 3) where dsRNA segment levels are not maintained in a steady state could also explain dsRNA segment variability in A.bisporus. Although explaining dsRNA segment variations and phenotypic changes in U.maydis (Koltin, 1986) and G.grammis (Ferault et al., 1979; McFadden et al., 1983), dynamic drift does not explain how disease symptoms are maintained with the apparent loss of the "standard" dsRNA segments in A.bisporus.

Further questions concerning the cause(s) of La France disease came from the discovery of high molecular weight dsRNA segments 15-6.6 Kbp in both diseased and "apparently healthy" isolates (Romaine et al., 1989; Harmsen et al., 1989; Chapter 3). Preliminary experiments (Chapter 4) indicated a degree of homology between these high molecular weight dsRNA segments and those of the "standard" pattern. Diseased cultures which showed changes in the "standard" dsRNA segments maintained these high molecular weight segments along with disease symptoms. The incomplete recovery from symptoms of these cultures following heat treatment could be due to host mutations as previously described or the maintenance of these high molecular weight dsRNA segments. It is possible that smaller "standard" dsRNA segments could come from the larger ones by internal deletions, as is thought to be the case in E.parasitica (Hiremath et al., 1986; Rae et al., 1989). Alternatively as they appear unencapsidated the high molecular weight dsRNAs may represent intermediates in "standard" dsRNA segment replication, Replicative structures similar to those found during T_4 and Lambda bacteriophage and ssRNA plant virus replication (Lewin, 1985; Matthews,1981) may be responsible for these high molecular weight dsRNA molecules. However, dsRNA replication systems studied so far do not appear to involve large replicative intermediates (Buck, 1979). High molecular weight dsRNA segments can also be found in "apparently healthy" cultures (Romaine et al., 1989; Harmsen et al., 1989), therefore if they are involved in the disease probably require other factors for disease expression. Results in Chapters 3 and 7 indicate that these high molecular weight dsRNA segments may be inducible in "apparently healthy" cultures with no history of dsRNA or La France disease. It is clear from the work in this thesis and previous research that the role of

dsRNA in La France disease, if any, is not a simple one. Further work is required especially on dsRNA/host interactions before dsRNA can be assigned a more positive role in the disease.

The significance of VLPs in the disease is also uncertain, mixed infections containing up to six morphologically distinct particles have been found in La France diseased material (Hollings, 1962; 1967; van Zaayan, 1979; Passmore & Frost, 1979). Studies by Last et al., (1974) indicated that levels of 29 nm particles have correlated with growth rate decreases in culture. VLPs have been shown to contain some dsRNA segments of similar Mr to the "standard" pattern although it has not proved possible to link individual dsRNA segments consistently with particle types (Chapter 3, Barton & Hollings, 1979; Wach & Romaine, 1983). Only the bacilliform particle, 50 x 19 nm, has been thoroughly characterised and shown to have a ssRNA genome (Tavantiz & Smith, 1980). As with the "standard" dsRNA segments VLPs have also been detected in "apparently healthy" material (Nair, 1972; Passmore & Frost, 1979).

In S.cerevisiae and G.graminis encapsidation of dsRNA in VLPs is believed to have a role in preventing the potential deleterious effects of naked dsRNA (Pennman & Summers 1965; Gray et al., 1984). High levels of naked dsRNA in A.bisporus could contribute to disease symptoms. Cell free infectivity studies using dsRNA segments of the "standard" pattern appeared to initiate disease symptoms (Chapter 7). In E.parasitica and O.ulmi VLPs have not been isolated (van-Alfen, 1986; Rogers et al., 1986). In both cases the presence of dsRNA was associated with a deleterious effect on fungal growth and reproductive fitness (Day et al., 1979; Rogers et al., 1986).

The association of the "standard" dsRNA segments with mitochondria of diseased isolates may also be significant in the production of disease symptoms. Results indicate that the majority of dsRNA within diseased tissue is associated with mitochondria. The replication and transcription of dsRNA within mitochondria may affect mitochondrial metabolic processes as well as inhibiting protein synthesis and mtDNA replication. Rogers et al., (1987) in O.ulmi showed the localisation of dsRNA within mitochondria to be associated with changes in cytochrome ratios in the electron transfer chain. Unfortunately, cytochrome levels were not studied in La France diseased cultures. The investigation of cytochrome levels, other respiratory complexes and in-vivo and in-vitro effects of dsRNA on mitochondrial protein synthesis are important areas for further research.

Transmission experiments, curing and differences in dsRNA segment profiles provide indirect evidence for the involvement of other non-dsRNA factors in the disease. Possible candidates include DNA plasmids and viruses, viroids, mtDNA rearrangements and host mutations. The 20 Kbp DNA segment detected following CF11 chromotography in diseased and "apparently healthy" isolates appeared identical when digested with restriction enzymes. Possible homology was also shown between this DNA segment and dsRNA segments of the "standard" pattern. The origin and function of the 20 kbp DNA segment is unclear and requires further RFLP and sequencing studies to determine its relevance to La France disease. Rearrangement of mitochondrial DNA was not shown to occur. Host control of dsRNA replication was not investigated, however, if abnormalities in host control are also responsible for La France disease this could explain how the presence of dsRNA/VLPs can occasionally have no effect on

A.bisporus.

It is fairly well established that in most cases mycoviruses have evolved along with their hosts to exist with no significant deleterious effects (Lemke, 1979; Buck, 1986). Fungal-virus host interactions have been extensively studied in yeast (Esteban & Wickner, 1987; Icho & Wickner, 1988; Uemura & Wickner, 1988). In yeast dsRNA/viral copy number is under the control of host-negative regulator genes (Ball et al., 1984). Mutations in these genes can cause conditions such as cold sensitivity where dsRNA replication escapes from host control and is deleterious to the host (Ridley et al., 1984). Alternatively mutations in dsRNA segments can also cause deleterious effects as in the case of suicide mutants in S.cerevisiae (Bruenn, 1986). Mutations in host genes or dsRNA segments may be responsible for the deleterious effects of dsRNA segments in A.bisporus.

In A.bisporus, extensive inbreeding may have increased the likelihood of a deleterious agent persisting. Some authors including Fritsche, (1977) believe that all white mushrooms grown today are derived from a chance cluster isolated in 1927 (Klingman, 1950). Recent genetic studies of A.bisporus have shown that cultivated strains including new hybrids show little difference in RFLP analysis (Castle et al., 1987; Loftus et al., 1988; Brooks et al., 1990). Also mushroom spawns used in mushroom production are taken from vegetative cultures, occasionally kept in this state for up to 30-40 years (Elliot, 1985). It is possible that during such an extensive culture period genetic changes in the host could occur introducing new agents or altering the properties of existing ones. As mushroom spawns have an entirely vegetative existence and are generally

maintained at low temperatures, such changes would probably not be selected against. The presence of dsRNA segments of the "standard" pattern in "apparently healthy" spawns (Deahl, 1986), could be considered evidence for this. Further changes to the dsRNA segments or host genes when the spawn is exposed to different conditions in the crop could facilitate a switch from a cryptic to a disease state.

REFERENCES

Ahmed, R. & Fields, B.N. (1981). Reassortment of genome segments between reovirus defective interfering particles and infectious virus: construction of temperature - sensitive and attenuated viruses by rescue of mutations from DI particles. Virology 111, 351-363.

Ainsworth, C.C. & Sharp, P.J. (1989). The potential role of DNA probes in plant variety identification. Plant Varieties & Seeds 2, 27-34.

Al-Aidroos, K., Somers, J.M., & Bushey, H., (1973). Retention of cytoplastmic killer determinants in yeast cells after removal of mitochondrial DNA by ethidium bromide. Mol. Gen. Genet. 122, 323-330.

Albouy, J. (1972). Etude ultramicrosopic du complexe viral de la "goutte niche" de carpophores d'Agaricus bisporus. Ann. Phytopathol. 4, 39-44.

Amersham. (1985). Membrane transfer and detection methods. Amersham International Limited.

Anderson, M.L.M., Young, B.D. (1987). Quantative filter hybridization. In "Nucleic Acid Hybridization a Practical Approach" (Haynes, B.D., & Higgins S.J. eds.), pp. 73-111 IRL Press.

Anognostakis, S.L., & Day, P.R., (1979). Hypovirulence conversion in Endothia parasitica Phytopathology 69, 1226-1229.

Anagnostakis, S.L., (1982). Biological control of chestnut blight. Science 215, 466-471.

Anagnostakis, S.L., (1982). Genetic analyses of Endothia parasitica: Linkage data for four single genes and three vegetative compatability types. Genetics 102, 25-27.

Arand, J.E., (1987), Preparation of nucleic acid probes. In "Nucleic Hybridization a Practical Approach" (Hames, B.D., & Higgims S.J., eds.), pp. 17-110, IRL press.

Asher, M.J.C., (1981). Pathogenic variation. In "Biology and Control of Take-all" (Asher, M.J.C. & Shipton, P.J., eds.), pp.199-218. Academic Press.

Atkey, P.T. & Barton, R.J, (1978). Club shaped virus-like particles. Rep Glasshouse Crops Res Inst. 1977, 132-133.

Atkins, R.A., & Lambowitz, A.M. (1984). The Poky mutant of Neurospora crassa a 4-base-pair deletion at the 5' end of the mitochondrial small rRNA Proc. Natl. Acad. Sci. U.S.A. 81, 3791-3795.

Atkey, P.T., Barton, R.J.J., Hollings, M. & Stone, O.K. (1975). Mushroom viruses. Rep. Glasshouse crops Res. Inst. 1973, 119-120.

Baker, K.F., (1970). Types of Rhizoctonia diseases and their occurrence. In "Rhizoctonia solani : Biology and Pathology" (Parmeter, J.R., ed.), Los Angeles, pp. 125-148 University of California Press.

Ball, S., Tiretiaux, C & Wickner, R. (1984) Genetic control of L-A and L-(BC) dsRNA copy number in killer systems of Saccharomyces cerevisiae. Genetics 107, 199-217.

Bartnicki-Garcia, S., Bracker, C.G., Reyes, E & Ruiz-Herrera, J., (1978). Isolation of chitosomes from taxonomically diverse fungi and synthesis of chitin microfibrils in-vitro. Exp Mycol. 2 73.

Barton, D. & Hollings, M., (1979). Purification and some properties of two viruses infecting the cultivated mushroom Agaricus bisporus. J. Gen. Virol. 42, 231-240.

Beilharz, M.W., Cobon, G.S. & Nagley, P. (1982). A novel species of double-stranded RNA in mitochondria of Saccharomyces cerevisiae. Nucleic Acids Res. 10, 1051-1071.

Ben-Zvi, B.S., Koltin, Y., Mevarech, M. & Tamarkin, A. (1978). RNA polymerase activity in virions from Ustillago maydis. Mol. Cell. Biol. 4, 188-194.

Bertrand, H. & Pittenger, T.H. (1972). Isolation and classification of extranuclear mutants of Neurospora crassa. Genetics 71, 521-533.

Biraghi, A., (1953). Possible active resistance to Endothia parasitica in Castanea sativa. Congress of the International Union of Forest Research Organisations. pp. 643-645.

Bockelmann, B., Osiewacz, H.D., Schmidt, F.R. & Schutte, E. (1986). Extrachromosomal DNA in Fungi-organisation and function. In **"Fungal Virology"** (Buck, K.W. ed.), pp. 237-283. CRC Press.

Bozarth, R.F., Koltin, Y., Weissman, M.B., Parker, R.L., Dalton, R.E., & Steinlauf, R., (1981). The molecular weight and packaging of dsRNA in the Mycovirus from Ustillago maydis killer strains. Virology 113, 492-501.

Bozarth, R.F. & Lentz, E.T., (1978). Physico-Chemical properties and serology of Ustillago maydis P1 and P4. 4th Int. Congr. Virology. The Hague. The Netherlands. pp. 172 (abstr.)

Bozarth, R.F., (1979). The physico-chemical properties of mycoviruses. In "Viruses & Plasmids in Fungi" (Lemke, P.A., ed.), pp. 44-87, Marcel Dekker Inc.

Brasier, C.M. (1984). Inter-mycelial recognition systems in Ceratocystis ulmi : their physiological properties and ecological importance. In The Ecology and Physiology of the Fungal Mycelium" (Jennings, D.M. & Rayner, A.D.M., eds.), pp. 451-497. Cambridge University Press, London.

Brasier, C.M., (1983). A cytoplasmicly transmitted disease of Ceratocystis ulmi. Nature (London) 305, 220-223.

Brooks, J.J., Moore, D. & Elliott, T.J. (1990). Pers. Comm.

Bruenn, J.A. & Brennan, V. E. (1980). Yeast Double-stranded RNAs have heterogeneous 3' termini. Cell 19, 923-933.

Bruenn J. (1986). The killer systems of Saccharomyces cerevisiae and other yeasts. In "Fungal Virology" (Buck. K.W., Ed.), pp.85-108. CRC Press.

Bruenn, J.A. & Kane, W. (1978). Relatedness of the double-stranded RNAs present in yeast virus-like particles. J. Virol. 26, 762-772.

Buck, K.W. (1979). Replications of double-stranded RNA mycoviruses. In "Viruses and Plasmids in Fungi. (Lemke, P. eds). pp. 93-160. Marcel Dekker Inc.

Buck, K.W., (1984). A new double-stranded RNA virus from Gaeumannomyces graminis. J gen Virol. 65. 987-990.

Buck, K.W., (1986). Fungal virology - An overview. In "Fungal virology" (Buck, K.W. ed.), pp. 1-84. CRC Press.

Buck, K.W., (1987). Viruses of plant pathogenic fungi. In "Genetics and Plant Pathogenesis" (Day, P.R. & Jellis, G.J. ed.), pp. 111-126. Blackwell Scientific Publication, Oxford.

Buck, K.W., (1986). Viruses of the Wheat take-all fungus Gaeumannomyces graminis var. Tritci. In Fungal virology (Buck, K.W. ed.), pp. 221-236. CRC Press.

Buck, K.W., Almond, M.R., McFadden, J.J.P., Romanos, M.A. & Rawlinson, C.J. (1981). Properties of thirteen viruses and virus variants obtained from eight isolates of the wheat take-all fungus Gaemannomyces gramanis. J. Gen. Virol. 53, 235-245.

Buck, K. W. (1990). Pers. Comm.

Buxton, F., Gwynne, D.I. & Davies, W.R. (1985). Transformation of Aspergillus niger using the arg B gene of Aspergillus nidulans. Gene 37, 207-214.

Carmichel, G.G. & McMaster, G. K. (1980). The analysis of nucleic acids in gels using glyoxal and acridine orange. Methods Enzymol. 65, 380-391.

Carusi, C.A. (1977). Evidence for blocked 5' termini in human adenovirus DNA. Virology 76, 380-394.

Castanho, B. & Butler, E.E. (1978a). Rhizoctonia decline studes on hypovirulence and potential use in biological control. Phytopathology 68, 1511-1514.

Castanho, B. & Butler, E.E. (1978b). The association of dsRNA with Rhizoctonia decline. Phytopathology 68, 1515-1519.

Castle, A.J., Horgen, P.A., Anderson, J.B., (1987). Restriction fragment length polymorphisms in the mushrooms Agarics brunnescens and Agarious bitorquis. Applied and Environmental Biology 53, 818-822.

Caten, C.E., (1972). Vegetative incompatibility and cytoplasmic infection in fungi. Journal of General Microbiology 72, 221-229.

Collins, R.A., Stohl, L.L., Cole, M.D. & Lambowitz, A.M. (1981). Characterisation of a novel plasmid DNA found in mitochondria of N.crassa. Cell 24, 443-452.

Cummings, D.J., Belcour, L. & Grandchamp, C. (1979). Mitochondrial DNA from Podospora anserina II. Properties of mutant DNA and multimeric circular DNA from senescent cultures. Mol. Gen. Genet. **171**, 239-250.

Dale, J.R., Manners, J.M. & Irvin, J.A.G. (1988). Colletotrichum gloeosporioides isolates causing different anthracnose disease on Stylosanthes in Australia carrying distinct double-standed RNAs. Trans. Br. Mycol. Soc. **91**, 671-676.

Davis, R.D. (1985). Factors affecting protoplast isolation. In **"Fungal protoplasts : Application in Biochemistry and Genetics,"** (Peberdy, J.F. & Ferenczy, eds.), pp. 45-72. New York, U.S.A., Marcel Dekker.

Dawe, V.H., & Kuhn, C.W. (1983). Isolation and characterization of a double-stranded DNA mycovirus infecting the aquatic fungus, Rhizidiomyces. Virology **130**, 21-28,

Day, P.R., Dodds, J.A., Elliston, J.E., Jaynes, R.A. & Anognostakis, S.L. (1977). Double-stranded RNA in Endothia parasitica. Genetics **67**, 1393-1396.

Day, P.R., & Anognostakis, S.L. (1973). The killer system in Ustilago maydis : heterokaryon transfer and loss of determinants. Phytopathology **63**, 1017-1018.

Deahl, K. & San-Antonio, J.P., (1986). Electrophoretic analysis of double-stranded RNA in stocks of cultivated mushroom Agaricus brunnescens. Plant diseases **71**. 430-433.

Del Vecchio, V.G. (1978). Immune electron microscopy of virus-like particles of Agaricus bisporus. Exp. Mycol. 2, 138-144.

Delhotal, P., Legrand-Pernot, F & Lecoq, H. (1976). Etude des virus de Colletotrichum lindemuthianum II. Transmission de particles virales. Ann. Phytopathol. 8, 437-448.

de-Viries, H., de Jonge, J.C., Van't Saint, P., Aggsteribbe, E. & Arnberg, A. (1981). A stopper mutant of Neurospora crassa containing two populations of aberant mitochondrial DNA. Curr. Genet. 3, 205-7.

Dickson, H. (1936). Ann. Bot., 50, 719-733.

Dhawale, S.S., Paietta, J.V. & Marzluf, G.A. (1984). A new, rapid and efficient transformation procedure for Neurospora. Curr. Genet. 8. 77-79.

Dodds, J.A. (1980). Association of type 7 virus-like dsRNA with club shaped particles in hypovirulent strains of Endothia parasitica. Virology 107, 1-12.

Dodds, J.A. (1980). Revised estimates of the molecular weights of dsRNA segments in hypovirulent strains of Endothia parasitica. Phytopathology 70, 1217-1220.

Edwardson, J.R.. Bond, D.A. & Christie, R.B. (1976). Cyloplasmic Sterility factors in vicia faba L. Genetics 82, 443-449.

Efstratiadis, A. Kafatos, F.C. Maxam, A. & Maniatis, T. (1976). Enzymatic in vitro synthesis of globin genes. Cell. 7, 279-288.

Elliott, T.J. (1985). Spawn-making and spawns. In the Biology and Technology of the Cultivated Mushroom (Flegg. P.B., Spencer, D.M. & Wood, D.A., eds.), pp 131-139. J. Wiley & Sons.

Elliston, J.E. (1978). Pathogenicity and sporulations of normal and diseased strains of Endothia parasitica. In American chestnut. Proc. American Chestnut Symp." pp. 95- , West Virginia University books, Morgantown.

El-Sherbeini, M. & Bostain, K.A. (1987). Viruses in fungi : infection of yeast with the K1 and K2 killer viruses. Proc. Natl. Acad. Sci. U.S.A. 84, 4293-4297.

Esser, K., Kuck, U., Lang-Hinrichs, G., Lemke, P., Osiewacz, H.D., Stahl, U. & Tudznski, P. (1986). Plasmids of eukaryotes. Fundamentals and applications. Springer, Berlin, Heidelberg, New York, Tokyo.

Esser, K. & Tudzynski, P. (1979). Genetic control and expression of senescence in Podospora anserina. In "viruses and plasmids in fungi" (Lemke, P.A., ed.), pp. 595-615. Marcel Dekker, New York.

Esser, K. & Keller, W. (1976). Genes inhibiting senescence in the ascomycete Podospora anserina. Mol. Gen. Genet. 44, 107-110,

Esser, K. & Tudzynski, P. (1980). Senescence in fungi. In "Senescence in plants." (Thiamann, K.V., ed.), pp. 67-83. CRC Press, Boca Raton, Florida.

Esteban, B. & Wicker, R.B. (1987). A new non-mendelian genetic element of yeast that increases cytopathology produced by M1 double-stranded RNA in SK1 strains. Genetics **117**, 399-408.

Fauron, C. & Havlik, M. (1989). The maize mitochondrial genome of the normal type and the cytoplasmic male sterile type Type T have very different organisation. Curr. Genet. **15**, 149-154.

Feinberg, A.P. & Vogelstein, B. (1983). A technique for radiolabelling DNA restriction endonuclease fragments to high specific activity. Anal. Biochem. **132**, 6-13.

Ferault, A.C., Tivoli, B., Lemaire, J.M. & Spire, D. (1976). Etude de l'evolution comparee du niveau d'agressvite et du contgendu en particles de type viral d'une souche de Gaeumannomyces graminis (sacc) Arx et Oliver (Ophiobolus gramminis sacc). Ann. Phytopatho. **11**, 187.

Finnegan, P.M. & Brown, G.G. (1986). Autonomously replicating RN in mitochondria of maize plants with S-type cytoplasm. Proc. Natl. Acad. Sci. USA. **83**, 5175-5179.

Field, L.J., Bruenn, J., Chang, T.H., Pinhani, O. & Koltin, Y. (1983). Two Ustilago maydis viral dsRNAs of different size code for the same product. Nucleic Acids Research **11**, 2765-2778.

Fink, G.R. & Styles, C.A. (1972). Curing of a killer factor in Saccharomyces cerevisiae. Proc. Nat. Acad. Sci. USA. **66**, 2846-2849.

Finkler, A., Koltin, Y., Barash, I., Sneh, B. & Pozniak, D. (1985). Isolation of a virus from virulent strains of Rhizoctonia solani. J. Gen. Virol. 66, 1221-1232.

Finnegan, P.M. & Brown, B.G. (1986). Autonomously replicating RNA in mitochondria of maize plants with S-type cytoplasm. Proc. Natl. Acad. Sci. USA. 83, 5175-5179.

Franklin, T.J., & Snow, G.A. (1981). Biochemistry of Antimicrobial Action. Chapman & Hall.

Fried, H.M., & Fink, G.R. (1978). Electronmicroscopic heteroduplex analysis of "Killer" double-stranded RNA species from yeast. Proc. Natl. Acad. Sci. USA. 75, 4224-4228.

Frick, L.J.. & Lister, R.M. (1978). Serotype variability in virus-like particles from Gaeumannomyces graminis. Virology 85, 504-517.

Fritsche, G. (1977). Breeding work on the newly cultivated mushroom: Agaricus bitorquis (Quel) Sacc. Mushroom Journal 50, 54-61.

Fullbright, D.W. (1984). Effects of eliminating dsRNA in hypovirulent Endothia parasitica. Phytopathology 74, 722-724.

Gandy, D.G., (1959). Mushroom Sci., 4, 411-415.

Gandy, D.G., (1962), Studies on die-back of Mushrooms. Mushroom Sci., 5, 468-479.

Gandy,D.G., (1960a). A transmissible disease of cultivated mushrooms (water stipe). Ann. Appl. Biol. 48, 427-430.

Gandy, D.G. (1960b). "Watery Stipe" of Cultivated Mushrooms. Nature (London) 185, 482-483.

Ghabrial, S.A., Sanderlin, R.S., & Calvert, L.A. (1979). Morphology and virus-like particle content of Helminthosporium victoriae colonies regenerated from protoplasts of normal and diseased isolates. Phytopathology 69, 312-315.

Ghabrial, S.A. (1986). A transmissible disease of Helminthosporium victoriae - evidence for a viral etiology. In "Fungal Virology," (Buck, K.W. ed.), pp. 163-176. CRC Press.

Gray, C.W., Page, G.A. & Gray, D.M. (1984). Complex of fd gene J protein and double-stranded RNA. Journal of Molecular Biology 175, 553-559.

Gril, L.K. & Barger, S. J. (1981). Identification and characterisation of double-stranded RNA associated with cytoplasmic male sterility in Vicia faba. Proc. Natl. Acad. Sci. USA, 78, 7043-7046.

Gunge, N., Tamaru, A., Ozawa, F. & Sakaguchi, K. (1981). Isolation and characterisation of linear deoxyribonucleic acid plasmids from Kluyveromyces lactis and the plasmid associated killer character. J. Bacteriol. 145, 383-390.

Hamlyn, P. F., Bradshaw, R.E.., Mellon, F.M., Santiago, C.M., Wilson, J.M. & Peberdy, J.F.., (1981). Efficient protoplast isolation from fungi using commercial enzymes. Enzymes and Microbial Technology. 3, 321-325.

Hankin, L & Puhalla, J.E. (1971). Nature of a factor causing interstrain lethality in Ustilago maydis. Phytopathology 61, 50-53.

Harmsen, M.C., van Griensven, L.J.L.D. & Wessels, J.G.H. (1989). Molecular analysis of Agaricus bisporus dsRNA J.Gen. Virol. 70, 1613-1615.

Hashiba, T., Homma, Y., Hyakumachi, M. & Matsuda, I. (1984). Isolation of a DNA plasmid in the fungus Rhizoctonia solani. Journal of General Microbiology 130, 2067-2070.

Herring, A.J., & Bevan, E.A. (1974). Virus-like particles associated with double-stranded RNA species found in killer and sensitive strains of the yeast Saccharomyhces cerevisiae. J. Gen. Virol.. 22, 387-394.

Hicks, R.G.T., & Haughton, K.L. (1986). Detection of double-stranded RNA in shake cultures of Agaricus bisporus affected by La France disease. Trans. Mycol. Soc. 86, 579-584.

Hillman, B., Rae, B., Tartaglia, J. & Nuss, D. (1989). Elucidating the structure and function of double-stranded RNA genetic elements associated with biological control of chestnut blight. Molecular Biology of Plant-Pathogen Interactions. 59-70.

Hinz, W. E., Mohan, M., Anderson, J.B. & Hargen, P.A. (1985). The mitochondrial DNA s of Agaricus bisporus : heterogeneity in A.bitorquis and homogeneity in .A.brunnescens. Cur. Genet. 9, 127-132.

Hinz, W.E., Anderson, J.B. & Horgan, P.A. (1968). Nuclear migratiion and mitochondrial inheritance in the mushroom Agaricus bitorquis. Genetics 119, 35-41.

Hiremath, S., L'Hostis, B., Chabrial, S.A. & Rhoads, R.E. (1986). Terminal structure of hypovirulence - associated dsRNA's in the chestnut fungus Endothia parasitica. Nucleic Acids Res. 14, 9877-9896.

Holland, J., Spindler, K., Horodyski, F., Grabau, A , Nichol, S. & Var depol, S. (1982). Rapid evolution of RNA GENOMES. Science 215, 1577-1585.

Hollings, M. (1962). Viruses associated with die-back disease of cultivated mushrooms. Nature 196, 962-965.

Hollings, M., Gandy, D.G. & Last, F.T., (1963). A virus disease of a fungi : die-back of cultivated mushroom. Endeavour 22, 112-117.

Hollings, M. (1967). Mushroom viruses. Rep. Glasshouse. Crops Res. Inst. 101.

Hollings, M. & Stone, O.M. (1971). Viruses that infect fungi. Ann. Rev. Phytopath. 9, 93-118.

Hollings, M. (1978). Mycoviruses : viruses that infect fungi. Adv. Virus. Res. 22, 1-53.

Hollings, M., Stone, O.M. & Atkey, P.T. (1968). Mushroom viruses, Rep. Glasshouse crops Res. Inst., 1967, 101-102.

Hollings, M. (1982). Mycoviruses and plant pathology. Plant Diseases. 66, 1106-1112.

Honeyman, A.L. & Currier, T.C. (1983). The isolation and characterisation of two linear DNA elements from G.graminis var. tritici. the causative agent of "wheat take-all" disease. Abstr 83rd Annu. meet Am. Soc. Microbiol., New Orleans, Amer. Soc. for Micro., Washington DC pp. 135.

Hunst, P.L., Latterell, F.M. & Rossi, A.E. (1986). Variation in double-stranded RNA from isolates of Pyricularia oryzae. Phytopathology 76, 674-678.

Inciarte, M.R., Lazaro, J.M., Sales, M., Vinuela, E. (1976).Structure of replicating RNA molecules of Bacillus subtillus bacteriophage 129. J. virol. 34, 187-199.

Icho. T. & Wickner, R.B. (1988). The Mak 11 protein is essential for cell growth and replication of M double-stranded RNA and is apparently a membrane-associated protein. Journal of Biological Chemistry 263, 1467-1475.

Inciarte, M.R., Lazaro, J.M., Salas, M., Vinuela, E. (1976). Structure of replicating DNA molecules of Bacillus subtillus Bacteriophage 029. J. Virol. 34, 187-199.

Itmar, D., Gronsky, R., Lebendiker, M., Kaempfer, R. (1964). The nature of the interactiion of eukaryotic initiation factor 2 with double-stranded RNA. Eur. J. Biochem. 145, 373-379.

Ito, M., Fukuda, Y., Murata, K & Kumura, A. (1983). Transformation of Intact Yeast Cells treated with Alkali Cations. J. Bacteriol. 153, 163-168.

Jamil, N., Buck, K.W. & Carlile, M.J. (1984). Sequence relationships between virus double-stranded RNA from isolates of Gaeumannomyces graminis in different vegetative compatibility groups. J. Gen. Virol. 65, 1714-1747.

Jinks, J.L. (1959). Lethal suppresive cytoplasm in aged clones of Aspergillus Glaucus. J. Gen. Microbiol. 21, 397-409.

Jodon, M.H., Royse, D.J. & Schisler, L.C. (1981). Effects of high temperature after casing on mushroom production. Can. J. Bot. 59, 735-741.

Kempken, F., Meinhardt, F. & Esser, K. (1989). In organello replication and viral affinity of linear extrachromosomal DNA of the ascomycete Ascobolus immersus.. Mol. Gen. Genet. 218, 523-530.

Kim, W.K. & Klassen, G.R. (1989). Double-stranded RNAs in mitochondrial extracts of stem rusts and leaf rusts of cereals. Curr. Genet. 15, 161-166.

Kiss, T., Posfai, J. & Solymosy, F. (1983). Sequence Homology between potatoe spindle tuber viroid and U3B SnRNA. FEBS. LETTERS **163**, 217-220.

Klingman, A.M. (1950). Handbook of mushroom culture 2nd edn. J. B. Swayne, Kennet Square, Pa, USA.

Koenig, R. An, D. & Burgermeister, W. (1988). The use of filter hybridisation techniques for the identification, differentiatiion and classification of plant viruses. Journal of Virological methods. **19**, 57-68.

Koll, F., Begel, O., Killer, A.A., Vierny, C. & Belcour, L. (1984). Ethidium bromide rejuvination of senescent cultures of podospora anserina : Loss of senescence - specific DNA and recovery of normal, mitochondrial; DNA. Curr. Genet. **8**, 127-134.

Koltin, Y & Day, P.F. (1976). Inheritance of killer phenotypes and double-stranded RNA in Ustilago maydis, Proc. Natl. Acad. Sci. U.S.A. **73**, 594-598.

Koltin, Y. (1986). The killer systems of Ustilago maydis. In "Fungal Virology" (Buck, K.W. ed.), pp. 109-141, CRC Press.

Koons, K., Raid, L.C. & Romaine, C.P., (1983). An analysis of the virus-like particles assocated with La France disease. Phytopathology **73**, 369 Abstr.

Koons, K., Schlagnhaufer, B. & Romaine, C.P. (1989). Double-stranded RNAs in mycelial cultures of Agaricus bisporus affected by La France disease. Phytopathology **79**, 1272-1275.

Kuntzel, H., Kockel, H.G., Lazarus, C.M. & Lunsdorf, H. (1982). Mitochondrial genes in Aspergillus. In **"Mitochondrial genes"** (Sionisnske, P.P., Borst, P. & Aattardi, G. eds.) pp. 349. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

Lapierre, H., Molin, G., Faivre-Smiok, A., Michon, E., Alboug, J., & Morand, J.C. (1971). Presence de particles a symetrie helicoidale dans une souch du champignon de couche cultive dans le nord de la France. Annales de Phytopathologie. **3, 538-539.**

Lapierre, H. (1973). Etude de l'influence des vires sur les champignons phytopathogenes du sol, in Perspectives de Lutte Biologique des champignons parasites des plantes cultivees et les pournitures des Tissues ligneaux, station Fedarale de recherches Agronomiques de Lauranne, Switzerland, pp. 62.

Last, F.T., Hollings, M. & Stone, O.M. (1967). Some effects of cultural treatments on virus diseases of cultivated mushrooms, Agaricus bisporus. Annals of Applied Biology **59, 541-562.**

Leaver, C.J. & Gray, M.W. (1982). Mitochondrial genome organisation and expression in higher plants. Ann. Rev. Plant Physiol. **33, 373-402.**

Lewin, B. (1985). In "Genes II". Wiley.

Last, F.T., Hollings, M. & Stone, O.M. (1974). Effects of cultural conditions on the growth of healthy and virus infected cultivated mushrooms of A. bisporus. Ann. Appl. Bio. **76, 99-111.**

Lemke, P.A. (1979). Co-evolution of fungi and their viruses. In "Proceedings in life sciences, fungal VIMS." GS 12th Int. Cong. of Microbiology, Micology, Munich, West Germany, Sept. 3-8, pp. 2-7. Molitoris et al, Co-evolution of fungi.

Lesemann, D.E., & Koenig, R. (1972). Association of club shaped virus-like particles with a severe disease of Agaricus bisporus. Phytopath. Z. 89, 161-169.

Levin, D.H., Petryskyn, R. & London, I.M. (1980). Characterisation of double-stranded RNA activated kinase that phosphorylates sub-unit of eukaryotic initiation factor 2 (elf-2) in reticulocyte lysates. Proc. Natl. Acad. Sci. USA. 77, 832-836.

L'Heritier, P. (1970). Drosophila viruses and their role as evolutionary factors. Evol. Biol. 4, 185-209.

Lhoas, P. (1971). Transmission of double-stranded RNA viruses to a strain of Penecillium stoloniferum through heterokaryosis. Nature (London) 230, 248-249.

Lhoas, P. (1971). Infection of protoplasts from Penicillium stoloniferum with dsRNA viruses. J. Gen. Virol. 13, 365-367.

L'Hostis, B.S.T., Hiremath, R.G., Rhoads, R.E. & Ghabrial, S.A. (1985). Lack of sequence homology between double-stranded RNA from Europe and American hypovirulent strains of Endothia parasitica. J. Virol. 66, 351-355.

Linberg, G.D. (1959). A transmissible disease of Helminthosporium victoriae. Phytopathology 49, 29-32.

Litzenberg, S.C. (1949). Nature of suseptibility to Helminthosporium victoriae and resistance to Puccinia coronata in Victoria oats. Phytopathology 39, 300-318.

Luisoni, E. (1972). Riv Patol veg ser 4 199-206.

Loftus, M.G., Moore, D. & Elliot, T.J. (1988). DNA polymorphisms in commercial and wild strains of the cultivated mushroom, Agaricus bisporus. Theor. Appl. Genet. 76, 712-718.

Lucas, J. (1989). Pers. Comm.

Luke, H.H. & Wheeler, H.E. (1955). Toxin production by Helminthosporium victoriae. Phytopathology 45, 453-458.

Makkouk, K.M., Katul, L. & Rizkallah, A. (1968). A simple procedure for the purification and antiserum production of bean yellow mosaic virus. J. Phytopathology 122, 89-93.

Marcou, D. & Schecroun, J. (1959). Cr. hebol. seanc. acad. sci., Paris, 1248, 280-283.

Marino, R., Saksena, K.N., Schuler, M., Mayfield, J.E. & Lemke, P.A. (1976). Double-stranded ribonucleic acid in Agaricus bisporus. Applied and Environmental microbiology 31, 433-438.

Matthews, R.E.F. (1981). In Plant virology second edition. pp. 190-273. Academic Press Inc. London.

Maxam, A.M. (1977). A new method for sequencing RNA. Proc. Natl. Acad. Sci., U.S.A., 74, 560-564.

McFadden, J.J.P., Buck, K.W. & Rawlingson, C.J. (1983). Infrequent transmission of double-stranded RNA virus particles but absence of DNA proviruses in single ascopore cultures of Gaeumannomyces graminis. J. Gen. Virol. 64, 927-037.

Meinhardt, F., Kempken, F. & Esser, K. (1986). Proteins are bound to the termini of a linear plasmid in the filamentous fungus Ascobolus immersus. Curr. Genet. 11, 234-246.

Molin, G. & Lapierre, H. (1973). L'acide nucleique des virus de champignons : cas de virus de l'Agaricus bisporus. Ann. Phytopathol 5, 233-240.

Moffit, E.M. & Lister, R.M. (1975). Application of a serological screening test for detecting double-stranded RNA mycoviruses. Phytopathol 65, 851-859.

Morris, T.J. & Dodds, J.A. (1979). Isolation and analysis of double-stranded RNA from virus infected plant and fungal tissues. Phytopathology 69, 854-858.

Munoz-Rivas, A., Specht, C.A., Drummond, B.J., Froelinger, E., Novotny, C.P. & Ullrich, R.C. (1986). Transformation of the basidiomycete, Schizophyllum commune. Mol. Gen. Genet. 205, 103-106.

Nair, N.G. (1972). Observations on virus disease of the cultivated mushroom A.bisporus in Australia. Mushroom Sci. 8,155-170.

Nair, N.G. (1973). Heat therapy of virus-infected cultures of the cultivated mushroom Agaricus bisporus. Aust. J. Agric. res. 24, 533-541.

Nuss, D.L. & Summers, D. (1984). Variant dsRNAs associated with transmission-defective isolates of wound tumour virus represent terminally conserved remnants of genome segments. Virology 33, 276-288.

Nyland, G. & Goheen, A.C. (1969). Heat therapy of virus diseases of perennial plants. Ann. Rev. Phytopath. 7, 331-354.

Page, N. (1987). Pers. Comm.

Pallet, I.H. (1976). Interaction between fungi and their viruses. In "Microbial and Plant Protoplasts," (Peberdy, J.F., Rose, A.H., Rogers, H.J. & Cocking E.C., eds.) pp. 107. Academic Press London.

Palukaitis, P. (1985). Preparation of and use of cDNA probes for the detection of viral genomes. Methods Enzymol. 188, 723-742.

Palukaitis, P. (1986). Preparation and use of cDNA probes for detection of viral genomes. Methods Enzymol. 118, 723-742.

Passmore, E.L. & Frost, R.R. (1974). The detection of virus-like particles in mushrooms and mushroom spawns. Phytopath. Z. 80, 85-87.

Passmore, E.L. & Frost, R.R. (1979). The detection and occurence of virus-like particles in extracts at mushroom sporophores. Phytopath. Z. 95, 346-353.

Passmore, E.L. & Frost, R.R. (1980). Virus-like particles in mushroom spawns. Phytopath. Z. 97, 68-81.

Paterson, I. (1988). Pers. Comm.

Paul, C.P. & Fulbright, D.W. (1988). Double-stranded RNA molecules from Michigan hypovirulent isolates of Endothia parasitica vary in size and sequence homology. Phytopathology 78, 751-755.

Peberdy, J.F. (1989). Presidential address : Fungi without coats - protoplasts as tools for mycological research. Mycol. Res. 93, 1-12.

Penman, S. & Summers, D. (1965). Effects of host cell metabolism following synchronous infection with polioviruses. Virology 27, 614-619.

Philliskirk, G. & Young, T.W. (1975). The occurence of killer character in yeasts of various genera. Antonie van Leeuwenhoek 41, 147-151.

Powell, W.A. & van Alfen, N.K. (1987). Differential accummulation of poly (A)+RNA between virulent and double-stranded RNA-induced hypovirulent strains of Cryphonectria (Endothia) parasitica. Molecular & Cellular biology 7, 3688-3693.

Pulhalla, J.E. (1968). Compatibility reactions on solid medium and interstrain inhibition in Ustillago maydis. Genetics **60**, 461-475.

Rae, B.P., Hillman, B., Tartaglia, J. & Nuss, D. (1989). Characterisation of double-stranded RNA genetic elements associated with biological control of chestnut blight : organisation of terminal domains and identification of gene products. The EMBO. Journal **8**, 657-663.

Raeder, U. & Broda, P. (1985). Rapid preparation of DNA from filamentous fungi. Letters in Applied Microbiology **1**, 17-20.

Rawlinson, C.J., Hornby, D., Pearson, M. & Carpenter, J.M. (1973). Virus-like particles in the take-all fungus, Gaeumannomyces graminis. Ann. Appl. Biol. **74**, 197-209.

Ridley, S. P., Sommer, S.S. & Wickner, R.B. (1984). Super killer mutants in Saccharomyces cerevisiae suppress exclusion of M double-stranded RNA by L-A-HN and confer cold sensitivity in the presence of M and L-A-HN. Molecular & Cellular Biology **4**, 761-770.

Rogers, H.J., Buck, K.W. & Brasier, C.M. (1986). The molecular nature of the D-factor in Ceratocystis ulmi. In **"Fungal Virology"**. (Buck, K.W. ed.), 209-220. CRC Press.

Rogers, H.J., Buck, K.W. & Brasier, C.M. (1987). A mitochondrial target for double-stranded RNA in diseased isolates of the fungus that causes Dutch elm disease. Nature **329**, 558-560.

Romaine, C.P. & Schlagnhaufer, B. (1989). Prevalence of double-stranded RNAs in healthy and La France disease-affected Basidiocarps of Agaricus bisporus. Mycologia **81**, 822-825.

Romaine, C.P., Srishautha, A., Wach, M.P. & Schlagnhaufer, B. (1987). La France disease of Agaricus bisporus : characterisation of a 35 nm spherical virus. In **"Developments in crop science."** (Wuest, P.J., Rayne, D.J. & Beelman, R.B., eds.), pp. 7-17. Elsevier Press Co., Amsterdam, Holland.

Ross, R.C., Brown, B.A. & Romaine, C.P. (1987). Recent experience in detecting viral double-stranded RNA in commercial mushroom crops and its effect on yield. In **"Development in Crop Science"** (Wuest, P.J., Royse, D.J. & Beelman, R.B., eds.), pp. 321-329, Elsevier Press Co., Amsterdam, Holland.

Rougeon, F. & Mach, B. (1976). Stepwise biosynthesis in-vitro of globin genes from globin mRNA by DNA polymerase of avian myeloblastosis virus. Proc. Natl. Acad. Sci. U.S.A. **73**, 3418-

Rubenstein et al. (1978). Reproductible alteration of cytoplasmic Polyhedrosis virus double-stranded RNA genome patterns in laboratory passage. Virology **84**, 195-198.

Sammons, D.W., Adams, L.D. & Nikizawa, E.E. (1981). Ultrasensitive silver-based colour staining of polypeptides in polyacrylamide gels. Electrophoresis **2**, 135-141.

Sanderlin, R.S. & Ghabrial, S.A. (1978). Physicochemical properties of two distinct types of virus-like particles from Helminthosporium victoriae. Virology **87**, 142-151.

Sanderlin, R.S. (1977). Biophysical properties of virus-like particles occurring in healthy and diseased isolates of Helminthosporium victoriae, Meehan & Murphy, PhD thesis, University of Kentucky, Lexington.

Sandford, G.B. & Skoropad, W.P. (1955). Canad. J. Microbiol., 1, 412-415.

Scalla, R., Duc, G., Rigaud, J., Lefebvre, A. & Meignoz, R., (1981) RNA containing intracellular particles in cytoplasmic male sterile faba bean (vicia faba L.) Plant. Sci. Lett. 22, 269-277.

Schardl, C.L., Lonsdale, D.M., Pring, D.R. & Rae, K.R., (1984). Linearization of maize mitochondrial chromosomes by recombination with linear ipsomes. Nature (London) 310, 292-296.

Scazzocchio, C. (1986). The natural history of fungal mitochondrial genomes. In "Evolutionary Biology of the Fungi." (Rayner, A.D.M., Brasier, C.M. & Moore, D., eds.), pp. 53-73. Cambridge University Press, London.

Scheffer, R.P., Nelson, R.R. & Ullstrup, A.J. (1967). Inheritance of toxin production and pathogenicity in Cochliobolus carbonum & Cochliobolus victoriae. Phytopathology 57, 1288-1291.

Schisler L.C., Sinden, J.W. & Sigel, E.M. (1967). Etiology, symptomatology and epidemiology of a virus disease of cultivated mushrooms. Phytopathology 57, 519-526.

Schisler, L.C., Sinden J.W., & Sigel, E.M. (1963). Transmission of a virus disease of mushrooms by infected spores. Phytopathology 53, 888 abstr.

Shelbourn, S.L. , Day, P.R., & Buck, K.W. (1988). Relationships and functions double-stranded RNA in a P4 killer strain of Ustillago maydis. J. Gen. Virol. 69, 975-982.

Sidwell, R.W. (1980). In Smith, R.A. & Kirkpatrick, W. eds. Ribavirin a broad spectrum antiviral agent, Academic Press, New York, pp. 23-42.

Silverstein, S.C., Christman, J.K. & Acs. G. (1976). The reovirus replicative cycle. Annu. Rev.. Biochem. 45, 375-408.

Sim, I.S. & McCullagh, K.G. (1985). Potential targets for selective inhibition of viral replication. In Approaches to Viral therapy.

Sinden, J.W. & Hauser, E. (1950). Report on two mushroom diseases. Mushroom Sci. 1, 96-100.

Smith, H.O. (1980). Recovery of DNA from gels. Methods Enzymology 42, 371-380.

Snider, P.J. (1965). In compatability in fungi (eds. Esser, K. & Raper J.R.) pp. 52-70, Springer, Berlin.

Snider, P.J. (1963). Genetic evidence for nuclear migration in basidiomycetes. Genetics 48. 47-55.

Stryer, L. (1981). In "Biochemistry". Freeman.

Stanway, C.A. & Buck, K.W., (1984). Infection of protoplasts of the wheat take-all fungus, gaeumannomyces graminis var. tritici with dsRNA viruses. J. Gen. Virol. 65, 2061-2065.

Stanway, C.A. (1985) . Double-stranded RNA viruses and pathogenicity of the wheat take-all fungus, Gaeumannomyces graminis, Var. tritici, PhD thesis, University of London.

Stohl, L.L., Collins, R.A., Cole, M.D. & Lambowitz, A.M. (1982). Characterisation of two plasmid DNAs found in mitochondria of wild type Neurospora intermedia strains. Nucleic acids Res. 10, 1439-1458.

Takai, S., Tizuka, T. & Richards, W.C. (1984). Discovery of plasmids in Ceratocystis ulmi. Phytopathology 74, 833 Abstr.

Tartaglia, J., Paul, C.P., Fulbright, D.W. & Nurse, D.L. (1986). Structural properties of double-stranded RNAs associated with the biological control of chestnut blight fungus. Proc. Natl. Acad. Sci. USA. 83, 9109-9113.

Tavantiz, S.M., Romaine, C.P. & Smith, S.H. (1980). Purification and partial characterisation of a bacilliform virus from Agaricus bisporus: a single-stranded RNA mycovirus. Virology 105, 94-102.

Tavantis, S.M. & Bandy, B.P, (1988). Properties of a mycovirus from Rhizoctonia solani and its virion associated RNA polymerase. J. Gen. Virol. 69, 1465-1477.

Thomas, P. (1983). Hybridisation of denatured RNA transferred or dotted on nitrocellulose paper. Methods in Enzymology 100, 255-266.

Trinci, A.P.J., (1971). Influence of the width of the peripheral growth zone on the radial growth rate of fungal colonies on solid media. J. Gen. Microbiol. 67, 325-344.

Tudzynski, P. & Esser, K. (1986).. Extrachromosomal genetics of Claviceps purpurea II plasmids in various wild strains and integrated plasmid sequences in mitochondrial genomic DNA. Curr. Genet. 10, 463-467.

Tudzynski, P. & Esser, K. (1979). Chromosomal and extrachromosomal control of senescence in the ascomycete Podospora anserina. Mol. Gen. Genet. 173, 71-84.

Turpen, T., Garger, S.J. & Brill, L.K. (1988). On the mechanism of cytoplasmic male sterility in the 447 line of Vicia faba. Plant Molecular Biology 10, 489-497.

Tzen, J.C., Sommers, J.M. & Mitchel, D.J.A. (1974). DsRNA analysis of suppressive mutnats of killer Saccharomyces cerevisiae. Heredity 33, 132 Abstr.

Uemmura, H. & Wickner, R.B. (1988). Suppression of chromosimal mutations affecting M1 virus replication in Saccharomyces cerevisiae by a variant of a viral RNA segments (L-A) that encodes coat protein. Molecular & Celluar Biology 8, 938-944.

Van Alfen, N.K., Jaynes. R.A., Anagnostakis, S.L. & Day, P.R. (1975). Chestnut blight: biological control by transmissible hypovirulence in Endothia parasitica.. Science 189, 890-891.

Van Alfen, N.K., (1986). Hypovirulence of Endothia (Cryphonectria) parasitical and Rhizoctonia solani. In "Fungal Virology" (Buck, K.W. ed.), pp.163-176. CRC Press.

Van Alfen, N.K., Hansen, D.R., Miller, S. & Barley, L. (1984). Cell-free transmission of the hypovirulent phenotype of Endothia parasitica. Phytopathology 74, Abst. 833.

van Zaayan, A. (1979). Mushroom viruses. In "Viruses and plasmids in Fungi" (Lemke, P.A. ed.), pp. 240-316, Marcel Dekker.

van Zaayan, A. (1976). Immunity of strains of A.bisporus to mushroom virus disease. Neth. J. Plant Pathol. 82, 121-131.

van Zaayan, A. (1972). Intracellular appearance of mushroom virus in fruiting bodies and basidospores of A.bisporus. Virology 47, 94-104.

van Zaayan, A., & Termmink, J.H. (1968). A virus disease of cultivated mushrooms in the Netherlands. Neth. J. Pl. Path., 74, 48.

Vodkin, M., Katterman, F. & Fink, C.R. (1974). Yeast killer mutants with doube stranded ribonucleic acid. J. Bacteriol. 117, 681-686.

Wach, M.P. & Romaine, C.P. (1983). Further characterisation of dsRNA associated with La France disease. Phytopathology 73, 376 abstr.

Wach, M.P., Sriskantha, A. & Romaine, C.F. (1987). Double stranded RNAs associated with La France disease of the commercial mushroom. Phytopathology 77, 1321-1325.

Wahl, G.M., Berger, S.L. & Kimmel, A.R. (1987). Molecular hybridisation of immobilised nucleic acids: Theoretical concepts and practical consideration. Methods in Enzymol. 152, 399-407.

Wesolowski, M. & Wickner, R. (1984). Two new doube-stranded RNA molecules showing non-mendelian inheritance and heat inductibility in Saccharomyces cerevisiae. Molec. Cell. Biol. 4, 181-187.

Wickner, R.B. (1974). Killer character of Saccaromyces cerevisiae: curing by growth at elevated temperature. J. Bac. 117, 1356-

Wickner, R.B. (1981). Killer systems in Saccharomyces cerevisiae. In "the molecular biology of the yeast Saccharomyces cerevisiae, life cycle and inheritance" (strathern, J., Jones, E. & Broach, J. eds.) pp. 415-444. Cold Spring Harbour Laboratory, New York.

Wickner, R.B. (1980). Plasmids controlling exclusion of the K2, killer double-stranded RNA plasmid of yeast. Cell 21, 217-226.

Wickner, R.B. & Leibowitz, M.J. (1977). Dominant chromosomal mutant bypassing genes needed for killer RNA plasmid replication in yeast. Genetics 87, 453-469.

Weislander, L. (1979). A simple method to recover intact high molecular weight RNA and DNA after electrophoretic separation in low gelling temperature agarose gels. Anal. Biochem. 98, 305.

Wood, H.A. & Bozarth, R.F. (1973). Heterokaryon transfer of virus-like particles associated with a cytoplasmically inherited determinant in Ustilago maydis. Phytopathology 63, 1019-1020.

Yang. R.J. et al (1979). Elution of DNA from agarose gels after electrophoresis. Methods in Enzymol. 88, 176-182.

Young, T.W. & Yagui, I. (1978). A comparison of the killer character in different yeasts and its classification. Antonie van Leewenhoke. 44, 59-77.

Zanzinger, D.H., Bandy, B.P. & Tavantiz, S.M. (1984). High frequency of finding double-stranded RNA in naturally occuring isolates of Rhizoctonia solani. J. Gen. Virol. 65, 1601-1605.

Zinn, K., Dimaio, D. & Maniatis, T. (1984). Identificatiion of two distinct regulatory regions adjacent to the Human B-interferon Gene. Cell 34, 865-879.

APPENDIX 1

ANALYSIS OF VARIANCE OF THE MEAN GROWTH RATES OF HEAT TREATED H6F3 USING BONFERONI INEQUALITY TEST.

		15°C		23°C		30°C		Mean Growth Rates mm/day ± S.D.
		Mid	Peri.	Mid	Per.	Mid	Peri.	
15°C	Mid							0.37 ± 0.08
	Peri.	t=1.37 n.s.						0.51 ± 0.01
23°C	Mid	t=0.09 n.s.						0.36 ±0.17
	Peri.		t=1.93 n.s	t=3.40 S*				0.7 ± 0.11
30°C	Mid			t=7.74 S*				1.12 ± 0.11
	Peri				t=7.0 S*	t=3.2 n.s.		1.46 ± 0.2

df 74

5% significance level > 3.215
1% significance level > 3.645

n.s. not significant at 5% level
S* significant at 5% level

APPENDIX 2

ANALYSIS OF VARIANCE OF MEAN GROWTH RATES OF HEAT TREATED 616 USING THE BONFERNONI INEQUALITY TEST

			No Treatment				Heat Treatment				Mean Growth Rates mm/day ± S.D.
			Mid		Peri		Mid		Peri		
			Initial	Final	Initial	Final	Initial	Final	Initial	Final	
No Treatments	Mid	In									0.48 ± 0.18
		Final									0.5 ± 0.15
	Peri.	In									
		Final									
Heat Treatment	Mid	In	t=5.96 S*	t=5.64 S*							1.25 ± 0.2
		Final					t=0 n.s.				1.25 ± 0.2
	Peri.	In	t=7.23 S*	t=6.9 S*							
		Final							t=0 n.s.		

df 44

5% significance level > 3.09
1% significance level > 3.645

n.s. not significant at 5% level
S* significant at 5% level

APPENDIX 3

ANALYSIS OF VARIANCE OF MEAN GROWTH RATES OF HEAT TREATED CULTURE F

		No Treatment	Heat Treatment		Mean Growth Rate mm per day S.D.
		Mid	Mid	Peri	
No Treatment					
	Mid				0.45 ± 0.08
Heat Treatment	Mid	t = 3.644 S*			0.70 ± 0.11
	Peri.	t = 3.634 S*	t = 0.38 n.s.		0.72 ± 0.19

df 54

5% significance level > 3.40
1% significance level > 3.825

n.s. not significant at 5% level
S* significant at 5% level

APPENDIX 4

ANALYSIS OF VARIANCE OF MEAN GROWTH RATES OF LA FRANCE DISEASE H6F3 AND CULTURE F FOLLOWING RIBAVIRIN TREATMENT

(i) H6F3	Ribavirin concentration ug/ml			
	MA	1	10	100
Mean Growth rate mm/day	0.53 ± 0.07	0.815 ± 0.90	0.56 ± 0.13	1.0 ± 0.0
MA	//	t = 4.49 S*	t = 0.37 n.s.	t = 6.8 S*

(ii) Culture F	Ribavirin concentration ug/ml			
	MA	1	10	100
Mean Growth rate mm/day	0.23 ± 0.0	0.45 ± 0.07	0.26 ± 0.03	0.53 ± 0.14
MA	//	t = 2,34 n.s.	t = 0.368 n.s.	t = 3,011 n.s.

13 df — 5% significance level > 3.116
1% significance level > 3.904

n.s. — not significant at 5% level
S* — significant at 5% level

APPENDIX 5

ANALYSIS OF VARIANCE OF MEAN GROWTH RATES OF LA FRANCE DISEASE H6F3 AND CULTURE F FOLLOWING CYCLOHEXIMIDE TREATMENT

(i) H6F3

	Cycloheximide concentration ug/ml				
	MA	5	15	25	50
Mean Growth rate mm/day	0.6 ± 0.07	0.36 ± 0.02	0.42 ± 0.09	0.73 ± 0.17	0.62 ± 0.03
MA		t = 1.48 n.s.	t = 1.44 n.s.	t = 1.38 n.s.	t = 0.234 n.s.

(ii) CULTURE F.

	Cycloheximide concentration ug/ml				
	MA	5	15	25	50
Mean Growth rate mm/day	0.2 ± 0.07	0.3 ± 0.05	//	0.27 ± 0.06	0.36 ± 0.04
MA		t = 1.28 n.s.	//	t = 0.906 n.s.	t = 2.43 n.s.

df 13 5% significance level > 3.116
1% significance level > 3.904

n.s. not significant at 5% level
significant at 5% level

APPENDIX 6

ANALYSIS OF VARIANCE OF MEAN GROWTH RATES FOLLOWING ATTEMPTED CELL FREE TRANSFORMATION OF APPARENTLY HEALTHY CULTURES

Treatment No. (1) Control

(2) Virus

(3) dsrna

(4) dsRNA/Virus

	Treatment No.				Mean Growth Rate mm/day ± S.D.
	(1)	(2)	(3)	(4)	
(1)					1.8 ± 0.46
(2)	t = 6.13 S*				1.05 ± 0.48
(3)	t = 3.715 S*	t = 2.17 n.s.			1.29 ± 0.41
(4)	t = 6.14 S*	t = 0.103 n.s.	t = 2.38 n.s.		1.02 ± 0.44

df 113 5% significance level > 2.69
1% significant level > 3.19

n.s. not significant at 5% level
S* significant at 5% level

APPENDIX 7

COMPARISON OF WHOLE CELL SECTORING DATA USING A STP NON PARAMETRIC TEST (BASED ON THE MANN-WITNEY TEST)

U = 623.7

At 5% significant level significant if > U.

Treatment No. (1) Control

(2) Virus

(3) dsRNA

(4) dsRNA/Virus

		TREATMENT			
		(1)	(2)	(3)	(4)
T	(1)				
R					
E	(2)	786			
A		S*			
T					
M	(3)	779	538		
E		S*	n.s.		
N					
T	(4)	795	526	466	
		S*	n.s.	n.s.	

n.s. not significant at 5% level

S* significant at 5% level