

DOUBLE-STRANDED RNA VIRUSES AND PATHOGENICITY OF THE
WHEAT TAKE-ALL FUNGUS, GAEUMANNOMYCES GRAMINIS
VAR. TRITICI.

A thesis submitted for the degree of Doctor of
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BY

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ABSTRACT

Double-stranded RNA (ds RNA) viruses have been investigated as a possible cause of hypovirulence in the wheat take-all fungus, Gaeumannomyces graminis var. tritici (Ggt). A collection of isolates of Ggt was made from wheat plants grown in field plots with known cropping histories. Total nucleic acid extracts were prepared from each isolate and the dsRNA fraction was analysed by polyacrylamide gel electrophoresis. The pathogenicities of isolates were compared by their effect on the dry weight of seedling shoots. Although a significant proportion of isolates appeared to be weakly pathogenic with this method of assay, there was no obvious correlation between low pathogenicity and the amount, numbers or molecular weight range of the ds RNA species in the isolates. In order to obtain direct evidence of the effect of virus infection on pathogenicity it was necessary to compare genetically related isolates with and without virus. This was achieved in two ways. Firstly, a number of isolates were cured of ds RNA infection by isolation of single conidia. Secondly, protoplasts from a ds RNA-free isolate have been infected with purified viruses and regenerated to yield isolates with novel infections. These virus-free and virus-infected isolates were assayed for pathogenicity using three more types of test; two of these were seedling disease indexes, while the third involved growing infected wheat plants to maturity. Many of the comparisons between virus-free and

virus-infected isolates demonstrated a change in pathogenicity, indicating that some ds RNA viruses probably reduce the pathogenicity of isolates of Ggt. The potential of ds RNA viruses for the biological control of take-all is discussed.

I would like to dedicate this thesis to my
parents and to Sally Adams, for their
constant support and encouragement.

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ABBREVIATIONS

The following non - standard abbreviations have been used:

Gg : Gaeumannomyces graminis

Gga : Gaeumannomyces graminis var. avenae

Ggg : Gaeumannomyces graminis var. graminis

Ggt : Gaeumannomyces graminis var. tritici

Og : Ophiobolus graminis

Pg : Phialophora graminicola

Pr : Phialophora radicicola

AbV : Agaricus bisporus virus

AfV : Aspergillus foetidus virus

AnV : Aspergillus niger virus

DrV : Diplocarpon rosae virus

GgV : Gaeumannomyces graminis virus

HmV : Helminthosporium maydis virus

PbV : Penicillium brevicompactum virus

PcV : Penicillium chrysogenum virus

Pc-fV : Penicillium cyaneo - fulvum virus

PsV : Penicillium stoloniferum virus

ScV : Saccharomyces cerevisiae virus

UmV : Ustilago maydis virus

" Where is the life we have lost in living ?

Where is the wisdom we have lost in knowledge ?

Where is the knowledge we have lost in information ? "

T.S. Eliot.

(From Choruses from "The Rock I")

INTRODUCTION

The disease of wheat (Triticum aestivum), known as take-all was first recognised in South Australia in 1852. By 1890 its cause had been identified as being a 'soil-borne' fungus, now called Gaeumannomyces graminis var. tritici. It infects the roots of wheat plants, causing vascular disruption and hence can seriously reduce grain production by the host plant. This active parasitic phase is followed by a saprophytic phase involving survival of the fungus on infected wheat straw (Garrett, 1981).

Take-all has been reported in most, if not all, regions of the world where wheat is grown, including Europe, America (North and South), the USSR and Australia (Garrett, 1981). As yet the best form of control for take-all is crop rotation, since no host breeding programmes have yielded any resistant varieties, nor have any fungicides been found to give an economically acceptable degree of control. However, rotation is often not ideal because there is not always a worthwhile non-graminaceous crop available to the farmer, to whom successful wheat monoculture is generally the most appealing option (Yarham, 1981). This outlines the problem faced by plant pathologists, breeders and farmers, i.e. none of the usual techniques for controlling plant disease appear to be feasible and thus other methods or new attitudes to old methods need to be investigated in an attempt to reduce the effect of take-all on the grain crop.

This study was undertaken to investigate the relationship between pathogenic variation in the take-all fungus and its double-stranded RNA viruses or possibly other cytoplasmic genetic determinants; with a view to developing an understanding of the nature of control of pathogenicity and possibly to provide a method of biological control of the take-all disease.

1.1 TAKE-ALL

1.1.1 The Organism.

In 1875 Saccardo described Raphidophora graminis Sacc. on a rotting culm base of Cynodon or Agropyron. This species was transferred to the genus Ophiobolus as O.graminis Sacc., an Ascomycete of the order Pleosporales, in 1881 by Roumeguere and Saccardo. Prillieux and Delacroix (1890), from a comparison of perithecia produced on wheat with the description given by Saccardo (1875), identified the cause of take-all as O.graminis (Sacc.). In 1952 Arx and Olivier, after studying the perithecia, asci and ascospores of the fungus responsible for take-all of wheat, placed it in a different order, the Diaporthales, and created a new genus, i.e. Gaeumannomyces. Later, following further studies, the wheat take-all fungus became known as Gaeumannomyces graminis (Sacc.). Arx and Olivier var. tritici Walker (Walker, 1972; 1981) which is often abbreviated to G.graminis var. tritici or Ggt. An early study of the wheat take-all fungus by Davis (1925) noted certain cultural characteristics; amongst these being that the fungus will grow between 4°C and ca. 30°C, in the pH range of 3 and 10, (optimum between 6 and 7), and that it is homothallic. The appearance of the fungus on agar is slightly variable but young colonies generally appear pale in colour, the hyphae being hyaline, later followed by the development of

darkly pigmented macrohyphae. Furthermore there is a tendency for the hyphae at the colony edge to curl back on themselves (Cunningham, 1975; 1981).

Other major taxonomic features of Ggt include: the production of perithecia on lower leaf sheaths and stems, unitunicate asci with slightly curved ascospores (70-105 μm x 2.5-3 μm), asexual spores of a type termed phialospores, production of runner hyphae on roots, formation of simple hyphopodia, (which are attachment and/or penetration structures), and a growth rate in culture at optimum temperature (20-25°C) usually in the range of 6-10 mm per 24 hours (Walker 1971; 1981).

Ggt contains fewer nuclei per hyphal compartment than other runner hyphae-forming fungi and certain other cer^eal root pathogens (Chambers, 1970b). Indeed hyphal tip cells of Ggt appear to be predominantly uninucleate, (average 1.3 ± 0.04 nuclei/cell). Similarly the asexual phialidic conidia produced in culture by flooding with water appear to be uninucleate (Deacon 1976; Deacon and Henry 1978a). These conidia are elliptical (6 x 3 μm) and tend to be germinable, whereas those produced in soil, which appear sickle-shaped, (3.7-7.5 x 1.0 μm), are not germinable (Brown and Hornby, 1971). Hence the asexual spores of Ggt are as yet of unknown significance in its life cycle. Pairs of nuclei in multinucleate cells become isolated and pass into ascogenous hyphae which are the origin of the asci. Nuclear fusion occurs in the young ascus with

four chromosomes observed in each nucleus. Eight ascospores are formed and these eventually each divide into a spore containing six uninucleate cells (Jones, 1926).

Ggt is essentially homokaryotic but with some genetic variation as a result of accumulated mutations (Asher, 1981). Synthesis of heterokaryons in culture has proved to be largely unsuccessful (Chambers and Flentje, 1967a; Rawlinson et al., 1973) and similarly little success was met in a series of crossing experiments using a genetic marker, (auxotrophy for PABA), for analysis (Blanch et al., 1981). However circumstantial evidence has been presented both by Deacon and Henry (1978a) and Naiki and Cook (1983a) that variation amongst progeny phenotypes may be due to nuclear type differences i.e. heterokaryosis.

Other fungi closely related to Ggt found in association with cereal roots in temperate regions include the two other varieties of G.graminis, vars. avenae, (Gga) and graminis (Ggg) and also species of the genus Phialophora (see Table 1). The varieties of the former genus are distinguishable by their host ranges and pathogenic effect on different hosts; Ggt can infect either wheat or barley, Gga is generally found on oats, (although it can also infect many other graminaceous hosts, including wheat), while the hosts of Ggg include stoloniferous grasses and rice (Deacon,

TABLE 1 (after Deacon 1981). Hosts and geographical distributions of the dark mycelial parasites most closely involved with take-all.

FUNGUS	DISTRIBUTION	CHARACTERISTIC HOSTS	DISEASE	SELECTED REFERENCES
<u>Gaeumannomyces graminis</u> var. <u>tritici</u>	Cosmopolitan	Wheat, barley	Take-all	Asher & Shipton (1981)
Var. <u>avenae</u>	Cosmopolitan	Oats, turf grasses	Take-all and Take-all patch	Walker (1972 & 1981) Nilsson & Drew-Smith (1981)
Var. <u>graminis</u>	Australia and other warm temperate/ subtropical soils	Stoloniferous grasses and rice	No disease Crown sheath rot	Walker (1972 & 1981) Ou (1972)
<u>Phialophora</u> sp. (lobed hyphopodia)	Europe Australia	Maize, other cereals Stoloniferous grasses	No disease No disease	Deacon (1981) & Walker (1981)
<u>Phialophora</u> sp. (unlobed hyphopodia)	France South Africa	Maize, wheat Maize	No disease No disease	Deacon (1981) & Walker (1981)
<u>P. graminicola</u>	Europe Australia	Grasses	No disease No disease	Scott (1970) Deacon (1973b & 1981)
<u>P. radicicola</u>	Canada	Maize	No disease	Cain (1952), McKeen (1952) Walker (1981)

1981). The representatives of the genus Phialophora that are found on the roots of cereals and grasses are very similar in morphology, cytology and conidiogenesis to Gaeumannomyces; the only differences being their relative root-invasive capacities (Deacon, 1981) and their inability to form perithecia. There are primarily three representatives distinguishable; Phialophora sp. (lobed hyphopodia), which resembles an imperfect state of Ggg, Phialophora sp. (unlobed/undefined hyphopodia) and Phialophora graminicola; believed to be the imperfect state of Gaeumannomyces cylindrosporus (Walker, 1981; Deacon, 1981). The first two types of Phialophora sp. fall into the moderately invasive category, i.e. they will only penetrate to the sixth cortical layer, next to the endodermis, of wheat seminal roots, but will sometimes penetrate the stele of wheat lateral roots, (which only have four cortical cell layers), hence causing localised vascular disruption which does not spread into the seminal root and does not cause any reduction in shoot growth (Deacon, 1974b; Deacon, 1981). P. graminicola is termed a poorly invasive parasite and will only penetrate to the sixth cortical layer of wheat roots following their natural senescence (Holden, 1976). It appears that P.graminicola is widespread in agricultural soils and is a successful member of the wheat rhizosphere population (Deacon, 1973a; 1973b; 1974a; 1974b; 1976b; Slope et al., 1978). Hence it is

apparent that there is a range of "dark mycelial parasites" found on the roots of the Gramineae, with Ggt being one of the most damaging to the host plant.

1.1.2 Infection and Pathogenicity.

The initiation of the infection process and the subsequent development of the infection of wheat roots by Ggt depends upon the inoculum potential or 'invasive force' of the fungus. This can be defined as 'the energy of growth of a parasite available for infection of a host at the surface of the host organ to be infected (Garrett, 1970). Two factors can be identified as being important in this respect; firstly, the cross-sectional area of the fungus in contact with the unit area of the host surface, and secondly, the relative degree of vigour of these hyphae. In the case of Ggt it appears that there are two sources of inoculum; ascospores and infected crop residues from a previous growing season (Brooks, 1965; Hornby, 1981). Ascospores are probably only of major significance in the case of infection at sites where take-all has not been previously observed (Hornby, 1981). Brooks (1965) records that ascospores of Ggt will not germinate in unsterile soil and will only germinate on or next to an exposed proximal part of a seminal root of a wheat seedling. Hence airborne Ggt ascospores can initiate infection, but are unlikely to be as significant as other sources of inoculum in the epidemiology of the

disease. Gerlagh (1968) and Rawlinson and Muthyalu (1976) have provided evidence for a pioneering function of ascospores, notably on newly cereal-cultivated land, where arrival of inoculum by other means seems intrinsically unlikely. Soil-borne inoculum seems to be the major source for the continued epidemic with infected crop residues being the major site of carry-over (Hornby, 1975; 1981). As these residues decompose, their population of Ggt declines, the pathogen having only limited competitiveness as a soil saprophyte (Garrett, 1970). Thus infectivity decreases and the distribution of inoculum becomes fragmented as the interval from the previous wheat crop lengthens. If a field that has been subject to wheat monoculture is left fallow for two years then the inoculum potential drops below detectable levels (Hornby, 1975). It has been suggested that another possible reservoir of soil-borne inoculum is grass weeds in and around agricultural soil. Many weeds are hosts to Ggt, e.g. Agropyron repens and Lolium perenne, and hence perpetuate the pathogen even in the absence of its main host (Hornby, 1981; Nilsson and Drew-Smith, 1981).

There appears to be no varietal variation in susceptibility of wheat to Ggt and hence the speed of infection and host response differs only in timing not nature, between different pathogen isolates. Such resistance as there is, is horizontal, sensu Van der

Plank (1968). Slope and Cox (1964) reported that all commercial varieties of wheat and barley are susceptible to take-all, while pointing out that even small differences in susceptibility could be important with respect to yields of wheat and barley grown as monocultures. Nilsson (1973) claimed to have observed substantial differences in resistance of eight winter wheat cultivars over a two month growth period, but these results have not been confirmed. Moreover Scott (1981) reiterates the view that there is a lack of natural resistance potential in wheat and barley with all known cultivars being susceptible to take-all under field conditions; wheat being highly susceptible and barley somewhat less susceptible. Scott and Hollins (1980) have observed that Triticale appears to be resistant to Ggt when mature, although it is susceptible as a seedling. However, Triticale is not, as yet, a commercially attractive alternative to wheat.

The infection process begins with the growth of hyphae of Ggt towards host roots (Brown and Hornby, 1971; Weste, 1972; Pope and Jackson, 1973; Wildemuth et al., 1984) followed by the formation of runner hyphae which are the result of fusion of 2 or 3 hyaline hyphae (Weste, 1972) on the surface of the root. The runner hyphae grow and spread mainly longitudinally over the root surface (Chambers and Flentje, 1967a), and off these are formed hyaline infection hyphae (see Figure 1). These hyphae can penetrate root hairs, epidermal

cells, or cells inside the root and they are also formed by germinating ascospores (Weste, 1972). On contact with a living host cell the hypha forms an appressorium-like swelling with a narrow infection of penetration peg (Weste, 1972; Walker, 1972). On initiation of penetration the host responds by deposition of lignin in the cell wall and successful penetration appears to depend on the enzymatic degradation of the components of the cell wall. (Holland and Fulcher, 1971; Pearson, 1974; Skou, 1981). Lignin deposition is relatively localized to the site of the penetration peg and the resulting appositions have been termed lignitubers. Skou (1981) described the balance between the production of the lignituber by the host and the rate of lignin degradation by the pathogen as a struggle on which depends the success or failure of the infection. Once the apex is penetrated by the infection hypha then the invaded cell soon dies, although no phytotoxic effect associated with Ggt has been observed (Weste and Thrower, 1971). It seems the endodermis is the most resistant layer of cells which only the most invasive of root pathogens can penetrate. Speakman and Lewis (1978) and Speakman, Garrod and Lewis (1978) observed that while Ggt could penetrate the endodermis of wheat roots and pass into the stele; Phialophora sp. (lobed hyphopodia) could penetrate cortical cells but not any further and P.graminicola

Figure 1 (from Skou, 1981)

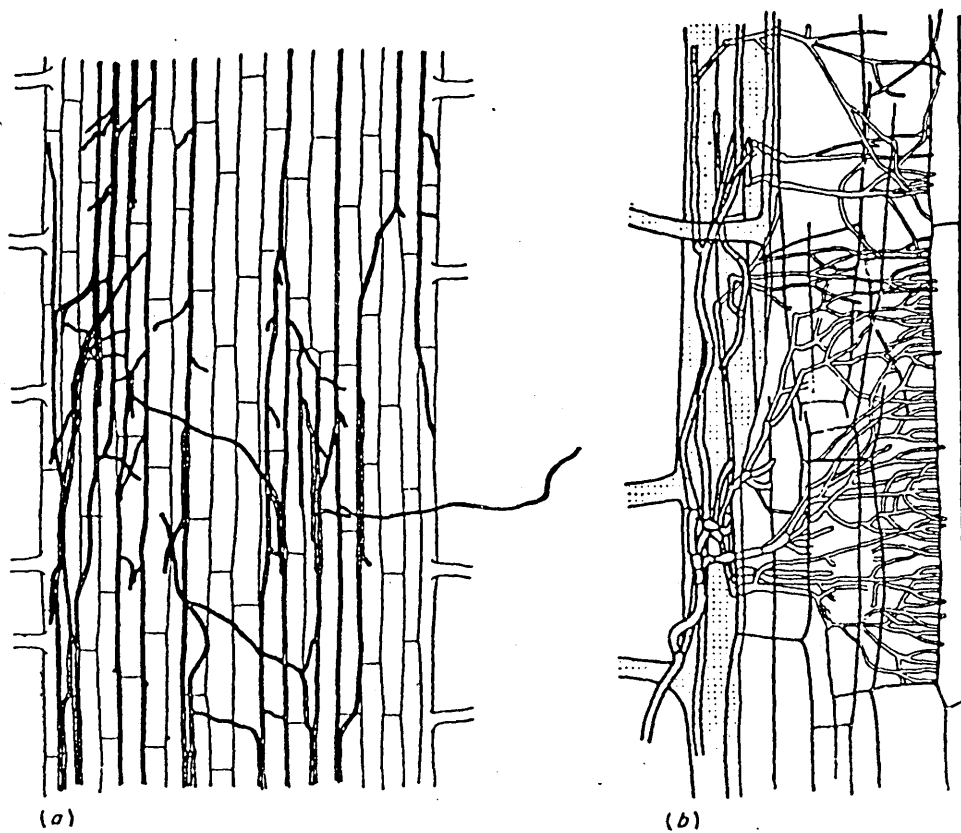


FIG. 1 Ectotrophic growth and infection of a wheat seedling root by *G. graminis*: (a) surface view, (b) longitudinal section with dark runner hyphae on the surface to the left and with hyaline infection hyphae spreading in the cortex to the right.

would not penetrate beyond the third layer of live cortical cells, its progress being stopped by lignituber formation. However, as previously discussed root cortex death (Holden, 1976) permits the passage of P.graminicola to the innermost layer of cortical cells which in healthy wheat plants remain alive. The endodermis being a naturally stronger barrier, with lignin and suberin deposited in its cell walls as a matter of normal development (Esau, 1977), is probably initially penetrated by Ggt through the plasmodesmata (Clarkson et al., 1975). However, once this layer of cells is overcome the fungus can invade and spread through the remainder of the stele in a fashion similar to the invasion of the cortical cells, causing disruption of the phloem and occlusion of the xylem vessels with the consequent disruption of host metabolism and development of the diagnostic morphological feature of black stelar lesions (Clarkson et al., 1975). Once this stage is reached the fungus spreads freely through host tissues up to the stem base causing extensive destruction and cell death finally producing perithecia on the stem base (Skou, 1981).

Once vascular disruption has begun, evidently host physiology is seriously affected. Asher (1972b) observed that the most significant effect of infection was the prevention of water transport, while there was also an increased translocation of assimilates from the shoots to the roots. However, both Clarkson et al.,

(1975) and Fitt and Hornby (1978) considered that the most crucial stage of infection was the disruption of the phloem since this caused reduced translocation of nutrients to the apical meristems, cessation of root elongation and cessation of ion flow into the xylem. Furthermore, Gilligan (1980), studying the spread of Ggt on wheat roots noted that a secondary infection distal to the primary infection is uncommon due to a reduced disease potential of the root, possibly because of a lowered nutrient status.

The physical effects on the plant caused by this disruption of its metabolism include; reduced biomass and leaf area, fewer tillers and retarded root growth, and either impaired grain development, (often termed 'whiteheads'), or even no grain development (Asher, 1972a; Chambers, 1971; Manners and Myers, 1981). If infection occurs when the plant is reasonably mature then often it can compensate for these deleterious effects to some extent by producing more adventitious roots; a mechanism called 'disease escape', or tolerance (Asher, 1972a; Garrett, 1981). Evidently the effect on grain yield is of the greatest consequence, however this often proved difficult to assess. Weste and Thrower (1971) examined the effect of twelve isolates of the take-all fungus on grain production and plant dry weight and in each case they saw a significant reduction in yield. Cunningham et al.

(1968) using field plots artificially infected with Ggt recorded decreases in grain yield of 50% and 24% for wheat and barley, respectively. A more widespread survey using statistical analysis allowed Stynes and Wallace (1974) to demonstrate that Ggt in their sample was responsible for an average yield reduction of 9% between flowering and harvesting without obvious symptoms, such as whiteheads being manifest. The problem of assessing yield losses accurately has not yet been solved though it is evident that take-all causes substantial yield losses (Clarkson and Polley, 1981; Lester, 1981).

There are two further topics which should be discussed here since although they are not as well understood as the infection process and its outcome, they would appear to influence infection directly. They are pathogenic variation and the phenomenon known as take-all decline.

Variation in levels of pathogenicity of isolates of the take-all fungus has been observed ever since the early studies of the disease, e.g. Davis (1925). However, as pointed out by Asher (1980) most of the early studies involved few isolates and gave little indication of how long they had been in culture. This could well be an important consideration since Chambers (1970a) and Naiki and Cook (1983a) have reported that some isolates appear to become less pathogenic on repeated subculturing. The studies of Rawlinson et al.

(1973) and Cunningham (1975) indicated however that amongst a large number of isolates of known culture history, a wide range of pathogenicities could be observed. Further to this Blanch (1977) and Asher (1980) studied freshly isolated populations of Ggt from wheat roots and concluded that the isolates examined did demonstrate variation in pathogenicity, as assessed by their effect on the shoot dry weight of wheat seedlings. The results of Cook and Naiki (1982), who also studied fresh field isolates, show less variation in pathogenicity, with the majority of their isolates being highly pathogenic and also some loss of pathogenicity on subculturing. Overall it would appear that there is a distribution of pathogenicity levels in natural populations of Ggt. Furthermore, two studies have observed variations in the pathogenicity of progeny populations. Chambers and Flentje (1967b) noticed that although all the progeny from a perithecium on host tissue were highly pathogenic, the progeny from a perithecium in culture were equally divided into strongly and weakly pathogenic populations. Secondly, isolates from conidia also differ in their ability to cause disease (Deacon and Henry, 1978a). At the cellular level Clarkson et al. (1975) observed that a less pathogenic isolate caused lesions similar to those of a highly pathogenic isolate, but more slowly and less frequently.

Potential sources of pathogenic variation include: mutation, sexual reproduction, parasexual recombination, heterokaryosis and cytoplasmic genetic determinants. In Ggt pathogenicity appears to be under polygenic control with possibly five to nine effective factors involved, as determined by genetic crosses (Blanch, 1977; Blanch, Asher and Burnett, 1981). It seems likely, therefore that isolates of Ggt do genuinely vary in pathogenicity and that this variation is due either to selective pressures on the controlling genes and accumulated mutations in these genes (Asher, 1981) or to the effect of cytoplasmic factors (Rawlinson and Buck, 1981), or a combination of both.

Take-all decline (TAD) is a phenomenon noticed with the onset of intensive cereal growing without rotation, a practice which is now widespread following the development of husbandry practices to a point where it can be very profitable (Yarham, 1981). The general pattern involved in the phenomenon can be summarised as follows: when soil new to cereal growing or following a number of fallow years is planted with consecutive wheat or barley crops there is a steady increase in yield loss due to take-all, up to the third and fourth crops followed by a recovery in yields to some degree and a stabilization at or about this intermediate level. Various authors, e.g. Slope and Cox (1964), Gerlagh (1968), Shipton (1972), Brown, Hornby and Pearson (1973) and Cunningham (1975), have observed

this pattern. Many possible causes for take-all decline have been proposed and these have been reviewed by Hornby (1979), who described TAD as 'a theorist's paradise'. Most popular are; firstly, that there is a development of an antagonistic microflora in the soil by the onset of decline (Brown, Hornby and Pearson, 1973; Rovira and Wildemuth, 1981), maybe involving antibiosis (Gerlagh, 1968), and secondly, that soil nitrogen levels have an effect on take-all disease severity, i.e. the greater the soil nitrogen level the lower the disease severity (Slope and Cox 1964; Brown, Hornby and Pearson, 1973; Huber, 1981). As will become apparent in the next section (1.1.3) these two hypotheses probably interact and hence contribute to take-all decline. Lastly, it should be noted that pathogenic variation within populations of Ggt does not appear to have any bearing on take-all decline (Asher, 1980; Cook and Naiki, 1982).

The process of infection of host roots and other tissues is evidently a major part, if not the major part of the life cycle of Ggt and it is well adapted to this role since wheat and barley roots offer little or no resistance to the progress of the fungus into its vascular tissue. Grain yield losses can be serious, but the host does not die until late in the growing season which is to the advantage of Ggt for its overwintering. Pathogenic variation and take-all

decline are presumably expressions of natural selection and the ecology of the whole of the life cycle of the take-all fungus.

1.1.3 Saprophytic Survival.

The ecology of Ggt will be discussed here in reference to its surrounding soil microflora and soil status. Saprophytic survival of take-all inoculum begins in crop residues at the time immediately post harvest and depends on a number of interacting factors including the competitive saprophytic ability (CSA) of the strain of take-all, microbial competition for the crop residues and the physical and nutrient condition of the soil (Shipton, 1981).

It is believed that Ggt survives as a saprophyte on crop residues in the main, but it has also been shown to exist in small fragments of organic debris in the soil (Hornby, 1975; Sivasithamparam and Parker, 1976). This phase of the life cycle of Ggt would seem to be divisible into two sections, these being firstly, possession of, and survival on an organic substrate and secondly, growth of hyphae from this nutritional base through soil towards living host roots.

The survival of Ggt, (and hence its fitness for growth later towards fresh host roots), is dependent on its (CSA) which has been defined as the summation of physiological characteristics that make for success in colonization of dead organic substrates in competition

with other microorganisms (Garrett, 1970). The CSA of Ggt appears to be limited, e.g. when compared with another root-rot pathogen such as Fusarium roseum f. sp. cerealis 'Culmorum', it appears to be a poor straw colonizer and much more susceptible to competition (Garrett, 1970). A crucial aspect of the survival of Ggt is the nitrogen level in the substrate. Garrett (1970) concluded that Ggt can die out prematurely in wheat straw from exhaustion of respiratory carbon reserves because it has not sufficient nitrogen to continue mycelial growth which would enable it to exploit further reserves of cellulose in the straw. Further to this, Garrett (1976) introduced the concept of cellulolysis adequacy index (CAI) as an indication of the extent to which a fungus decomposes cellulose at a rate sufficient to maintain saprophytic survival in straw buried in soil with no nitrogen supplement. The CAI of Ggt appears to be intermediate with respect to other soil fungi. Hence Ggt tends to survive best presumably in soils with an average level of nitrogen, since at low levels its rate of cellulolysis would be insufficient to support mycelial growth, while in soils of high nitrogen levels microbial competition increases, which is detrimental to the take-all fungus.

The phenomenon of fungal growth inhibitor production by many isolates of Ggt (Romanos et al., 1980; McGinty et al., 1984) may well also play a role in saprophytic survival of, and substrate possession

by, Ggt. The inhibitor, produced on media in the pH range 3.5-5.0 is inhibitory to the producer strain, to other isolates of Ggt and to certain other species of fungi. It is possible therefore that at soil pHs below 5, that self inhibition and inhibition of the microflora would decrease the rate of straw decomposition, hence aiding the survival of the Ggt isolate. Alternatively it may be detrimental to growth on, and infection of, wheat roots and therefore be involved in TAD (Smiley and Cook, 1973; Rawlinson and Buck, 1981). Furthermore, Naiki and Cook (1983b) have suggested a correlation between increased duration in culture of a Ggt isolate, decreased pathogenicity and increased potential for inhibitor production. However if production of the inhibitor is detrimental to the survival of the fungus, it is odd that so many Ggt isolates maintain the capacity to produce inhibitor (McGinty et al., 1984) apparently contrary to selection pressure. Therefore the former theory would seem more plausible.

Survival of Ggt is detrimentally affected if the infested soil is left fallow or used to grow 'break crops' i.e. non-host Gramineae (e.g. oats) or other non-hosts such as legumes (Hornby, 1975; Shipton, 1981). Kollmorgen, Griffiths and Walsgott (1983) have compared grain yields from a wheat crop after a non-graminaceous crop, such as field peas (Pisum sativum

L.) or oilseed rape (Brassica napus L.) and a graminaceous crop such as wheat, barley or triticale. They found grain yields to be considerably higher after the non-cereal crop, (or fallowing), than after the cereal crops; indicating that in the former case there appears to have been a reduction in inoculum level of Ggt due to its relatively poor ability to survive long periods in the soil without living host roots to infect.

Although such loss in inoculum potential appears to occur rapidly, Shipton (1972 and 1981) has recorded that soil used for growing non-susceptible break crops could still cause take-all up to 66 months after the last susceptible crop. Three potential explanations could be forwarded for this and similar observations. Since it seems unlikely from the CSA of Ggt, that mycelial inoculum residing in organic debris could be responsible, such inoculum could arise from a resting spore stage in the soil, from ascospores causing re-infection or graminaceous weeds acting as an inoculum reservoir. Sivasithamparam and Parker (1976) have reported the formation of chlamydospores and microsclerotia by Ggt in culture under stress and although these have not been observed in nature; soil particles of diameter less than 0.42 mm were shown to carry Ggt inoculum indicating a possible biological role for microsclerotia and chlamydospores, although this is as yet unproven.

Methods of stubble cultivation also have an effect on the survival of Ggt although it appears that a particular method can have varying results in differing soils and climates. For example the practice of direct drilling wheat seed with no cultivation of the stubble^b (or 'zero tillage') has produced lower levels of take-all incidence in winter wheat in England (Brooks and Dawson, 1968) and reduced take-all in spring barley in Scotland (Lockhart et al., 1975); while Moore and Cook (1978), Rovira and Venn (1983) and Kollmorgen and Walsgott (1984) have reported increased take-all in the Pacific Northwest USA and Victoria, Australia due to zero tillage. Brooks and Dawson (1968) attributed their results to the reduced spread of the fungus because the soil was less disturbed; but both Moore and Cook (1978) and Kollmorgen and Walsgott (1984) believe that increased take-all incidence was due to greater quantities of surviving inoculum. Furthermore the Australian authors noted that deep ploughing of the stubble was effective in reducing take-all survival in their study. Hence the method of tillage chosen has an evident effect on inoculum and varies between differing soils and climates.

The physical and chemical status of the soil in which Ggt may be found also has a bearing on inoculum potential. Generally speaking a higher soil pH, often due to liming causes increased carry-over of take-all

and this probably links to some extent, with the observation that nitrogenous fertilizer in the form of ammonium ions reduces take-all since it reduces the rhizosphere pH (Cook, 1981; Trollidenier, 1981). The level at which soil pH influences the saprophytic stage of Ggt is unclear; however Cook (1981) has postulated three separate, or possibly linked mechanisms. Firstly, low pH shifts the CO_2/HCO_3 balance to the more gaseous side and is more inhibitory to Ggt. Secondly, more acid soil increases the potential for microbial antagonism; and thirdly, certain plant nutrients such as manganese and other trace elements are less available in alkaline soils causing the plant to be more susceptible to take-all. (This last point has been confirmed by Rovira, Graham and Asher, 1983; Reis, Cook and McNeal, 1983). Huber and co-workers have studied the effect of ammonium fertilizer ($\text{NH}_4^+\text{-N}$) applied to soil infested with take-all at some length. In 1972 Huber reported that $\text{NH}_4^+\text{-N}$ applied in autumn led to increased take-all, while if applied in spring it decreased take-all. Huber et al. (1980) observed that if an inhibitor of nitrification, such as nitrapyrin was applied with the fertilizer then yields were not affected by take-all, and Huber (1981) propounds the view that much of the yield loss due to take-all, and perhaps due to other diseases of wheat, could be alleviated by maintaining a better nutrient balance in the soil, especially nitrogen, phosphorous

and potassium. This could be achieved by avoiding alkaline soils. More recently Huber and Mburu (1983) reported that application of $\text{NH}_4^+\text{-N}$, (stabilized with nitrapyrin), was correlated with lower populations of manganese oxidizing bacteria in the soil, (permitting more manganese uptake by the host plant) and decreased severity of take-all. Powelson *et al.* (1983) have also noted that addition of chloride to the $\text{NH}_4^+\text{-N}$ fertilizer further reduces incidence of take-all. Hence the status of the soil appears not only to be important with regard to the inoculum potential of Ggt but also it is important to the host plant regarding its fitness of resistance to the pathogen.

The influence of the soil microflora on inoculum potential of Ggt has been studied extensively and there appear to be a number of groups of natural soil inhabitants that reduce the vigour and survival of Ggt, their combined effect often being recorded as producing a soil that is suppressive to the take-all fungus. Wildemuth, Rovira and Warcup (1979) and Rovira and Wildemuth (1981) have reviewed the nature and mechanism of this suppression, and they have made a number of significant observations. Firstly, it appears that the reduction in infective hyphae of Ggt reaching and colonizing wheat roots in such soil is due to a decrease in the emergence of hyphae from inoculum sources and also lysis of some of these hyphae. Hence

the site of suppression is the rhizosphere soil and not the root surface. Secondly, there appear to be two major types of suppression; general suppression which is the sum of microbial activity in the soil and is non-transferable to other soils and specific suppression, which is associated with cereal monoculture, it can be destroyed by heating the soil to 60°C for 30 minutes and is also transferable to other soils. This specific suppression they suggest is associated with non-sporing, heat sensitive, rod-shaped bacteria, which act mainly around the plant residues, reducing Ggt hypha emergence and causing lysis of emerging hyphae. There have been a number of reports of accumulation of such a specific suppression factor with these characteristics, e.g. Shipton, Cook and Sitton (1973); Sivasithamparam, Stukeley and Parker (1975); MacNish (1976); but as yet no one single organism type has been identified as being the cause, although there are three possibilities, and of course it may be an interaction of all three. Fluorescent pseudomonads and possibly other bacteria such as Bacillus cereus var. mycoides and Streptomyces lavendulae have been observed to have detrimental effects on Ggt survival and vigour, both on agar and in the soil (Rovira and Campbell, 1975; Cook and Rovira, 1976; Sivasithamparam, Parker and Edwards, 1979; Tschudi and Kern, 1979; Campbell and Faull, 1979; Campbell and Ephgrave, 1983). Furthermore, Smiley

(1978a; 1978b) observed lower hyphal vigour in soil amended with NH_4^+ -N fertilizer and increased numbers of Pseudomonads spp. and streptomycetes which appeared to be directly inhibiting infection of wheat roots by Ggt. Potential fungal inhibitors of Ggt have been investigated by Sivasithamparam and Parker (1980a; 1980 b) and Wildemuth (1982). Fusarium oxysporum was observed, amongst other fungi, to be capable of inhibiting growth of Ggt both in the soil and on nutrient-poor agar. In general these inhibitory fungi also appear to be restricted to organic matter for their saprophytic survival and hence would appear to act on Ggt in a different manner from the inhibitory bacteria discussed above. Lastly, certain soil inhabiting amoebae have been reported to be capable of attacking hyphae of Ggt causing localized lysis of the hyphae presumably in a parasitic fashion (Homma et al., 1979; Chakraborty and Warcup, 1984). Therefore it appears that there is a range of soil microorganisms capable of antagonising Ggt in its saprophytic phase and it is probably a balance of these that make up the specific antagonism described by Rovira and Wildemuth (1981).

It is evident therefore that the saprophytic phase of Ggt involves various factors ranging from the CSA of the fungus in the crop residues, the surrounding nutrient balance, especially nitrogen and its form, the

soil micro-flora and the cultivation techniques, if any, the soil is subjected to. All these factors interact to influence the inoculum potential of Ggt to reinitiate infection.

1.1.4 Potential Control Methods.

Control of the take-all disease of wheat has not as yet been successfully accomplished in conjunction with intensive cereal farming, but there are a number of promising methods and these are discussed below.

a) Cultivation techniques.

Crop rotation is believed by some authors to be at present the only form of successful control (Slope and Etheridge, 1971; Yarham, 1981). As Slope and Etheridge (1971) point out, the first wheat crop after the break is safe from take-all and should be high-yielding, but the next few wheat harvests are those that are most susceptible to take-all attack. Furthermore, Yarham (1981) discusses the problem of loss of income for the farmer involved in crop rotation. He suggests that where ~~non-graminaceous~~ crops are economically worthwhile, e.g. oilseed rape, they should be used as alternate crops with wheat to eradicate Ggt.

New crop production methods have also been investigated for their effect on take-all incidence, the best example being direct drilling. As discussed in the previous section (1.1.3) this has shown mixed results, giving some disease control for Brooks and

Dawson (1968) and Lockhart, Heppel and Holmes (1975), but increased take-all severity for Moore and Cook (1978), Rovira and Venn (1983) and Kollmorgen and Walsgott (1984). It seems probable that this is an expression of different soil types, (success being found in firmer, heavier soil that restricts pathogen movement), and hence could be adopted in some parts of the world to lessen take-all incidence.

The other promising cultivation technique revolves around better use of fertilizers and soil ammendments (discussed in 1.1.3).

b) Use of fungicides.

Until recently no fungicides had been reported as being biologically active against take-all. Bateman (1983; 1984) and Rovira and Whitehead (1983) have achieved reductions in take-all severity using benomyl primarily, and also PCNB and nuarimol, all as soil drenches. Furthermore, Bateman (1983; 1984) noticed an increase in grain yield with benomyl of the order of 12% in the first crop; however, this was not seen in the second successive crop, possibly due to decreased microbial antagonism. Bockus (1983) using triadimenol-treated wheat seeds reported reduced take-all severity in artificially and naturally infested field soil to the extent that grain yield losses due to take-all were decreased by up to 60 to 75%. If this treatment is economically viable and effective in soils other than the Kansas soil in which these trials were performed,

then this could be the first effective control measure for Ggt in cereal monoculture.

c) Breeding for resistance.

Scott (1981) has outlined the lack of natural resistance potential to Ggt in wheat and barley. Those members of the Gramineae that are resistant do not, unfortunately, form fertile hybrids with wheat or barley. Hence breeding for resistance would have to involve mutagenesis; presumably on single cell cultures or protoplasts, followed by regeneration to whole plants, (which has not been successful as yet), in an attempt to produce useful variation. Or long term screening projects for polygenic resistance may produce a variety with increased resistance. Holden (1980) has confirmed that Ggt cannot infect oats due to the production by the host of the inhibitor compound avenacin. Furthermore he suggested that if the ability to produce avenacin could be transferred to wheat and barley, either by conventional ^{on} breeding techniques or perhaps by genetic manipulation then this would confer resistance to Ggt. Overall though, it appears that the prospect for producing resistant cultivars of wheat and barley to take-all is at present a long way off.

d) Disease forecasting.

Disease forecasting has been a successful technique employed against many fungal foliar pathogens of crops and has rendered fungicide treatment of these

diseases more effective and economic. As Clarkson and Polley (1981) have discussed, little is yet known of the climatic, soil and cropping factors that would be essential to produce an accurate forecasting system for outbreaks of take-all. However, if this were achieved, accurate forecasts at present would be of little value since there is not much that can be done to prevent the disease. However, two possibilities are evident; application of NH_4^+ -N fertilizer, which appears to reduce take-all severity (Huber, 1981) and application of benomyl as a soil drench (Bateman, 1983; 1984; Rovira and Whitehead, 1983). Hence the development of an efficient forecasting system would be valuable, provided that other methods of control continue to show promise and become operational.

e) Cross-protection by Pg and Ggt.

In the ectotrophic growth stage of Ggt, prior to infection of the stele, a number of other fungi taxonomically and ecologically similar to Ggt, though lacking its ability to cause grain yield loss, are also present (Balis, 1970; Holden, 1976; Deacon, 1981). Of these fungi Phialophora graminicola (Pg) and Gaeumannomyces graminis var. graminis (Ggg) are especially interesting as potential biological control agents.

Death of the host plant root cortex plays an important role in the mode of survival of these two avirulent fungi, since it has been observed that,

unlike Ggt, they cannot penetrate living cortex cells and hence depend upon the release of cell contents for their nutrition (Holden, 1976; Deacon and Henry, 1978b; Deacon, 1981). On the other hand though it is apparent from Kirk (1984) that Ggt can also benefit from the senescence of the root cortex, providing it with greater invasive force for the remaining root cell layers. Hence it is a delicate balance between Ggt and these avirulent fungi. For the avirulent fungi to restrict the spread of Ggt on and into the root they must colonize the root heavily first. The mode of action of protection is unclear but probably involves not only possession of the ecological niche but also induction of lignin deposition in the cortical cell walls (Speakman and Lewis, 1978). Cowan (1978) reported that not only was there an increase in lignification induced by avirulent invasion, but also a qualitative change in the lignin involving deposition of caffeic acid in the compound matrix which may adversely affect the progress of Ggt through the cell wall. Deverall, Wong and McLeod (1979) developed a bioassay to determine sensitivity of Ggt to antifungal compounds and found that Ggt is sensitive to many phytoalexins. However, they could find no evidence for production of such compounds in wheat roots when either challenged by Ggt or cross-protected by Ggg. So it seems unlikely that cross-protection in this situation

involves phytoalexins.

In the field, many workers have observed that reduced take-all severity can result from a high population level of Pg (Deacon, 1973a; 1976b; Slope et al., 1978; Slope et al., 1979). However, this tended to be effective for only a few wheat crops following breaks using grass leys. Rawlinson, Hornby and Muthyalu (1977) noted that the problem with using Pg as a biological control agent would be accumulating and maintaining a sufficiently high population level of Pg in soil used in cereal monoculture.

Wong (1975), and Wong and Southwell (1980) achieved reduction of take-all severity in glasshouse and field trials, respectively, using Ggg and Ggg/Phialophora sp. mycelium as a soil amendment. However, Wong (1981) admits that this technique does not achieve complete control, nor is it likely to. Perhaps though, if isolates of either Pg or Ggg could be selected that persist between cereal crops and compete better with Ggt then this method would form an effective technique for take-all control. In one respect this has been achieved; Wong (1980) selected isolates of Ggg that grow better at temperatures below 15°C and hence these would compete with Ggt more effectively early in the wheat growing season.

f. Antagonism by bacteria.

General and specific antagonism of Ggt by bacteria was discussed in the previous section on saprophytic

survival of Ggt and has also been reviewed by Rovira and Wildermuth (1981). Specific antagonism appears to act both around crop residues and also in the rhizosphere of growing wheat plants. Hence attempts have been made to adapt this natural phenomenon to create a method of biological control.

Faull (1978) and Campbell and Faull (1979) studied the effect of an isolate of Bacillus cereus var. mycoides and reported disease suppression using artificially inoculated soil in both glasshouse and field trials. However, as often observed with biological control methods, the antagonist does not persist in the soil between crops at an effective level and problems were encountered with producing sufficient bulk of the bacterium for a large scale operation (Campbell and Faull, 1979).

The use of pseudomonad bacteria perhaps appears to be more promising as a control agent. Wong and Baker (1981) and Weller and Cook (1981) both reported disease suppression of take-all using soil amendments of the bacteria in greenhouse tests. Following a mass-screening of pseudomonads isolated from wheat roots for capacity to suppress take-all (Zhang, Weller and Cook, 1983), Weller (1983) and Weller and Cook (1983) have achieved improved grain yields of up to 27% in natural soil by pre-treating winter wheat and spring wheat seeds with one or other of two especially active

strains of pseudomonad. This method of control would appear to be potentially successful on a widespread basis provided it is not too difficult or costly to pretreat all commercial wheat seed liable to encounter Ggt as it grows into a mature plant.

g. Viruses of Ggt.

The potential of virus infection for the control of Ggt will be discussed in section 1.2.3.

h. Conclusion.

At present there appear to be a number of promising methods by which take-all may be controlled. Yarham (1981) suggests that an integrated approach would yield the best results, i.e. partial control being supplied by a number of the topics discussed, adding up to an overall control effect.

1.2 MYCOVIRUSES AND EXTRACHROMOSOMAL ELEMENTS OF FUNGI

1.2.1 Mycoviruses; General Characteristics.

Since the original demonstration of virus particles in the cultivated mushroom, (Agaricus bisporus), associated with 'die-back disease' (Hollings, 1962) many other viruses or virus-like particles (VLP) have been observed in various fungi, hence the name, mycoviruses. Further interest was generated by the discovery that isometric virus particles found in Penicillium stoloniferum were associated with the interferon-inducing capacity of statolon, a fermentation product of P.stoloniferum (Ellis and Kleinschmidt, 1967). Similarly, helenine, another interferon inducer and fermentation product of Penicillium funiculosum was demonstrated to be double-stranded RNA (dsRNA) in nature and probably of viral origin (Lampson et al., 1967). Following this Banks et al. (1968) showed that both P.stoloniferum and P.funiculosum contained isometric virus particles with ds RNA genomes and that the ds RNA was capable of inducing interferon production. Various other closely related fungi were soon observed as harbouring similar virus particles, e.g. Penicillium chrysogenum (Banks et al., 1969a), Penicillium cyaneo-fulvum (Banks et al., 1969b) and Aspergillus foetidus and Aspergillus niger (Banks et al., 1970). Banks, Buck and Fleming (1971) produced a large scale isolation technique for the

active ds RNA which is an indication of the commercial potential of these viruses at that time. Hence mycoviruses became of major interest as interferon inducers (reviewed by Buck, 1979a) and as extrachromosomal genetic information in fungi of interest to science and commerce such as P.chrysogenum.

Bozarth (1972) has made an assessment of the number of fungal species that are liable to carry a viral infection. Random sampling of fungal species at that time indicated that 10 to 15% contain VLP from electron microscopy studies. Buck (1980) points out that this is probably at the low end of the range since some infections may fall below the level of detection by this method, or may not be observed due to preparation techniques. Furthermore, over 100 species of fungi from more than 60 genera throughout the classes of the Fungal Kingdom have been seen to contain VLP (Buck, 1980), and many of these are plant pathogens (see Table 2).

There has been some discussion in the literature over the manner of describing the particles observed in fungi. Many authors feel that due to a lack of any lytic effect, with capacity to reinfect, caused by the particles concerned, they should be termed 'virus-like particles' (VLP). However, as Buck (1979b) has discussed, many fungal VLP have been shown to have nucleoprotein structure, with their nucleic acid component encoding the capsid polypeptide. Furthermore

many VLP have been shown to be serologically related to VLP from other strains or closely related species; and lastly certain VLP have been shown to be capable of infection in extracellular preparation (Hollings, 1962; Ghabrial and Mernaugh, 1983; Stanway and Buck, 1984). Hence it would seem justifiable to use the term virus rather than VLP, where most or all of these criteria are satisfied.

By far the most common particle morphology and composition is isometric (with a diameter in the range of 25 to 50 nm) and dsRNA respectively for known mycoviruses (Yamashita, Doi and Yora, 1975; Moffit and Lister, 1975; Buck, 1980). However, there have been various other types also observed. For instance filamentous, bacilliform and bacteriophage particle types have been recorded from a wide range of fungi (Weber 1979; Kazama, 1979) and also pleomorphic particles, apparently membrane enveloped, have been reported in Agaricus bisporus and Endothia parasitica (Lesemann and Koenig, 1977; Dodds, 1980b). Similarly there have been a number of reports of differing genome compositions. The best verified of these include the bacilliform virus of A.bisporus, the genome of which appears to be single-stranded (ss) RNA (Tavantzis, Romaine and Smith, 1980) and the viruses of Thraustochytrium sp., (an Oomycete), and Rhizidiomyces sp., (a Chytridiomycete) whose genomes appear to be

TABLE 2. Plant pathogens containing virus particles and/or ds RNA. (¹ nd = not determined)

HOST SPECIES	PARTICLE(S)		GENOME	REFERENCES
	SHAPE	SIZE		
<u>Ceratocystis ulmi</u>	nd ¹	nd	ds RNA	Pusey & Wilson (1982)
<u>Colletotrichum lindemuthianum</u>	Isometric	30 nm	ds RNA	Rawlinson, Carpenter & Muthyalu (1975)
<u>Diplocarpon rosae</u>	Isometric	34 nm	nd	Bozarth, Wood & Goenaga (1972)
<u>Endothia parasitica</u>	Pleomorphic	300 nm	ds RNA	Day <u>et al.</u> (1977); Dodds (1980b)
<u>Erysiphe graminis</u> var. <u>agropyri</u>	Isometric	40 nm	nd	Yamashita, Doi & Yora (1975)
<u>Fusarium roseum 'culmorum'</u>	Isometric	25, 40 nm	nd	Lapierre <u>et al.</u> (1975)
<u>Gaeumannomyces graminis</u>	Isometric	29, 35, 40 nm	ds RNA	Buck <u>et al.</u> (1981a); Buck (1984)
<u>Helminthosporium maydis</u>	Isometric	35, 40, 48 nm	ds RNA	Bozarth, Wood & Nelson (1972); Bozarth (1977)
<u>Helminthosporium victoriae</u>	Isometric	35-40 nm	ds RNA	Sanderlin & Ghabrial (1978)
<u>Periconia circinata</u>	Isometric	32 nm	ds RNA	Dunkle (1974)
<u>Puccinia graminis tritici</u>	Isometric	38 nm	nd	Rawlinson & MacLean (1974)
<u>Pyricularia oryzae</u>	Isometric	25, 32 nm	nd	Ferault <u>et al.</u> (1971)
<u>Rhizoctonia solani</u>	Isometric	25-31 nm	ds RNA	Finkler <u>et al.</u> (1983)
<u>Thielaviopsis basicola</u>	Isometric	40 nm	nd	Bozarth & Goenaga (1977)
<u>Ustilago maydis</u>	Isometric	41 nm	ds RNA	Koltin & Day (1976)

dsDNA (Kazama, 1979; Dawe and Kuhn, 1983a and b). The virus of Thraustochytrium sp. resembles herpes virus in size and is hence far larger than other mycoviruses, while the virus of Rhizidiomyces sp. is 60 nm in diameter and therefore of similar size to other mycoviruses.

a. Taxonomy.

Until recently the mycoviruses have been largely unclassified. However a series of attempts have been made to rectify this, (Hollings, 1979; Matthews, 1982), followed by the proposal of six groups of ds RNA mycoviruses (Buck et al., 1984), (see Table 3). (Many mycoviruses are known by abbreviations. For their full names please see abbreviations page). These groups are distinguished by the number and size of ds RNA components there are in the genome, the major capsid polypeptide size, particle diameter and serological relationships. Groups A and B have undivided genomes, groups C, D and E bipartite genomes and group F tripartite genomes. These groups have been further classified by the recognition of two families of dsRNA mycoviruses; the Totiviridae (undivided genomes) and the Partitiviridae (divided genomes) (Brown, 1985). These two families are comprised thus:

- i) TOTIVIRIDAE (ds RNA mycoviruses with undivided genomes)

One genus: Totivirus

Type species: Saccharomyces cerevisiae virus L1

(ScV-L1; synonym, SCV-LA)

Other member: UMV-Pl-H1

ii) PARTITIVIRIDAE (ds RNA mycoviruses with divided genomes).

Genus: Partitivirus

Possible Genus: Penicillium chrysogenum virus group

Partitivirus (Bipartite genomes)

Type Species: Gaeumannomyces graminis virus O19/6-A (GgV019/6A).

Other Members: PsV-S, GgV-T1-A, AbV-4.

Penicillium chrysogenum virus group. (Number of genome components uncertain).

Type Species: Penicillium chrysogenum virus.

Other Members: PbV, Pc-fV.

Replication in both the Totiviridae and Partitiviridae is believed to occur asynchronously. Conservative replication is believed to be the case in the Totiviridae, while replication in the genus Partitivirus is semi-conservative. Replication in the PcV group has not been elucidated. It is feasible that viruses that appear to classify in the PcV group are truly members of the Partitivirus genus. This would be the case provided that the extra genome components of such viruses as PcV were satellite viruses (Mossop and Frankci, 1978) or defective interfering viruses (Huang

and Baltimore, 1977).

b. Virion structure and composition.

As already discussed most ds RNA mycoviruses are isometric in structure, with diameters in the range of 25 to 50 nm. The capsid structure of such mycoviruses appears to be uniform in being T₁, i.e. an icosahedron with 60 structure units. Examples of this structure are provided by Aspergillus foetidus 'slow' (S) and 'fast' (F) viruses (Buck and Ratti, 1975), PcV (Buck and Girvan, 1977), PsV-S PsV-F (Buck and Kempson-Jones, 1974) HmV (Bozarth, 1977), ScV (Bostian, Sturgeon and Tipper, 1980) and UmV (Bozarth et al., 1981). This is distinct from the small isometric plant viruses, many of which show T = 3 symmetry (Bozarth, 1979). In the majority of mycoviruses, the capsid comprises one polypeptide species, and it is possible that where more than one species has been seen, this could be due to detection of a cleavage product or a virally-encapsidated polymerase (Buck, 1977). The molecular weight of the capsid polypeptide ranges from 25,000 to 130,000 depending upon the virus concerned (Buck, 1980). It appears that the ds RNA components of a particular virus are singly encapsidated, e.g. PcV (Wood and Bozarth, 1972). In PsV-S four classes of particle were distinguished, empty, ss RNA-containing, ds RNA-containing and ss- and ds RNA containing (Buck and Kempson-Jones, 1973). The ss RNA and ss and ds

TABLE 3. Taxonomically important properties of members and probable members of the six groups ds RNA mycoviruses.

GROUP VIRUS	DIAMETER (nm)	S ₂₀ , W (S UNITS)	ds RNA SPECIES (Kbp)	MAJOR CAPSID POLYPEPTIDE mwt x 10 ⁻³	OTHER VIRUSES TO WHICH SEROLOGICALLY RELATED	REFERENCES ²
A						
ScV-L1	35-40	161	4.8	88	ScV-L2	a, b
ScV-L2	35-40	161	4.8	88	ScV-L1	a, b
UmV-P1-H1	41-43	172	6.1	75	--	c, d
B						
HmV	48	283	8.3	121	nd ³	e
C						
PsV-S	30-34	101	1.6, 1.4	42	Dr-V	f, g, h
DrV	34	nd	nd	nd	PsV-S	j
D						
GgV-019/6-A	35	126	1.8, 1.7	60	OgA-B	k
GgV-OgA-B	35	125	1.9, 1.8	55	019/6-A	k
OgV-F6-C	35	128	1.8, 1.7	54	OgA-B	k

continued over

TABLE 3 continued

GROUP VIRUS	DIAMETER (nm)	S ₂₀ , W (S UNITS)	ds RNA SPECIES (Kbp)	MAJOR CAPSID POLYPEPTIDE mw _t x 10 ⁻³	OTHER VIRUSES TO WHICH SEROLOGICALLY RELATED	REFERENCES ²
E						
GgV-F6-B	35	133	2.3, 2.1	73	T1-A, OgA-A	k
GgV-T1-A	35	133	2.2, 2.1	73	OgA-A, F6-B	k
GgV-OgA-A	35	133	2.2, 2.0	68	T1-A, F6-B	k
AbV-4	35	140-145	2.2, 2.0	69	nd	l
F						
PcV	35-40	140-145	3.2, 3.0, 2.9	125	PbV, Pc-fV	m, n
PbV	35-40	147	3.2, 3.0, 2.9	125	PcV	n, p
Pc-fV	35-40	145	3.2, 3.0, 2.9, 2.8	125	PcV	m

¹ Values for particles containing ds RNA.

² References: a = Field et al. (1982); b = Sommer & Wickner (1982); c = Bozarth et al. (1981); d = Field et al. (1983); e = Bozarth (1977); f = Bozarth et al. (1971); g = Buck & Kempson-Jones (1973); h = Buck & Kempson-Jones (1974); j = Bozarth et al. (1972a); k = Buck et al. (1981); p = Wood et al. (1977).

³ nd = not determined.

RNA-containing capsids are believed to be replicative intermediates. Five different particle sizes have been found to be associated with AfV-F, determined by the ds RNA species encapsidated, i.e. particle type E_0 contains the 1.24×10^6 ds RNA species whilst the E_1 (1.44×10^6), E_2 (1.70×10^6), E_3 (1.87×10^6), E_4 (2.70×10^6) (Buck and Ratti, 1975). Bozarth et al. (1981) report that the different species of 'heavy' ds RNA in UmV are separately encapsidated, while the 'medium' ds RNAs are encapsidated in sets of one, two or three. Both UmV and ScV appear to provide examples of helper and satellite virus. In each case the M ds RNA species depend on the larger ds RNA species, (H and L respectively), to provide its capsid polypeptide (Bozarth et al., 1981; Bostian, Sturgeon and Tipper, 1980).

Little is known of mycovirus virion assembly. Edmondson and Gray (1983) have observed that both the polypeptide and the ds RNA components of PcV must undergo conformational change on assembly.

c. Replication.

The genomes of mycoviruses are believed to encode both their own capsid polypeptide and a virion-associated RNA polymerase (Buck, 1980). These would seem to be the minimum requirements for a virus to promote its own replication. Koltin (1977) has shown by mutation studies that UmV ds RNA carries the genetic information for its capsid polypeptide, while ScV has

been shown both by in vitro translation of denatured ds RNA and by translation of in vivo-produced transcripts, to encode its own capsid polypeptide (Hopper et al., 1977; Bostian et al., 1983). Similarly both PcV and GgV 38-4-A have been shown to encode their own capsid polypeptides (K.A. Bostian, J.E. Hopper, R.F. Bozarth, cited in Buck, 1980; Romanos, Buck and Rawlinson, 1981). A number of mycoviruses have been observed to possess a virion-associated RNA polymerase, however as yet no definite proof of their genetic origin has been presented. Mycoviruses with this associated function include: PsV-S (Buck, 1975), PsV-F (Chater and Morgan, 1974), PcV (Nash et al., 1973), AfV-S and AfV-F (Ratti and Buck, 1975), ScV (Herring and Bevan; 1977), UmV (Ben-Tzvi et al., 1984), Allomyces arbuscula virus (Khandjian and Turian, 1977) and viruses from G.graminis (Buck et al., 1981b). Recently a new virus group was reported in G.graminis which would appear to lack sufficient genome size to encode both its capsid polypeptide and an RNA polymerase (Buck, 1984). It is possible that this virus is a satellite virus (requiring the replicase of another virus), or it has two ds RNA components of the same size but differing sequence, or that the one component has multiple reading frames (Buck, 1984). All of these proposals are equally plausible and hence require further study to elucidate the genome organisation of this virus.

Replication of mycoviruses has been reviewed by Buck (1979b; 1979c; 1980). On an ultrastructural and developmental level particle assembly appears to occur in the cytoplasm of the host, (evidence for this is provided by various electron microscopy studies, e.g. Yamashita, Doi and Yora, 1973 and 1975; Rawlinson et al., 1973) and in parallel with host growth with high virus yields being the result of continued virus replication once the host is in stationary phase. This has been compared with the 'relaxed' mode of replication of certain bacterial plasmids which are produced at a controlled copy number during exponential bacterial growth, but in stationary growth their numbers increase considerably (Timmis, Cabello and Cohen, 1974; Lewin, 1977; Buck, 1980). Yamashita, Doi and Yora (1973) observed aggregates of more than 100,000 particles of PcV in aged cells of P.chrysogenum associated with degeneration or disappearance of host cytoplasm and hence presumably cellular aberrations.

The mode of action of the virion-associated polymerases that have been studied appears to be initially to produce full-length single strand transcripts. This has been reported in vitro for PsV-S (Buck, 1975), AfV-S and AfV-F (Ratti and Buck, 1975), ScV (Bruenn et al., 1980; Welsh and Leibowitz, 1980) and various G.graminis viruses (Buck et al., 1981b). PsV-S has been observed to replicate by a semi-conservative mechanism, since incorporation of 5-bromo-

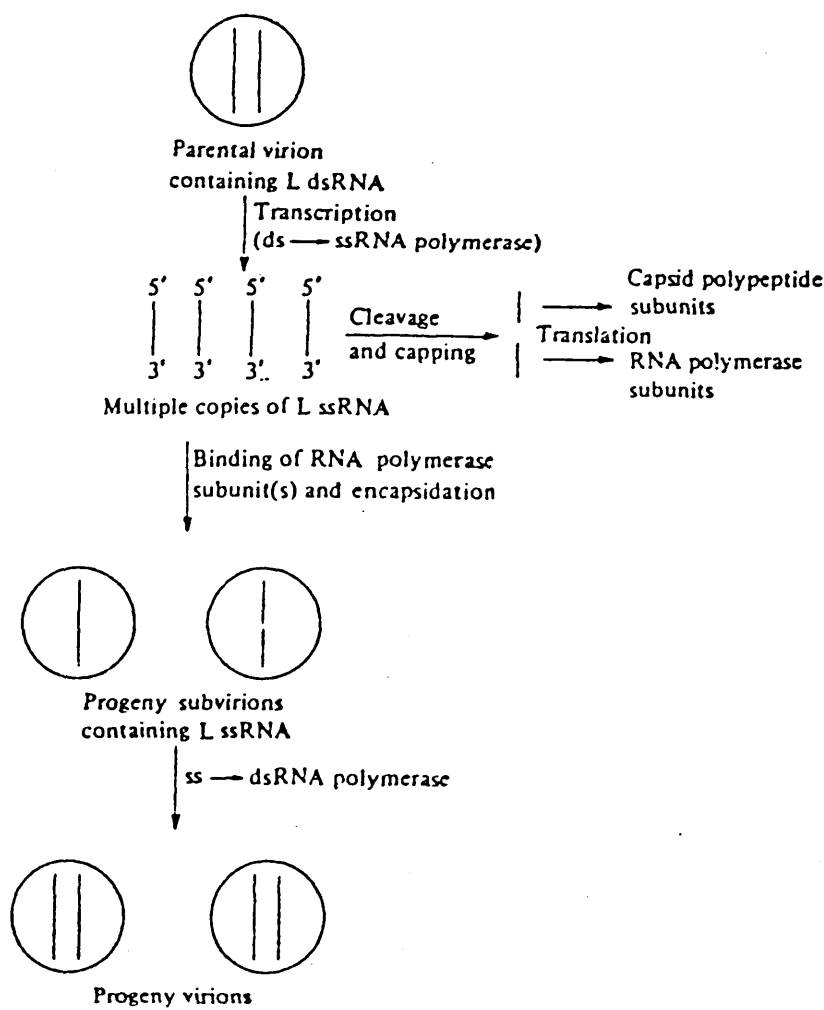
uridine triphosphate by PsV-S during replication in vitro followed by caesium sulphate centrifugation indicated the formation of ds RNA molecules of hybrid density (Buck, 1978). Hence it has been suggested that replication in the Partitiviridae, of which PsV-S is a member, is asynchronous and semi-conservative. Replication of ScV-L1, (the type species of the genus Totivirus of the family Totiviridae), and ScV-M1 (a satellite virus of the former) is believed to be asynchronous and conservative. Originally Herring and Bevan (1977) demonstrated that ScV RNA polymerase has both ds RNA-ss RNA, and ss RNA-ds RNA activity. However, both Welsh and Leibowitz (1980) and Bruenn et al. (1980) have shown that the yeast viral RNA polymerase in vitro acts predominantly as a transcriptase. In accordance with this later observation Buck (1980) has suggested a model for the replication of ScV-L1, (see Figure 2). In this model the process begins with the synthesis of a new plus strand, the progeny strand being displaced into the cytoplasm where it is either, capped and cleaved to give rise to subgenomic mRNAs separately encoding capsid polypeptide subunits and RNA polymerase subunits, and RNA polymerase subunits, or bound by RNA polymerase subunits and encapsidated, following which the progeny plus strand acts as a template for minus strand formation and a ds RNA virus particle is

completed. Recently Sclafani and Fangman (1984) have confirmed the proposal that ScV replicates conservatively, and hence also strengthening the suggestion that replication in the Totiviridae is asynchronous and conservative.

The replication of ScV is complicated by two subjects. Firstly, the dependence of the killer phenotype and M ds RNA on at least 30 host nuclear loci (Tipper and Bostian, 1984) and secondly, the discovery of exclusion mechanisms involving different species of L and M ds RNA (Wickner, 1983). The nuclear loci that are involved in M ds RNA maintenance (SPE 2, SPE 10, PET 18, MAK 1 and MAK 3-28) are often marked by a single allele, and it has been estimated that up to 100 such loci may exist. They are not clustered and have been mapped to 15 of the 16 known yeast chromosomes (Tipper and Bostian, 1984). A mutation at any one of these loci causes the loss of M ds RNA and the killer phenotype. It should be noted that KEX mutants lose the killer phenotype but retain their immunity (Wickner, 1979).

Recently it has been realised that L ds RNA consists of a number of molecular types, of essentially the same molecular weight and basic functions, but differing in other functions. Bobek et al. (1982), El-Sherbeini, Bevan and Mitchell (1983) and Thiele, Hannig and Leibowitz (1984) have all demonstrated by biochemical methods, (such as electrophoresis and

Figure 2. A model for the replication of L virions of Saccharomyces cerevisiae (from Buck, 1980).



homology studies), that there may be up to three distinct classes of L ds RNA called L-A, L-B and L-C (Sommer and Wickner, 1983) and these are probably separately encapsidated (Thiele, Hannig and Leibowitz, 1984). This discovery has helped to explain the phenomenon of virus exclusion reported by Field et al. (1982). When diploids are formed between two different killer strains, (i.e. M_1 and M_2), above pH 5 to inactivate their toxins, then M_2 ds RNA is lost, presumably via loss of L_2 ds RNA on which it is dependent. Furthermore, three different forms of L-A ds RNA carrying different combinations of the genes [HOK], [NEX] and [EXL] have been identified by Sommer and Wickner (1982), Wickner and Sommer (1983) and Wickner (1983). [EXL] codes for the exclusion of M_2 ds RNA, [NEX] prevents exclusion of M_2 ds RNA by [EXL] unless there is a certain nuclear mutation (i.e. mkt⁻), while [HOK] provides a helper function for S ds RNA of M_1 ds RNA. This division of L_A ds RNA into classes has also demonstrated that replication of L_A ds RNA is in fact dependent on some of the host maintenance genes (e.g. MAK1 1, 3, 8, 10 and 27, PET 18, SPE 2 and 10), that have been observed to affect M ds RNA replication. The function of L_{BC} is as yet unelucidated. However, Ball, Tirtiaux and Wickner (1984) have shown firstly that M ds RNA lowers L_A copy number but not L_{BC} copy number, and secondly, that SKI gene products, (the mutant phenotype called 'superkiller'), lower copy

number of L_A , L_{BC} , M_1 and M_2 .

Hence replication and maintenance of ScV is more complex: it is dependent on host nuclear functions and the various components of ScV also interact in this process. Replication of other mycoviruses may also be dependent on host genes, but no comparable studies have yet been carried out.

d. Transmission.

There have as yet been no reports of ds RNA mycoviruses being released from the host cell through lysis, and these released viruses being capable of renewing infection. There have been reports of cell lysis involving the release of virions (Lemke, Nash and Pieper, 1973; Mehta, Nash and Bozzola, 1982), but there is no evidence that this can cause in vivo horizontal transmission of the viruses concerned. Hence transmission of mycoviruses would appear to be limited to intracellular routes.

Virus particles have been observed in a variety of asexual and sexual spores, (i.e. vertical transmission), and there is evidence that transmission also occurs in some fungi via anastomosis, (i.e. horizontal transmission).

Asexual spores such as conidia have been seen to contain virus particles in various Penicillium spp. (Banks et al., 1968; Hooper et al., 1972; Sansing et al., 1973; Lemke, Nash and Pieper, 1973), in

Aspergillus flavus (Wood et al., 1974), in Pyricularia oryzae (Ferault et al., 1977) and in G.graminis (McFadden, 1982; McFadden, Buck and Rawlinson, 1983). In P.stoloniferum, Bozarth et al. (1971) observed 12 out of 12 monoconidial isolates from an infected culture to be infected, while Crosse and Mason (1974) recorded an average transmission rate of 9 out of 10 for P.chrysogenum. Boissonnet-Menes and Lecoq (1976) achieved a 100% success rate of transmission of virus in P.oryzae. While De Marini et al., (1977) in studying PsV-S and PsV-F transmission through a series of generations noted that of 43 monoconidial isolates selected at the end of the experiment, 2 contained neither PsV-S or PsV-F, while 3 lacked PsV-F in total. The remaining 40 isolates contained varying levels of PsV-F, some higher and some lower, than the original strain. Therefore mycovirus transmission through asexual spores appears to be frequent and widespread.

With respect to sexual spores, there appears to be a division between basidiospores and ascospores. For the former, virus transmission has been observed in basidiospores of Agaricus bisporus (Lecoq et al., 1979), Lentinus edodes (Ushiyama and Nakai, 1982), Coprinus lagopus (Shahriari et al., 1973). On the other hand, ascospores largely seem to prevent virus transmission, presumably by an exclusion mechanism during spore formation. Three fungal species that have been studied, G.graminis, Endothia parasitica and

Helminthosporium maydis have all been shown to produce ascospore progeny from virally infected or ds RNA-containing parental strains, that are apparently virus and ds RNA-free (Rawlinson et al., 1973; Day et al., 1977; Bozarth, 1977). In the case of E.parasitica, it should be noted that ds RNA-containing strains do not produce protoperithecia; hence crosses were achieved by fertilizing protoperithecia from a ds RNA-free isolate with conidia from a ds RNA-containing isolate, i.e. crosses between two ds RNA-containing isolates have not been achieved (Day et al., 1977). More recently McFadden, Buck and Rawlinson (1983) while studying two conidial cultures of isolate 3bla of Ggt showed that six out of eight single ascospore progeny of the conidial isolates were virus-free using a gel transfer hybridization technique, (which gave a 1,000 fold increase in sensitivity compared with PAGE). The two progeny isolates that were not virus-free had both transmitted only one of the four viruses present in the parental isolates and in both cases the ds RNA components of the transmitted virus had been slightly reduced in size. It is suggested that this virus shows greater resistance to exclusion and that presumably small identical deletions of the genome must have occurred. Lapierre et al. (1970) also observed virus exclusion on production of ascospores by G.graminis. However, in contrast to these results Tivoli et al.

(1979) have reported re-emergence of virus in ascospore cultures, after initially observing them to be virus-free. This has not been observed in the 3bla ascospore isolates of McFadden, Buck and Rawlinson (1983). Lecoq et al. (1979) suggest that the results of Tivoli et al. (1979) may represent the presence of a 'transmission gene' in the relevant strains that can transmit virus. Alternatively it was postulated by Buck (1980), that this may be due to the presence of a provirus-capacity in Ggt on the circumstantial evidence of Adler (1979) relating to Histoplasma capsulatum and the more direct homology studies of Vodkin (1977), concerning ScV-L and yeast. This provirus-capacity would explain the observation of Rawlinson et al. (1973) that isolates of Ggt from first year cereals are mostly free from detectable virus particles. This would be in line with the likelihood that most of these isolates would have arisen from ascospore infections (Brooks, 1965). Furthermore, Rawlinson and Muthyalu (1976) saw a gradual appearance and increase in virus particles found in isolates of Ggt after further wheat crops. However the work of Vodkin (1977) has proved impossible to verify (Wickner and Leibowitz, 1977; Hastie, Brennan and Bruenn, 1978) and seems spurious. Moreover, McFadden, Buck and Rawlinson (1983) could find no DNA provirus forms in the Ggt isolate 3bla progeny by hybridisation studies. Hence transmission of viruses through ascospores of Ggt is most likely a rare event

with no provirus involvement. Brewer and Fangman (1980) contrary to these observations with respect to Ggt and other Ascomycetes, have achieved 70% transmission of ds RNA viruses of Saccharomyces cerevisiae through ascospores, and they suggest that this represents preferential inclusion of the viruses into the spores. Why yeast should differ from other Ascomycetes in this way remains unknown.

Horizontal transmission via anastomosis has been reported in various fungi but mostly only in vitro. For example Lhoas (1971a) achieved transmission of viruses of P.stoloniferum via heterokaryosis using spore colour mutation to identify the newly infected strain. In Ustilago maydis Wood and Bozarth (1973) used mating type to define virus transfer. Schisler et al. (1967) demonstrated that in vivo transmission of virus frequently occurs in A.bisporus where contact is made between an infected spore germ tube and healthy mycelium. Anagnostakis (1981a and b) studying E.parasitica, the cause of chestnut blight, has noted that cytoplasmic determinants (which are associated with ds RNA and which are responsible for a phenomenon known as hypovirulence) can be transmitted in vitro and that stable heterokaryons can be formed by strains of E.parasitica from different vegetative compatibility (v-c) groups. In the field it does appear that the great variety of v-c groups does restrict the spread of

hypovirulence. However, transfer of the cured phenotype and also of ds RNA can occur between isolates that form weak barrage reactions; i.e. they are mildly incompatible (Anagnostakis, 1983). Rawlinson et al. (1973) reported transfer of Ggt viruses between isolates of the same v-c group, when anastomosed in culture. Similarly results from G.graminis, produced by Jamil, Buck and Carlile (1984) using hybridisation studies, indicate that transfer of viruses between incompatible isolates of this fungus probably also occur. Furthermore, Jamil and Buck (1984) have recorded that there appears to be an identical virus in a field isolate of Ggt and an isolate of Phialophora sp. (lobed hyphopodia), which implies that it could be feasible for viruses to cross species barriers, although it should be noted that these two fungi are very closely related. If virus transfer between these two wheat rhizosphere fungi does arise then it is possible that the observations of Rawlinson et al. (1973) and Rawlinson and Muthyalu (1976) regarding the appearance of viruses in isolates of Ggt, (discussed previously), may be the result of Phialophora sp. (lobed hyphopodia) acting as a source of infection. Caten (1972) has observed a decrease from 100% to 15% transfer of a cytoplasmic factor in incompatible interactions of Aspergillus amstelodami. Successful transfer only occurred where incompatibility was not polygenic.

In an attempt to demonstrate that more mycoviruses are capable of horizontal transmission and even infection as extracellular agents, protoplast studies have been employed by various researchers. Initially though, it should be noted that Hollings (1962) obtained a low level of virus transmission when purified mushroom virus preparations were injected into healthy young A.bisporus sporophores, demonstrating that some, if not all, of the cultivated mushroom viruses are capable of initiating infection in an extracellular form. Lhoas (1971b) reported the infection of virus-free protoplasts of P.stoloniferum with PsV-S, although the newly infected strain only had a virus titre equivalent to 10% of the donor strain. Pallett (1976) records successful infection of P.chrysogenum, Marasimius androsaceus and Mucor hiemalis with PcV, however with very low titres compared with naturally infected P.chrysogenum; and further to this the levels of virus fell below detection after three years of subculturing. A number of workers, using essentially the same techniques as Lhoas (1971b) and Pallett (1976) have failed to achieve infection. Bozarth (1975) did not observe virus transmission when trying to infect healthy protoplasts of three fungi each with their own natural viruses, these being: P.stoloniferum (PsV-S and PsV-F); P.chrysogenum (PcV) and Helminthosporium maydis (HmV). In similar cases Lai and Zachariah (1975)

reported failure with Penicillium claviforme, Bevan and Mitchell (1979) were unable to infect S.cerevisiae and Boissonnet-Menes and Lecoq (1976) failed to infect Pyricularia oryzae. Hence doubt is cast on the results of Lhoas (1971b) and Pallett (1976) in the terms that they represented true infections. It may be that their observations were of residual inoculum or infection by virus particles that were very restricted in their replication by their new environment.

Successful transmission of virus using protoplast fusion, (the recipient strain carrying a fungicide resistance marker) has been reported by Boissonnet-Menes and Lecoq (1976) using healthy and infected strains of P.oryzae. Furthermore Lecoq et al. (1979) confirmed that this new infection was stable since it had not decreased in virus titre over a number of subcultures in three years. Ghabrial (1980) and Ghabrial and Mernaugh (1983) have achieved infection of Helminthosporium victoriae healthy protoplasts using purified virus preparations in the presence of polyethylene glycol, albeit at only a frequency of about 1% in a small proportion of experiments. Hence it appears that under certain conditions purified preparations of mycoviruses are infective, although success in such transmission attempts is still rather limited.

Due to the limited nature of the transmissibility of mycoviruses in terms of horizontal transmission it

may be expected that the host range of particular viruses must be very restricted. Presumably the wide distribution of mycoviruses is due to infection of fungal ancestors and as these have evolved and diverged so have their indigenous viruses. This would explain observations of serologically related viruses in fungi that appear to be reasonably close in evolutionary terms, for instance the viruses of P.chrysogenum, P.cyaneo-fulvum and P.brevi-compactum (Wood and Bozarth, 1972; Buck and Givran, 1977), or the viruses of Aspergillus foetidus and Aspergillus niger (Buck, Givran and Ratti, 1973). (It is interesting to note that Peberdy et al., (1977) have formed interspecific hybrids between P.chrysogenum and P.cyaneo-fulvum using protoplast fusion). Also this theory could be forwarded to explain the occurrence of serologically related virus particles in distantly related fungi. Such a relationship has been observed between PsV-S and the virus of Diplocarpon rosae (Bozarth, Wood and Goenaga, 1972) and also between PsV-S and virus particles in Aspergillus ochraceus [unpublished results of R.F. Bozarth quoted by Buck (1980)]. It should be noted that serological relationships are not necessarily a complete indication of how closely related any two viruses are since they represent only a small part of the viral genomes concerned. Hence the work of Jamil, Buck and Carlile

(1984) is of particular interest since they studied sequence homologies in viruses in strains of G.graminis from different v-c groups. They report that several viruses were thus shown to be closely related although their strains of origin were incompatible. Anagnostakis (1983) has also noticed that ds RNA in E.parasitica can be transferred in unsuccessful anastomoses, (i.e. fusion between isolates of differing v-c groups, giving a weak barrage reaction), where the incompatibility is not too great. Moreover Jamil and Buck (1984) have suggested from their observation of an apparently identical virus in Ggt and Phialophora sp. (lobed hyphopodia), that virus transmission may occur between these two closely related species. Therefore in addition to the theory of mycovirus spread via fungal evolution, it is perhaps possible to propose that where partial anastomoses (i.e. such as those described by Anagnostakis, 1983), may occur, mycovirus transmission may also occur. This is obviously closely linked to the phylogenetic relationship of the fungi concerned and hence broadens the original hypothesis.

e. Other ds RNA viruses.

There are also a number of other ds RNA viruses known, but these are mostly clearly different from mycoviruses. Matthews (1982) distinguishes two other ds RNA virus families, the Reoviridae, (containing the genera Reovirus, Orbivirus, Rotavirus, Phytoreovirus and Cytoplasmic polyhedrosis virus group), and the

Cystoviridae (containing the genus Ø6 phage group, with its only species, 'phage Ø6). There has been another ds RNA family established, the Birnaviridae, (the bisegmented ds RNA animal virus group), the type species being Infectious Pancreatic Necrosis Virus (IPNV) of fish (Brown, 1985). Lastly, a number of plant cryptic viruses have recently been shown to be ds RNA-containing (Boccardo, Lisa and Milne, 1983) and hence may form another ds RNA virus family.

The Reoviridae have certain general characteristics that are worthy of mention. The viral genome consists of 10-12 linear, monocistronic ds RNA segments that are encapsidated as a whole genome in a capsid that has an inner and outer protein coat. Virions are isometric, 60 to 80 nm in diameter, and on entering the host cell, the outer protein coat is removed, leaving the core, or subviral particle which has RNA polymerase activity (Joklik, 1974; Silverstein, 1976). Reovirus replication is conservative, involving the synthesis of progeny RNA strands which act as templates for complementary strand formation, as well as being potential mRNAs (Silverstein, 1976). The host of the Reoviridae include vertebrates, insects and other arthropods, and dicotyledonous plants. Orbiviruses and Phytoreoviruses require insect vectors for their transmission, while the other genera do not (Matthews, 1982). The hosts, when infected, are

noticeably diseased, in some cases, e.g. Blue-tongue virus, (an Orbivirus of sheep), infection can be fatal (Wood, 1973).

Recently there have been reports of successful cloning of full length components of Reo- and Rotaviruses (Imai et al., 1983; McCrae and McCorquodale, 1983; Antczak et al., 1983). This should facilitate a better understanding of their genome organization, and provided their techniques can also be applied to mycoviruses, should furnish more detail concerning genome structure and function of fungal viruses.

The Cystoviridae has only one genus and one type species, the ds RNA bacteriophage Ø6 of Pseudomonas phaseolytica (Van Etten et al., 1973; Matthews, 1982). Particles of this virus are isometric, (ca. 75 nm in diameter), comprising a core (ca. 60 nm in diameter), surrounded by a lipoprotein envelope derived from the host membrane. Like the Reoviridae, the genome is divided, in this case into three components, each ds RNA segment being polycistronic, and as with all ds RNA viruses previously studied, an RNA polymerase with transcriptase activity is associated with the virion (Van Etten et al., 1973; Matthews, 1982). Van Etten et al. (1980) report that unlike the Reoviridae, replication of Ø6 appears to proceed by a semi-conservative mechanism.

Dobos et al. (1979) have documented the

occurrence of five animal viruses with bisegmented ds RNA genomes. These viruses have now been classified and constitute the family Birnaviridae (Brown, 1985), with the one genus Birnavirus and type species IPNV. Typical features of members of this family include: isometric particles of 58 to 60 nm in diameter, the virions consisting of multiple capsid polypeptides, forming a single protein coat. Mertens et al. (1982) demonstrated the presence of an RNA polymerase activity associated with the virions of IPNV and showed that it is responsible for transcription of the virus genome, probably by a semi-conservative mechanism. Of greater interest was the report of Mertens and Dobos (1982) that the mRNA of IPNV is functionally polycistronic, which is rare among eukaryotic mRNAs.

A number of small isometric viruses have been observed in plants that appear to have no effect on their hosts, hence they have been termed cryptic viruses. Examples of cryptic viruses include: Beet cryptic virus (BCV) (Kassanis, White and Woods, 1977); Vicia cryptic virus (VCV) (Kenten, Cockbain and Woods, 1980); Carnation cryptic virus (CCV) (Lisa, Luisoni and Milne, 1983). The virus particles of all these viruses appear to be about 30 nm in diameter and both CCV and RCV have been observed to contain ds RNA genomes (Lisa, Boccardo and Milne, 1981; Boccardo, Lisa and Milne, 1983). It is interesting to note that the plant

cryptic viruses bear a resemblance to the mycovirus family Partitiviridae in their particle size and morphology, and size of ds RNA components; although it should be noted that some of the plant cryptic viruses do possess more than two ds RNA species (Boccardo, Lisa and Milne, 1983).

In summary, it may be said that ds RNA viruses are also found in hosts of many other phyla. These other ds RNA viruses have some features in common, (e.g. possession of a virion-associated RNA polymerase), but also there are evidently considerable differences between ds RNA families. It is of interest that so many viruses have evolved with ds RNA as their genetic information and they are worthy of study simply in this respect.

1.2.2 The Viruses of Gaeumannomyces graminis.

Virus particles have been observed and studied in the take-all fungus (G.graminis), especially in the variety that infects wheat (var. tritici, or Ggt) and also in closely related species such as Phialophora sp. (lobed hyphopodia).

The first recorded observation of virus particles in this group of fungi came from Lapierre et al. (1970) whilst studying Ggt. According to their assessment by electron microscopy the particles were isometric with a diameter of 29 ± 2 nm. The occurrence of virus particles in Ggt was confirmed by Rawlinson and Pearson

(1972), who found six out of ten isolates to contain isometric particles that were 35 nm in diameter. They also found an isolate of G.graminis var. avenae (Gga) to contain apparently identical particles. In a study of 156 isolates of G.graminis, Rawlinson et al. (1973) discovered two isometric virus particle types, with diameters of 27 and 35 nm, sedimentation coefficients of 110S and 148S respectively and u.v. absorption spectra typical of nucleoprotein structures. The discrepancy between the virus particle sizes was resolved when Lapierre (1973) revised earlier measurements and reported that the virus particles reported in 1970 were 35 nm in diameter. The nucleic acid composition of the virus particles of G.graminis was demonstrated as being ds RNA by both Carpenter and Rawlinson (1974) and Lapierre et al. (1974). Later Rawlinson and Muthyalu (1975, 1976) discovered a further size of virus particle, 40 nm in diameter, in isolates of Ggt.

Further analysis of the ds RNA components of the virus particles of Ggt, employing polyacrylamide gel electrophoresis (PAGE), demonstrated that many of these viruses are multicomponent in their nucleic acid composition, and a wide variety of distribution patterns were observed in a collection^c of Ggt isolates (Almond, Buck and Rawlinson, 1977, 1978). Frick and Lister (1978) using a serological assessment also found virus particles to be widespread and diverse in their

nature in Ggt. They noted various serological groupings and degrees of relatedness between the virus particle types and suggested that such sero-type variation could reflect biotype variation.

Buck et al. (1981a) and Buck (1984) have studied the virus particles from a selection of Ggt isolates, examining their particle size, sedimentation coefficient, size of their ds RNA species, capsid polypeptide size and serological relationships. As a result of this, four groups of viruses infecting G.graminis have been proposed, (see Table 3a). Jamil, Buck and Carlile (1984) have found, using a gel transfer hybridization technique, that there was no sequence homology between viruses from these different groups, confirming them as accurate divisions. Two viruses have been observed that do not classify into these groupings (Rawlinson and Buck, 1981); these being F3-A and Fl0-A, (see Table 3a). The particles of these two viruses are 40 nm in diameter, however neither their ds RNA species nor their major capsid polypeptides are similar in size range to those of the viruses of group III (i.e. the 40 nm particle group). Hence viruses F3-A and Fl0-A are possibly members of fifth group of Ggt viruses.

The virus groups of Ggt, (see Table 3a), appear to fit into the taxonomic scheme for ds RNA mycoviruses (Brown, 1985), provided certain considerations are

TABLE 3a Properties of the viruses of G.graminis and their classification into groups I and IV (from Buck et al. 1981a; Buck, 1984; Rawlinson and Buck, 1981; Jamil and Buck, 1984).

GROUP	PARTICLE DIAMETER (nm)	SEDIMENTATION COEFFICIENT (s)	SIZE OF ds RNA SPECIES (Kbp)	Mwt OF CAPSID POLYPEPTIDE (x 10 ³ d)	A ₂₆₀ /A ₂₈₀	BOUYANT DENSITY IN CsCl g/ml	GROUP MEMBERS (VIRUS CODES)
I	35	109-128	1.59-1.89	54-60	1.4-1.5	1.36-1.37	019/6-A, 38-4-A, 01-1-4-A, 45/9-A, OgA-B, 3b1a-C, F6-C, 45/101-D
89 II	35	133-140	2.02-2.33	68-73	1.4-1.5	1.35-1.37	T1-A, F6-B, OgA-A, 3b1a-B, 45/101-A, 45/101-B
III	40	159-163	4.66-6.26	78-87	1.6	1.40-1.41	3b1a-A, F6-A 87-1-H
IV	29	127	1.8	66	1.7	1.40	45/101-C
<u>Ungrouped</u>	40	--	7.13-9.17	94-125	--	--	F3-A, F10-A

taken into account. Groups I and II evidently belong in the Partitiviridae, virus Ol9/6-A, (a member of group I), being the type species of the Partitivirus genus; whilst virus Tl-A, (a member of group II), is also a recognized member of the Partitivirus genus. However, it should be noted that there are viruses in both groups I and II that appear to have more than two components to their genome, which would place these viruses in the Penicillium chrysogenum virus group of the Partitiviridae. If this division of the Partitiviridae on the basis of number of ds RNA species comprising the genome is misleading, (due to the occurrence of satellite or defective viruses), then this discrepancy would be resolved. By Tl oligonucleotide fingerprint analysis of the ds RNA components of two serologically related viruses of G.graminis, Romanos, Buck and Rawlinson (1981) have demonstrated that one of three ds RNA species in virus 38-4-A is probably a satellite RNA. Since virus 38-4-A is a member of Ggt virus group I, this suggests that the distinction between bipartite and multipartite genomes in the Partitiviridae may not be firmly based. The group III viruses, (and the ungrouped viruses), would appear to belong to the Totiviridae on the basis of their particle size. Furthermore the single ds RNA species of 87-1-HV has been observed to hybridize with both ds RNA components of 3bla-AV, indicating that the latter virus does in fact have an undivided genome with

a smaller defective RNA species. This argument could also be the case for F6-AV and F3-AV, and hence they could be members of the Totiviridae. Lastly virus 45/101-C, (the group IV representative), would appear from its virus particle size and ds RNA species size to be a member of the Partitiviridae. However this presumes that in fact 45/101-C contains two, (or more), ds RNA species that are apparently identical in size, (as assessed by mobility in PAGE). This assumption seems quite feasible since the $A_{260}:A_{280}$ ratio and CsCl density values of 45/101-c are high in comparison with the viruses of Ggt groups I and II, suggesting a higher nucleic acid:protein ratio in the former compared with the latter. Moreover, ScV-L1 has been shown to contain ds RNA components that originally appeared identical in size, (since they had the same mobility in one gel system), but these have now been separated by high voltage electrophoresis (Thiele, Hannig and Leibowitz, 1984). Hence virus 45/101-C quite possibly contains more than one ds RNA species, and would therefore be a member of the Partitiviridae.

A number of the Ggt viruses of groups I and II have been shown to possess virion-associated RNA polymerase activity, but no activity for group III viruses has been detected; while group IV virus has not been examined in this respect (Buck et al., 1981b; Buck, 1984). Where activity was observed, full-length

ds RNA transcripts were produced for up to 48h by the in vitro system, with the mode of transcription probably being semi-conservative.

Virus particles containing ds RNA have been isolated from Phialophora sp. (lobed hyphopodia), Phialophora graminicola and G.graminis var. graminis by McGinty, Buck and Rawlinson (1981) and Buck, McGinty and Rawlinson (1981). These essentially demonstrate similar characteristics to the viruses of Ggt; most of the virus particles have diameters of 35 or 40 nm, only the virus particles from P.graminicola being 30 nm in diameter. The ds RNA components are similar in size range, and many of the viruses are again of divided genome (with only the larger, 40 nm, viruses being possibly undivided). Indeed on the range of characteristics used to classify the viruses of Ggt (Buck et al., 1981a; Buck 1984), two serologically unrelated viruses of Phialophora sp. (lobed hyphopodia) appeared to be very similar to the Ggt viruses of groups I and II. However none of the viruses from Ggt isolates, many of them group I and II viruses, reacted serologically with either of these Phialophora viruses (Buck, McGinty and Rawlinson, 1981). On the other hand, it should be noted that serological relationships have been demonstrated between virus particles from Phialophora sp. (lobed hyphopodia), and from Ggg, with those from Ggt (Rawlinson and Muthyalu, 1975, 1976).

No absolute evidence for horizontal transmission

of G.graminis viruses has been obtained in vivo. although the evidence of Rawlinson et al. (1973) and Jamil, Buck and Carlile (1984) discussed in section 1.2.1.d, indicates that such transmission probably does occur. Similarly the possibility of virus transfer between G.graminis and Phialophora spp. has been discussed in section 1.2.1.d, in the light of the report of Jamil and Buck (1984). Lastly, vertical virus transmission in G.graminis has also been discussed in section 1.2.1.d.

The study of the viruses of G.graminis and related fungi has contributed considerably to the body of knowledge relating to the ds RNA mycoviruses. It has also assisted the study of the biological significance of the viruses of Ggt, discussed in section 1.2.3.

1.2.3. Biological Significance of Mycoviruses.

Many mycoviruses appear to be non-aggressive towards their hosts since the infection does not result in disease symptoms. Ghabrial (1980) suggests that rather than describe such infections as 'latent', (which implies in virological terms that virus particles are not demonstrable in the host), they should be described as avirulent. This definition, of course, involves the host-virus interaction and since most, if not all, mycoviruses do not appear to exist extracellularly and hence do not interact with a number of possible hosts. Perhaps therefore, it is a better

description to say that the virus is non-aggressive or non-pathogenic, or that the host is tolerant of the infection. Lemke (1979) has speculated that the majority of mycoviruses have evolved, or are evolving towards a life cycle that is simple and in concert with their hosts, a hypothesis analogous to that proposed by Doolittle and Sapienza (1980) and Orgel and Crick (1980), i.e. their proposal of 'selfish DNA'. Moreover it would seem to be true from the studies of Still et al. (1975) that non-aggressive mycoviruses such as PsV-S and PsV-F do not have any detrimental effect on their hosts' metabolism, including host macromolecule synthesis, largely because the vast majority of virus replication occurs after the fungus has completed its exponential growth phase; although this may be due to containment by the fungus, rather than an example of host-parasite co-evolution. Evidence for this is provided by the studies of Border et al. (1972), Border (unpublished results quoted in Buck, 1977) and Yamashita, Doi and Yora (1973) who all observed that virus particles in Penicillium sp. may either be free in the cytoplasm or contained in vesicles or vacuoles. The latter could regulate the overall level of virus particles in the fungus, since it is presumed that only free virus particles are capable of replication.

There are also a number of mycoviruses that have a definite effect on their host, either detrimental or

potentially beneficial. These can be divided into three categories of effect; degeneration or lysis of the host mycelium; production of a 'killer' factor; and hypovirulence or reduced pathogenicity of the host without other symptoms. These categories will be discussed in the following sections.

a. Degenerative mycelial diseases.

Formation of lytic plaques in fungi has rarely been observed and generally only under specific conditions. Lemke, Nash and Pieper (1973) reported localised plaques in certain mutant strains of Penicillium chrysogenum, when grown on an unbuffered high lactose-containing medium. Lemke (1975) further observed that this phenomenon could be cured by heat treatment of mycelium and reintroduced by heterokaryosis, (associated with transfer of ds RNA virus particles), and that wild type strains carry a nuclear gene, (which is dominant in diploids), which encodes resistance to lysis. Two other species of Penicillium; P.citrinum and P.variable have been shown to form plaques on similar media. In both cases, wild type ds RNA virions containing strains were observed to exhibit the phenomenon (Benigni, Ignazzitto and Volterra, 1977; Borre et al., 1971). More recently, the yeast, Candida albicans has been induced to produce lytic plaques by the inclusion of a fungal cell wall synthesis inhibitor, Aculeacin A, at subinhibitory levels in the growth medium (Mehta, Nash and Bozzola,

1982). Isometric virus particles of 12, 18 and 28 to 39 nm diameters were observed both in ultra thin sections of cells and also in the plaque lysate. The plaque lysate was apparently capable of initiating new infection, but this ability was lost after treatment with u.v. light, heat treatment at 80°C for 10 minutes or being maintained at pH2 for 30 minutes. Hence the virus particles seem to be associated with infective plaque formation.

The degenerative disease of Agaricus bisporus, most commonly called 'die-back', but also known as 'watery stipe', 'La France disease' and 'X-disease' is characterised by sparse mycelial growth, suppression of development, rapid necrosis, dwarfing and early maturity of sporophores (Gandy, 1960; Hollings, 1962; Schisler, Sinden and Sigel, 1967). According to Dieleman-van Zaayen (1979) most mushroom scientists are now of the opinion that all these various names relate to only one disease of A.bisporus. Both Hollings (1962) and Schisler, Sinden and Sigel (1967) have shown that 'die-back' can be transmitted, and involves the transfer of virus particles. The former reported that cell-free virus preparations were infective, while the latter authors reported that heterokaryosis between an infected germ tube of a germinating basidiospore and healthy mycelium led to disease and virus particle transmission. There are a number of types of virus

particle found in A.bisporus, and as yet there is not direct evidence concerning which is/are the causative agent(s) of the disease. Barton and Hollings (1979) studied the two ds RNA-containing isometric particle types, (25 and 35 nm diameter particles), AbV-1 and AbV-4 respectively. These two viruses are serologically unrelated; AbV-1 has two ds RNA components both of about 1.4×10^6 d. Tavantzis et al. (1980) studied the bacilliform virus of A.bisporus, and reported that its genome comprises a single ss RNA component of 1.4×10^6 d. In a more recent survey, Wach and Romaine (1983a and b) observed that ds RNA was associated with decreased yields and deformed sporophores, while no ds RNA was ever seen in healthy sporophores. Diseased mycelium appeared to contain from 3 to 12 ds RNA species with molecular weights ranging from 0.21 to 4.3×10^6 . Wach and Romaine (1983a and b) propose that 'die-back' of A.bisporus is associated with multiple virus genotypes.

'Die-back' disease of mushrooms appears to be a disease that has appeared only in the last 30 to 40 years. Lemke (1979) suggests that this may be the result of over-cultivation, while Buck (1980) suggests that it may be due to virus mutation or complementation of partial genomes following heterokaryosis. This last suggestion in the light of the evidence of Schisler, Sinden and Sigel (1967) and Wach and Romaine (1983a and b), perhaps also involving

virus mutation, seems the most likely explanation for the appearance of the disease.

Two plant pathogens have also been seen to exhibit a degenerative disease of the mycelium; these being Helminthosporium victoriae and Rhizoctonia solani. The disease of H.victoriae was first recorded by Lindberg (1959); who noticed that of a range of cultures freshly isolated from diseased oats, certain of the fungal colonies were abnormally stunted, sectorised frequently and sporulated poorly. Later Ghabrial, Sanderlin and Calvert (1979) observed that the hyphae at the edge of diseased colonies appear swollen with distorted cell walls and their cytoplasm is disorganised. Lindberg (1959) demonstrated that the disease was transmissible to most healthy isolates by anastomosis and sometimes by treatment of the healthy mycelium with a homogenate of the diseased mycelium. Lindberg (1971a and b) also reported similar diseases of two other species of Helminthosporium; H.oryzae and H.maydis. Sanderlin and Ghabrial (1978) found two serologically distinct types of virus particles in H.victoriae; the first type was an isometric particle (35 to 40 nm in diameter) with a sedimentation coefficient of 190S and a single ds RNA of 3.0×10^6 d. The second type of virus particle was also isometric of a similar size to the first, but with an S value of 145S and four distinct ds RNA species with molecular weights of 2.4, 2.2, 2.1 and 2.0×10^6 d.

The 190S virus occurred in both healthy and diseased isolates; while the 145S virus was only found in diseased isolates along with the 190S virus. Ghabrial, Sanderlin and Calvert (1979) investigated the effect of regenerating protoplasts from originally diseased isolates. They found that the new colonies could be separated into three categories; a) healthy vigorous colonies (Type 1), b) colonies that resembled the original diseased isolate (Type 2) and, c) severely stunted colonies with little or no aerial mycelium (Type 3). Colonies of Type 1 were shown to contain only the 190S virus with none or only trace amounts of the 145S virus. Colonies of Types 2 and 3 contained both viruses and the severity of disease appeared to correlate with the level of the 145S virus. Hence it seems that the 145S virus is the causative agent of the disease, but the 190S virus is not deleterious to the host, though it may be necessary for replication of the 145S virus. Ghabrial (1980) and Ghabrial and Mernaugh (1983) have achieved transmission of the disease by infecting healthy protoplasts of H.victoriae with purified virus preparations containing both viruses, in the presence of polyethylene glycol. Disease transmission only occurred at a very low level, i.e. it was detected in only 2 out of 5 experiments at a frequency of about 1%. The newly infected isolates exhibited the same disease symptoms as expected and contained both types of viral particle. Therefore, the

viruses found in H.victoriae have been shown to be closely associated with, if not the causative agents of, the disease, and to be capable of infection as extracellular agents.

The degenerative disease of Rhizoctonia solani or 'Rhizoctonia decline', is characterised by slow growth, production of few or no sclerotia, irregular mycelial appearance including a change in colour to white-tan, (rather than brown). Isolates can be cured of their diseased condition by hyphal tipping, while the disease can be introduced by anastomosis of a diseased strain with a healthy strain (Castanho and Butler, 1978a). Three isolates of R.solani out of thirteen were found to contain ds RNA, (but no viral particles), by Castanho, Butler and Shepherd (1978), and all three exhibited disease symptoms, while the other ten isolates were healthy. Diseased isolate 189a contained three ds RNA segments with molecular weights of 2.2, 1.5 and 1.1×10^6 d. A healthy hyphal tip isolate of 189a, termed 189HT5 did not contain any ds RNA and when 189a and 189HT5 were anastomised, the latter was returned to the diseased state and it also regained the ds RNA species. Hence it was proposed by Castanho, Butler and Shepherd (1978) that ds RNA was associated with and perhaps the cause of Rhizoctonia decline.

Castanho and Butler (1978b) reported that the virulence of isolate 189a was very much reduced as a

result of its diseased condition. Moreover they observed that in co-inoculation tests with isolate 189HT5, (a virulent isolate), it prevented sugar beet seedling damping off to a significant extent. Hence they proposed that this weakly virulent or hypovirulent isolate, 189a, may be useful in the control of plant disease caused by R.solani.

Finkler et al. (1983) and Zanzinger et al. (1984) have also studied the effect of ds RNA on pathogenicity of R.solani. They observed that highly pathogenic isolates may contain higher numbers and concentrations of ds RNAs than weakly pathogenic isolates and therefore it is doubtful if ds RNA is responsible for either low pathogenicity of Rhizoctonia decline. It is worthy of note that Finkler et al. (1983) also found isometric virus particles of 25 to 31 nm associated with the ds RNAs.

It is possible that another cytoplasmic determinant is responsible for Rhizoctonia decline, i.e. a linear ds DNA plasmid, (this is discussed in section 1.2.4.).

In all cases of degenerative mycelial diseases discussed it would be beneficial to the study of ds RNA viruses if the control of the diseases at the gene product level could be identified since this would further the understanding of the nature of the viruses involved.

b) Production of killer factors.

There are two fungal species that have been extensively studied with respect to killer factor or killer toxin production and these are Saccharomyces cerevisiae and Ustilago maydis, though it should be noted that a number of other yeast species also exhibit this phenomenon. Killer strains of these species are characterised by secretion of a toxin to which they themselves are insensitive but which is lethal to other strains of the same or related species, termed sensitives.

Woods and Bevan (1968) found that certain strains of S.cerevisiae were capable of producing an extracellular killer factor which appears to be proteinaceous in nature. Palfree and Bussey (1979) purified this killer factor and showed that it was a protein of approximately 11,500d and that it was active in this purified form especially in the pH range 4.2 to 4.6. From genetic studies involving crosses, the formation of diploids and the analysis of the progeny from the diploids, it is apparent that the killer factor, and immunity to it, in S.cerevisiae is under the control of cytoplasmic genetic determinants (Somers and Bevan, 1969; Bevan and Somers, 1969). These cytoplasmic genetic determinants were inferred by various studies to be associated with ds RNA viruses found in killer strains (Vodkin and Alianell, 1979; Bevan and Mitchell, 1979). Originally Bevan, Herring

and Mitchell (1973) observed that killer strains contained two species of ds RNA, L and M, (with molecular weights of 2.5×10^6 and 1.4×10^6 , respectively). Later the molecular weight of the larger ds RNA species was re-estimated as 3.3×10^6 (Bevan and Mitchell, 1979). Certain strains of S.cerevisiae only carry the larger ds RNA species and these strains are sensitive to killer factor (Bevan and Mitchell, 1979).

The two ds RNA species are separately encapsidated in virions approximately 40 nm in diameter that consist of the same major capsid polypeptide (Bostian, Sturgeon and Tipper, 1980). By in vitro translation Hopper et al. (1977) demonstrated that L ds RNA encodes the major capsid polypeptide, indicating that M ds RNA is a satellite virus of L ds RNA.

There have been a number of variant killer phenotypes observed; among these being; neutral mutants, (that contain M ds RNA, but do not secrete killer and yet are immune); superkiller mutants, (that contain up to 2.5 times more M ds RNA than usual and whose toxin is often 3 to 4 times more stable than toxin from a normal killer strain and may require a nuclear mutation, known as ski), diploid dependent killer mutants, (these diploid factors for its own replication), (Vodkin and Alianell, 1979; Bevan and Mitchell, 1979; Buck, 1980).

Another class of ds RNAs have been observed in yeast and these are related to the suppressive non-killer phenotype (Vodkin and Alianell, 1979). Strains of this type carry S ds RNA which is dominant over M ds RNA; when two such strains are crossed most of the diploids are non-killers and all of the meiotic progeny are non-killers and sensitive, but contain L and S ds RNA. Bruenn and Kane (1978) showed using T_1 fingerprint analysis that two different species of S ds RNA, S_1 (0.92×10^6 d) and S_3 (0.46×10^6 d), are both derived from M ds RNA and that in fact S_1 is a duplication of S_3 . This was confirmed by Fried and Fink (1978) by heteroduplex mapping and strengthened the argument that S virions are defective interfering viruses of M virus, and that presumably the suppressive non-killer phenotype following an S x M cross is the result of S ds RNA outcompeting M ds RNA in the replication process (Ridley and Wickner, 1983).

The first direct evidence that M ds RNA encodes the killer toxin was provided by the study of Hopper et al. (1977). They showed by in vitro translation that M ds RNA carries the genetic information for two proteins, one of which was later shown to contain the killer toxin by peptide mapping and cross-antigenicity (Bostian et al., 1980; Welsh and Leibowitz, 1982; Thiele and Leibowitz, 1982). These two polypeptide products originate from different regions of the M ds RNA which can be separated by S1 nuclease digestion

(Welsh and Leibowitz, 1982). In vivo transcripts have been isolated from cells of S.cerevisiae (Bostian et al., 1983) and these were a full-length and a partial-length ss RNA from both L ds RNA and M ds RNA. The two transcripts of L ds RNA have in vitro M RNA activity to produce SCV-Pl, (the 88,000d capsid polypeptide); while the two transcripts of M ds RNA encode a 32,000d polypeptide, which is a precursor of the killer toxin and is called the pre-prototoxin. M ds RNA has also been shown to contain a large stem and loop structure in the 5' terminus of the plus strand, (and conversely in the 3' terminus of the negative polarity strand) which is presumably a transcription and/or replication start signal, while the plus strand has been shown to be responsible for programming the synthesis of the preprototoxin, (Thiele and Leibowitz, 1982). Moreover, Hannig, Thiele and Leibowitz (1984) have reported the presence of a polyadenylated tract in the M ds RNA, and they suggest that this may well be an alternative to transcript polyadenylation.

Recently Skipper, Thomas and Lau (1984) and Bostian et al. (1984) have cloned and sequenced the preprototoxin gene of M₁ ds RNA. This demonstrated the presence of an open reading frame in M₁ ds RNA that equates to a 34.7 Kd protein of 316 amino acids, which is similar in size to the preprototoxin. From this study Bostian et al. (1984) deduced a model for the assembly

of the toxin molecule, taking into account the discovery of an unstable 42 Kd, membrane associated, glycosylated protein in killer cells, which is believed to be the protoxin (Bostian, Jayachandran and Tipper, 1983). By this model, toxin would consist of two dissimilar subunits α and β , which are separated in preprotoxin by a large glycosylated segment, γ ; there being the possibility that γ is responsible for immunity. The final subunit of the preprotoxin, δ , is the N-terminal segment.

Bostian et al. (1984) and Bostian, Jayachandran and Tipper (1983) suggest that processing of the preprotoxin occurs as it is transported and secreted by the host cell endoplasmic reticulum and plasma membrane. Bussey et al. (1983) have demonstrated that host functions related to secretion are crucial for processing the 42 Kd membrane-bound protoxin. These functions may be equivalent to the two previously identified host nuclear genes KEX1 and 2, needed for killer expression and the host nuclear gene REX, which is involved in immunity expression (Vodkin and Alianell, 1979).

Lolle et al. (1984) have achieved expression of cDNA clones of M_1 preprotoxin gene in yeast strains were able to produce preprotoxin, secrete killer toxin, and were immune to the killer toxin they were secreting. Hence this appears to prove that the preprotoxin gene of M_1 ds RNA encodes both the killer

and immunity factors.

Killer toxin interacts with a yeast cell initially via a cell wall receptor site thought to be a (1-6)- β -D-glucan (Hutchins and Bussey, 1983). Next it is presumed to contact the plasma membrane where it is believed it causes the disruption of the sensitive cell. The primary effect is the leakage of potassium ions and ATP through the plasma membrane (Wickner, 1979). It was reported by de la Pena et al. (1980) that 40 minutes after a culture of sensitive cells was exposed to killer, 50% of the cells were dead due to an inhibition of the proton pumping ability of the plasma membrane which would presumably lead to the leakage of potassium ions and ATP, as previously observed. The site at which the immunity factor operates is as yet unelucidated, but presumably it must either be in the cell wall, or the plasma membrane. Al-Aidroos and Bussey (1978) have found certain yeast strains that are resistant to killer toxin, due to a chromosomal mutation. The nature of this resistance is believed to lie in these strains lacking a cell wall receptor site for the killer toxin. Hence perhaps the immunity factor could well act by blocking the cell wall receptor site of the producer strain.

Various strains, species and genera of yeast have been found to have the capacity to produce killer toxin. Rogers and Bevan (1978) originally classified a selection of these into four groups on the basis of

their killing/immunity reaction to one another. Young and Yagiu (1978) conducted a more wide-ranging survey observing killer strains from various genera including Saccharomyces, Candida, Hansenula, Pichia, Torulopsis and Kluyveromyces. They identified 13 groups of killer specificity on the same grounds as Rogers and Bevan (1978), but noted that ds RNA could only be extracted from members of three of these groups, which are the groups into which only the toxin-producing Saccharomyces spp. classify. Hence although all killer producing strains have the common characteristic of producing a protein-based toxin, many killer yeasts do not carry the genetic information for their toxin on ds RNA. It is worthy of note that recently the yeast Yarrowia (Saccharomycopsis) lipolytica has been shown to contain a ds RNA virus and also secrete killer toxin (Groves, Clare and Oliver, 1984). Whether Y.lipolytica would classify with the Saccharomyces spp. toxin producing groups or not would be of interest; and also this result indicates that killer production is even more widespread among yeasts than previously believed. Of the killer producers that do not contain ds RNA two different methods of encoding the toxin have been identified. In Torulopsis glabrata evidence has been found for the killer determinant being chromosomally located (Sriptra-kash and Batum, 1984); while in Kluyveromyces lactis the genetic information for killer

production is carried on one of two linear DNA plasmids that appear to be interdependent (Gunge, Murata and Sakaguchi, 1982; Stark et al., 1984). The mode of action of the toxin of Pichia kluyveri, (which lacks ds RNA), appears to be remarkably similar to the action of S.cerevisiae toxin (Kagan, 1983), whereas the mode of action of K.lactis toxin is different since it appears to inhibit adenylate cyclase activity in sensitive cells and cause their arrest at the G₁ phase in the cell cycle (Sugisaki et al., 1983).

The other fungal species known to produce and secrete a similar killer factor to that produced by yeast is Ustilago maydis. Puhalla (1968) reported that certain U.maydis strains were capable of inhibiting the growth of other strains and that this ability was inherited extrachromosomally. The killer phenotype in U.maydis was correlated with ds RNA containing isometric virus particles, 43 nm in diameter, by heterokaryosis (Wood and Bozarth, 1973; Koltin and Day, 1976; Bozarth et al., 1981). The ds RNA components range in size from 4.2×10^6 to 0.30×10^6 and have been classified into three groups; heavy (H), medium (M) and light (L) (Koltin and Day, 1976; Koltin, 1983). Three different strains have been recognised and studied as killer producers in U.maydis, and these are designated P1, P4 and P6, with their respective killer factors designated KP1, KP4 and KP6 (Koltin and Day, 1976; Koltin and Levine, 1979). All three killer

factors are proteins with molecular weights of about 10,000. However they are not identical since they show differing migration patterns in gel electrophoresis at pH8.3, they differ in thermolability, pH dependence and biological activity. Furthermore KP1 and KP4 do not cross react with antiserum to KP6 (Kandel and Koltin, 1978). As in S.cerevisiae killer strains are immune to their own killer factor, and also certain non virus-containing strains of U.maydis have been shown to be resistant to killer toxins; this characteristic being encoded by a recessive nuclear allele (Koltin and Day, 1976; Koltin and Levine, 1979). The activity of these killer factors is not restricted to U.maydis; Koltin and Day (1975) demonstrated that there is widespread sensitivity to them amongst other cereal and grass smuts, although most other fungi and all bacteria appear to be unaffected by the toxin. From this observation Koltin and Day (1975) postulated that if the genetic information for killer production and immunity could be transferred to cereals and expressed appropriately, then this would constitute a means of disease control of various smuts.

The ds RNA species are encapsidated in identical isometric virus particles, 43 nm in diameter, the capsid polypeptide being encoded by the H ds RNA segments (Koltin, 1977). Bozarth et al. (1981) observed that the H ds RNA are separately encapsidated,

while the M ds RNAs are encapsidated with up to three components per virus particle. As seen with other mycoviruses there is a virion associated RNA polymerase, the reaction products being full length ss RNAs from in vitro transcription (Ben-Tzvi et al., 1984).

From a series of genetic studies using mutagenesis and crossing, the different ds RNA segment classes have been assigned various functions. It is important to note that although each killer strain contains a different ds RNA species pattern, (there being a number of H and M type components), the viral functions are the same (Koltin and Levine, 1979; Koltin, 1983). Both the capsid polypeptide and replication function, (presumably the polymerase itself), are coded for by the H ds RNA components (Koltin 1977; Koltin, Mayer and Steinlauf, 1978); while the functions related to the killer phenotype are associated with the M and L ds RNA components (Koltin, Levine and Peery, 1980), with the L ds RNA species responsible specifically for the immunity factor (Peery, Koltin and Tamarkin, 1982).

Through the analysis of the products of in vitro translation, Simon, Gorecki and Koltin (1981) demonstrated that only from virus complexes containing M ds RNA components was a protein antigenically related to killer toxin produced. This protein was larger than the extracellular purified toxin, (19,000d compared with 10,000d). It could also be produced by

translating poly A m RNAs from toxin producing U.maydis cells.

By analysis of sequence homology between L and M ds RNA species, Field et al. (1983) found that in fact L ds RNA is homologous to one end of M ds RNA and hence it would appear that M ds RNA codes for both the killer toxin and the immunity factor and presumably the L ds RNA species must have arisen by the replication and packaging of a sub-genomic RNA.

In a large survey of strains of U.maydis isolated from infected corn samples, Day (1981) found that ds RNA is common in U.maydis. Apart from four isolates, termed 'brownners', that appeared to carry a cytoplasmic character that causes sensitive U.maydis cells to turn brown, (notably all four 'browner' isolates contained the same ds RNA pattern), the only biological effect of ds RNA viruses in U.maydis that Day (1981) observed was the production of killer factor. No effect on host pathogenicity was observed.

It has been reported, as in the case of S.cerevisiae, that as a result of crossing two strains with different killer phenotypes, (in conditions where the toxins are inactive), there is an exclusion mechanism that prevents the co-existence of two killer types in the one cell (Koltin and Day, 1976; Koltin, 1983). It appears that one type of H ds RNA species, H4, (1.8×10^6 d), found in strain P4, is capable of

modifying the exclusion, so that progeny from a P1 x P4 fusion may be P1, P4 or P1/P4. In other cases though the exclusion of the other strain is absolute, e.g. as a result of a P1 x P6 cross, all the progeny are non-killers, but still contain H ds RNA. Hence it is presumed that it must be the H ds RNA that is involved in controlling the exclusion mechanism (Koltin, 1983).

The secreted killer toxin of U.maydis has an in vitro nuclease activity that is apparently capable of digesting ss RNA, ss DNA and ds DNA, but not ds RNA (Levine, Koltin and Kandel, 1979). Hence it cannot be involved in the exclusion mechanism and presuming it can enter sensitive cells, it is quite probably responsible for inhibiting cellular metabolism. However, further work, especially in vivo, is needed to demonstrate that it is actually active in this fashion.

The genome organisation and biology of the ds RNA viruses of U.maydis appears to bear many similarities with those characteristics of the ds RNA viruses of S.cerevisiae, e.g. the large ds RNA components being the helper virus; the smaller, satellite ds RNA components coding for the killer toxin and immunity factor; the need to process the in vivo translated toxin to produce the in vivo form and the presence of an exclusion mechanism. However the mode of action of the two killer toxins does appear to differ considerably. On the other hand, as Buck (1980) has discussed, fungal killer toxins do appear to resemble

bacteriocins, and Hardy (1978) has distinguished 3 classes of colicin (i.e. a bacteriocin produced by Eshcerichia coli). Colicin E1 disrupts energy dependent reactions in the plasma membrane, E2 possesses DNase activity, while E3 is capable of inactivating ribosomes. Hence the killer proteins of S.cerevisiae and U.maydis could be said by comparison to fall into two of these classes and therefore, the difference in their mode of action is not so surprising.

The production of killer factors by the two fungi, S.cerevisiae and U.maydis has yielded a great deal of information regarding the activity of the fungal viruses concerned and widened our knowledge of mycoviruses in general. However the significance of killer factor production in vivo has yet to be understood; for instance, it appears that U.maydis either does not produce killer factor whilst in contact with maize, or the killer factor is inactivated in this situation (Day and Dodds, 1979).

c) Hypovirulence.

Endothia parasitica, the fungal pathogen of chestnut trees and the cause of chestnut blight has been observed to exhibit the phenomenon known as hypovirulence. A number of characteristics of hypovirulence have been outlined by Anagnostakis (1978) and these include: a reduction in the pathogenicity of the fungus E.parasitica, but not in its saprophytic

vigour; it is controlled by cytoplasmic genetic determinants; these cytoplasmic genetic determinants appear to be associated with ds RNA. Hypovirulence was observed as a natural phenomenon in Italy in the 1950s, where chestnut trees with cankers caused by E.parasitca, instead of dying were seen to recover. Strains of E.parasitica, isolated from the bark of these cured trees were seen to have reduced virulence, were called hypovirulent (H) strains. H strains have now been isolated in France and in the USA, but unlike Italy, natural spread of hypovirulence has not been observed in the latter two countries (Anagnostakis, 1978).

Puhalla and Anagnostakis (1971) reported that cytoplasmic determinants were responsible for hypovirulence and Day et al. (1977) observed that all H strains examined carry ds RNA, whereas most virulent (V) strains do not. (This has been confirmed by Anagnostakis, 1981a; Van Alfen, 1982). Furthermore all H strains appear to contain a ds RNA species in the size range 5 to 7 x 10⁶d. Other than this, their banding patterns on polyacrylamide gel electrophoresis do not have any common features (Day et al., 1977; Dodds, 1980a). Van Alfen, Jaynes and Bowman (1978) found that H strains could revert to V strains in culture through growth and U.V. irradiation, which tends to lend credence to the hypothesis that a cytoplasmic factor is responsible for hypovirulence.

The ds RNA species found in E.parasitica range from 4.3 to 7×10^6 in molecular weight (Dodds, 1980a; Van Alfen, 1982). In certain European strains the ds RNA species appear to be associated with pleomorphic club-shaped particles, up to 300 nm long with spherical heads, 50 to 90 nm in diameter (Dodds, 1980b). These particles contain virtually no protein and are probably membrane enclosed (Gillies and Van Alfen, 1981). Hence it is most likely that they are not virions, but replication structures. It is interesting to note that similar particles have also been observed in Agaricus bisporus (Lesemann and Koenig, 1977).

Van Alfen (1982) states that pathogenicity in E.parasitica is a continuum from low to high, implying that to designate a strain hypovirulent is perhaps too clear-cut. However, it would seem feasible in E.parasitica to distinguish between an H and a V strain. For instance Havir and Anagnostakis (1983) have reported that V strains produce oxalate in culture at a rate of 10-40 mg/g dry weight of mycelium, while H strains produce no detectable oxalate. It is important to note that oxalate is believed to be active in pathogenicity in two ways; firstly, it combines with host calcium, thus weakening the host, and secondly, it lowers the host pH which aids the action of various degradative enzymes of the pathogen, e.g. polygalacturonase. Furthermore conversion from V to H

has been achieved by Anagnostakis and Day (1979) for a number of strains by anastomosis; transfer has been successful even where the strains are vegetatively incompatible. Conversion of a collection of V strains from a number of vegetative compatibility (v-c) groups, using both an Italian and a French H strain, was associated with faithful transfer of the major components of the original ds RNA patterns, which also were stable through subsequent subculturing (Anagnostakis, 1981a).

Biological control of E.parasitica through hypovirulence spread naturally in Italy, but not in France or the USA. The evidence presented by Anagnostakis (1977, 1981b, 1983) indicates that in the USA this is due to the large number of v-c groups found in E.parasitica populations that will naturally slow the spread of the cytoplasmic H factor. Use of mixtures of H strains from a number of v-c groups has proved to be a way of overcoming this problem and has led to effective biological control of the disease in France; while in the USA, as yet, this method achieves control of individual cankers but has not induced natural spread (Anagnostakis, 1982; Jaynes and Elliston, 1980; Anagnostakis and Waggoner, 1981).

The phenomenon of hypovirulence demonstrated by E.parasitica is very closely associated with, if not actually caused by, the ds RNA species found within it. As yet no molecular evidence has been produced

relating to the biological functions of these ds RNAs, as has been achieved with the ds RNA viruses of yeast. If this were so, it would present both a greater understanding of the variety of activity of mycoviruses and an insight into the molecular interactions between the virus and the fungus, i.e. how it affects the control of pathogenicity of the fungus.

Dutch Elm Disease, caused by the fungus Ceratocystis ulmi, is also possibly similarly influenced. Isolates of C.ulmi have been recognised as falling largely into two categories; aggressive and non-aggressive, both in Britain and America (Gibbs, Houston and Smalley, 1979). This division was investigated by Brasier and Gibbs (1976) and Brasier (1977) with the conclusion that aggressivity is under polygenic control and that a cytoplasmic factor is not involved in the non-aggressive character. However, recently Brasier (1983) has reported the transmission of a cytoplasmic factor between aggressive strains of C.ulmi that causes a disease of mycelium characterised by severely reduced growth and lowered reproductive fitness. The cause of the disease is unknown, however the fact that it is not transmitted through ascospores suggests that it may be associated with ds RNA. There have been a number of reports of ds RNA occurring in C.ulmi, however not relating to diseased isolates, only relating to 'more aggressive' (MA) and 'less

aggressive' (LA) isolates (Pusey and Wilson, 1979, 1981, 1982). Species of ds RNA have been observed in both isolate types, (with molecular weights ranging from 0.4 to 2.0×10^6). The LA isolates generally contain more ds RNA components and those components that are unique to the LA isolates are perhaps involved in influencing their phenotype (Pusey and Wilson, 1982). However at present, if hypovirulence is affecting Dutch Elm disease it is not obvious from the studies of Pusey and Wilson (1982); while the report of Brasier (1983), (if the cause is ds RNA-associated), indicates a condition more akin to a mycelial disease, such as Rhizoctonia decline (Castanho and Butler, 1978a; Castanho et al., 1978) than that of hypovirulence in E.parasitica, where the saprophytic vigour of the pathogen is not always affected (Anagnostakis, 1978).

Another fungal pathogen that has been studied as demonstrating hypovirulence due to ds RNA virus infection is the take-all fungus of wheat, Ggt. The first observations by Lapierre et al. (1970) that certain virus containing strains of Ggt were weakly pathogenic, but that highly pathogenic cured strains, (without virus), could be obtained by isolating single ascospores from the former strain types, were indicative of ds RNA virus-associated hypovirulence occurring in Ggt. Lemaire et al. (1970) further suggested that the phenomenon of take-all decline, (see

section 1.1.3), was the result of virally infected isolates of Ggt. Apparently confirming that this change in pathogenicity level is related to virus infection, Tivoli, Ferault and Lemaire (1979) achieved virus transmission via anastomosis, (identified by use of fungicide resistance markers), which was associated with loss of pathogenicity in previously highly pathogenic isolates. Furthermore, Tivoli et al. (1979) recorded the reappearance, after some years, of virus particles in an ascospore isolate of Ggt strain cured of infection and with the reappearance of the infection it also became weakly pathogenic.

In a series of experiments these same workers looked at the potential of these weakly pathogenic isolates of Ggt as biological control agents. Lemaire et al. (1971) and Lemaire et al. (1975) observed that certain isolates of Ggt, (i.e. the virally infected isolates discussed above), appeared to be able to control the disease potential of pathogenic isolates when co-inoculated in greenhouse trials. (Asher, 1978b has reported similar results using weakly pathogenic isolates of unknown viral content). Brun, Tivoli and Carpentier (1976) attempted to study the mechanism of control and concluded that induced resistance, such as is probably responsible in the examples of cross-protection by Pg and Ggg, is possibly involved. Lemaire et al. (1976) described the results of a four

year field trial using weakly pathogenic strains of Ggt to prevent take-all. They proposed that their results demonstrated that control could be achieved in this fashion and that transmission of 'hypoaggressivity' could occur between isolates of Ggt in the soil and lead to lower levels of take-all incidence and severity.

However, Rawlinson and Pearson (1972) observed that six isolates of Ggt and one isolate of Gga which were infected with virus were not necessarily weakly pathogenic or associated with TAD. In a survey of 156 isolates of G.graminis, 114 of which contained virus particles, Rawlinson et al. (1973) examined the relationship between virus infection and reduced isolate pathogenicity. They found no association between virus infection, (either with 27 nm or 35 nm particles, or with both particle types), and weak pathogenicity, association with TAD, unusual isolate growth, or loss of potential to produce perithecia. Furthermore, as reviewed by Rawlinson and Buck (1981), it has quite often been the case with other viruses of fungi that an effect on the host caused by infection has been prematurely and erroneously claimed. However, following further study of the viruses of Ggt it has become clear that there is considerable variation amongst them (Buck et al., 1981a), and that a particular class of viruses, or mutant virus, may be responsible for the reports of Lapierre et al. (1970)

etc., and that in the large screening programme of Rawlinson et al. (1973) this could have been masked by the other viral types present. Hence a survey with more emphasis on the genome composition of viruses found in Ggt might yield an explanation for the contradictory results concerning hypovirulence and viral infection in Ggt (Rawlinson, 1978; Rawlinson and Buck, 1981).

The biological significance of mycoviruses can be described in four categories: non-aggressive, (or host-tolerated); production of killer factors; association with degenerative mycelial diseases; and association with hypovirulence.

1.2.4 Extrachromosomal Elements of Fungi.

There have been many observations in the literature that certain unusual phenotypes of fungi are the result of the influence of extrachromosomal genetic elements. Some of these may be attributable to the influence of a mycovirus infection of the particular fungus; but many altered fungal phenotypes have other causes and it is intended to discuss these other extrachromosomal elements divided into two classes; mutant mitochondrial genomes, and plasmids with no sequence homology with either the mitochondrial or nuclear genome of the host.

The first significant example of mitochondrial mutant genomes producing an altered phenotype was the

'petite' phenotype of Saccharomyces cerevisiae causing respiratory deficiency. These mutants lack the mitochondrial genome, either in part or totally and hence cannot code for essential respiratory enzymes e.g. succinate oxidase and cytochrome oxidase. The result being that the colony formed by cells of this phenotype are much smaller than wild type cells on respirable carbon sources, hence the name 'petite' (Fincham, Day and Radford, 1979). Both the 'poky' and 'stopper' phenotypes of Neurospora crassa have been shown to be related to mitochondrial genome defects (Manella et al., 1979; Bertrand et al., 1980). In both cases the mitochondrial (mt) DNA appears to consist of a segment of the wild type mitochondrial genome, e.g. 'stopper' mt DNA consists predominantly of defective molecules of a particular 16×10^6 d segment of the wild type 40×10^6 mt DNA (Bertrand et al., 1980). These defective genomes, like the petite genome of S.cerevisiae, are lacking the sequence for cytochrome oxidase and hence their phenotypes are largely explained. A similar example is also presented by the 'ragged' phenotype of Aspergillus amstelodami. Here a small segment of the wild type mt DNA, only 1200 bp of a total 40,500 bp normal genome has been amplified as tandem repeats to yield certain large, extra mt DNA molecules that are found in conjunction with wild type mt DNA (Lazarus et al., 1980). Both A.amstelodami and

Aspergillus glaucus have also been found to exhibit a syndrome known as vegetative death (Caten, 1972; Caten and Handley, 1978). Caten (1972) showed that this was caused by a cytoplasmic factor, transmissible by anastomosis; while Caten and Handley (1978) demonstrated that it could be only maternally inherited in a sexual cross, indicating that vegetative death syndrome in Aspergillus spp. is probably a mitochondrial defect similar in cause to those discussed above.

The phenomenon of senescence in Podospora anserina is caused by a cytoplasmic factor that may only be maternally inherited in a sexual cross (Esser and Tudzynski, 1979). The onset of senescence in P.anserina appears to be influenced by a plasmid-like DNA (ca. 4.6 kbp) found in senescent mycelium (Stahl et al., 1978). Esser and Tydzynski (1979) have suggested that the most plausible explanation of this phenomenon of ageing is that a strain of P.anserina is predisposed to senescence according to its specific nuclear genotype, and ageing per se depends on the presence and activity of this extranuclear DNA plasmid. Vierny et al. (1982) identified this extranuclear DNA plasmid as being of mt DNA origin, with one region (2.6 kbp) being most frequently excised, circularised and often amplified. This region of DNA and its plasmid form were termed Sen-DNA. Wright and Cumming (1983a) have demonstrated that this 2.6 kbp region (α -sen DNA) and

another region of 9.8 kbp (β -sen DNA) appear to be involved in senescence with α -sen DNA being predominant. During senescence both α -sen RNA and β -sen DNA are transported to the nucleus and integrated into the nuclear DNA (Wright and Cumming, 1983a). The α -sen DNA appears to code for two mRNAs in vivo, these being 2.4 and 2.5 kb long (Wright and Cumming, 1983b). However, the nucleotide sequence of α -sen DNA only identifies one open reading frame (Osiewacz and Esser, 1984). Perhaps the different mRNAs are only variants of each other in leader sequence, or termination site. The α -sen DNA has been shown to be an intron of cytochrome oxidase (subunit 1), which is possibly excised from the mt DNA and thus becomes the mobile element involved in the senescence process in P.anserina (Osiewacz and Esser, 1984).

Other than extrachromosomal elements that are derived from fungal mt DNA; which may be called plasmids since they appear to be capable of autonomous replication, (such as the examples discussed above); there are also a number of DNA elements found in various fungal species that do not have any homology with nuclear or mitochondrial DNA and are also capable of autonomous replication, (see Esser et al., 1983, for review of occurrence).

Some of these plasmids, though not derived from mt DNA, are associated with mitochondria. For instance,

Collins et al. (1981), Stohl et al. (1982) and Nargang et al. (1984) have discovered four different DNA plasmids in two species of Neurospora. Strain 'Mauriceville lc' of N.crassa and the strains 'Labelle', 'Fiji' and 'Varkud' of N.intermedia each contain a closed circular DNA plasmid in the size range 3 to 5.5 kbp. None of these plasmids show any sequence homology with Neurospora mt DNA, while the plasmids 'Varkud' and 'Mauriceville' are closely related, but are unrelated to either 'Labelle' or 'Fiji' plasmids (Collins et al., 1981; Stohl et al., 1982; Nargang et al., 1984). Tudzynski, Duvell and Esser (1983) have isolated two linear DNA plasmids (6.6 and 5.3 kbp), from mitochondria of Claviceps purpurea, which are not homologous with mt DNA of the host, but do show homology with each other. Similarly two linear DNA plasmids (7.4 and 8.5 kbp) have been isolated from mitochondria of Ggt (Honeyman and Currier, 1983). Unlike the plasmids of Neurospora sp. and C.purpurea, which do not appear to affect their host phenotype, preliminary evidence suggests that the two DNA plasmids of Ggt may be involved in affecting pathogenicity in the strain of Ggt carrying these plasmids (Honeyman and Currier, 1983).

The 2 μ m plasmid of Saccharomyces cerevisiae is a circular DNA plasmid of 6.3 kbp that appears to exist in the nuclei of its host for at least part of the cell cycle (Guerineau, 1979; Gunge, 1983). Other than the

functions it requires of its own replication, 2 μ m plasmid does not appear to encode any functions that are either detrimental or beneficial to its host.

Various other fungi have been shown to contain latent plasmids of differing size and uncertain cellular location, e.g. Ascobolus immersus (Francou, 1981), Cephalosporium acremonium (Minuth *et al.*, 1982) and Morchella conica (Meinhardt and Esser, 1984). Guardiola *et al.* (1982) reported the isolation of a 46.7 kbp circular DNA plasmid from Fusarium oxysporum that carried resistance to nifuroxine and enabled the host to express an exopolygalacturonase activity, which in the case of one strain examined, was essential for the fungus to infect tomatoes. However, serious doubt has been cast on the nature of this molecule, since Marriott, Archer and Buck (1984) have demonstrated that mt DNA of F.oxysporum is a 46.5 kbp circular molecule. Hence the molecule reported by Guardiola *et al.* (1982) is probably the mt DNA of F.oxysporum. Hashiba *et al.* (1983, 1984) have isolated a 2.6 kbp linear DNA plasmid from Rhizoctonia solani. This plasmid was isolated from three strains of R.solani 1668 RI-1, 1271 RI-64 and 1272 RI-1. Each of these three plasmid-containing strains is weakly pathogenic, while the three respective parental strains, (1668, 1271 and 1272) are strongly pathogenic and plasmid-free. The three weakly pathogenic, plasmid-containing strains were isolated

from soil, initially infested with the parental strains, (1668 RI-1, 1271 RI-64 and 1272 RI-1). Hence it appears plausible that the 2.6 kbp plasmid found in the weakly pathogenic strains (1688 RI-1, 1271 RI-64 and 1272 RI-1) is associated with loss of pathogenicity. Two linear DNA plasmids have also been shown to exist in Kluyveromyces lactis of 8.4 and 13.1 kbp (Gunge et al., 1982). These two molecules have been shown to confer a killer phenotype upon the host strain similar to that conferred by the ds RNA viruses ScV-L and ScV-M of Saccharomyces cerevisiae (Vodkin and Alianell, 1979), with the larger plasmid being responsible for maintenance functions, the small plasmid encoding the killer factor and immunity factor (Gunge et al., 1982; Stark et al., 1984). An interesting feature of the DNA plasmids of K.lactis is that they have proteins bound to their 5' termini (Kikuchi et al., 1984), which is possibly analogous to the VPg structures seen in certain viruses (e.g. Picornaviruses, Fraenkel-Conrat and Kimball, 1982).

As a result of the discovery of these plasmids of fungi, especially those that appear to be latent, the potential for creating vectors for the genetic manipulation of fungi has been investigated (Esser et al., 1983). 2 μ m plasmid of yeast has been extensively studied in this respect (Gunge, 1983); while of the plasmids of filamentous fungi progress has been achieved, for example, with both plasmid DNA from

P.anserina (Stahl et al., 1982) and plasmid DNA from C.acremonium (Tudzynski and Esser, 1982, 1983; Skatrud and Queener, 1984) towards functional vectors.

DNA plasmids found in fungi may be derived from the host mt DNA or they may be unrelated to either the nuclear or mitochondrial genome. In the first case the result is generally detrimental to the host since its respiratory metabolism tends to be impaired. In the second situation, the plasmid may either be apparently latent or it may encode a specific effect which alters the host metabolism either beneficially or detrimentally. Hence it is quite plausible that many cytoplasmically inherited effects of fungal phenotype attributed to ds RNA mycoviruses may be the result of an undetected plasmid, or vice versa.

1.3 BIOLOGICAL CONTROL OF PLANT PATHOGENS

The need for biological control of plant pathogens became apparent with the realisation that wholesale use of chemical control methods can endanger and pollute the environment, can be expensive, and can cause the development of resistant strains of bacteria, fungi and insects that render the use of such chemicals worthless. Biological control, as envisaged by Baker and Cook (1974) should be more enduring and subtle, providing a more stable and natural ecosystem. Cook and Baker (1983) have defined biological control as 'the reduction of the amount of inoculum or disease-producing activity of a pathogen accomplished by, or through, one or more organisms, other than man'. This, they point out has the potential of improving crop yields within existing resources, while avoiding environmental pollution and pathogen mutation to a resistant genotype. Soilborne fungal diseases have attracted considerable attention in this context since conventional measures often give inadequate or erratic control. As reviewed by Papavizas and Lumsden (1980), little real progress has been made for the biological control of such diseases; since there is a large gap between laboratory work, field trials, and eventually an operative system. More attention needs to be paid to the whole ecosystem and also the possibility of using a biological control agent along with cultural methods to develop an integrated control system.

Biological control systems that have been developed and appear to be successful, at least to some extent, may be separated into three distinct sections; control by cross-protection; control by protection of the infection court and pathogen suppression, and control by viral infection.

a) Cross-protection and induced resistance.

This phenomenon has been examined for both plant pathogenic viruses and fungi. Fletcher (1978) observed that virulent strains of a number of plant viruses, such as TMV, citrus tristeza virus and peach mosaic virus, could be prevented from infecting their natural hosts by prior inoculation with avirulent strains of the same virus. Matthews (1981) reviewed the use of plant viruses in such a manner and suggested that the mode of action may involve hypersensitive response molecules, occupation of virus-specific multiplication sites or adsorption of incoming particles by aggregates of particles of the avirulent strain. However he pointed out that viral cross-protection has not proved to be a particularly effective biological control method since many of the so-called avirulent strains often cause 5 to 10% yield loss; while the infected crop may also act as a reservoir of infection and the interaction of virulent and avirulent strains may lead to more serious disease through mutation or recombination.

Cross-protection of cotton with strains of Verticillium albo-atrum, a soil-borne fungus, has been achieved in field trials (Schnathorst and Mathre, 1966). Soil naturally infested with an aggressive strain, (estimated to be at a concentration of 5×10^3 viable propagules/g soil). Plants subsequently grown in this soil were protected; up to 94% of the plants only showing mild symptoms, instead of a 100% rate of severe wilt symptoms where there was no cross-protection. However, obviously to produce sufficient viable propagules of the mild strain would be difficult, and hence unless the mild strain is capable of persisting in the soil, which in the case of V. albo-atrum seems not to be the case, this type of control method is not, as yet, satisfactory.

Vascular wilt of cucumber caused by V.albo-atrum has been controlled by application of high molecular weight preparations from culture filtrates of the pathogen to the leaves of susceptible host plants (Tjamos, 1982). Systemic protection induced thus, was observed as limited symptom development. Protection was only achieved if application of the inducing preparation preceded the inoculation challenge by four days.

The foliar pathogens of cucumber, Colletotricum lagenarium and Cladosporium cucumerinum have also been controlled by cross-protection (Caruso and Kuc, 1977; Staub and Kuc, 1980). Prior inoculation of the host

plant with a mild strain of either fungus is required to induce systemic protection. In both cases only the first leaves need to be inoculated to protect the mature plant.

Cross-protection may not prove to be particularly practical, since it appears to be labour intensive; but if the mechanism of induced resistance could be understood, it holds great potential for elucidating the mechanisms of plant-pathogen recognition and response.

b. Protection of the infection court and pathogen suppression.

Examples of biological control that fall into this category involve either an occupation of the infection court by a non-pathogenic microbe (i.e. ecological competition), or an interaction between the pathogen and a non-pathogen, which is detrimental or antagonistic to the former. Two examples of control by infection court protection will be dealt with first.

The pathogen of certain forestry trees, Heterobasidion annosus has been effectively and economically controlled by use of the saprophytic fungus, Peniophora gigantea (Rishbeth, 1963). H.annosus accumulates inoculum potential for infection of living trees by initially colonizing and overgrowing tree strumps. It was found that if a tree stump was inoculated with 10^4 oidia of P.gigantea, immediately

after felling, then the stump was rapidly decomposed and H.annosus was unable to penetrate the already colonized tissue. Furthermore the oidial suspensions are easy and cheap to produce (Rishbeth, 1963). Hence this is a very simple and successful example of biological control.

Disease of tomatoes caused by Fusarium oxysporum f. sp. lycopersici is controllable by F.oxysporum f. sp. pisi (Langton, 1968). This depends on the presence of live F.oxysporum f. sp. pisi cells on the roots and lower stem of the tomato plants, which implies that they prevent invasion of the host by the F.oxysporum f. sp. lycopersici. This example has not been taken beyond this initial descriptive stage.

There are many examples of antagonism being potentially effective in biological control, although in most cases the mechanism by which control is exerted has not been precisely identified. The identification and introduction of antagonists of soil-borne plant pathogens has been reviewed by Kommedahl and Windels (1981), Papavizas and Lewis (1981) and Brown and Beringer (1983). Hence only a selection of antagonists will be discussed here, with the emphasis on soil-borne plant pathogens, but also drawing on other examples.

Apple canker, caused by Nectria galligena can be controlled by antibiotic producing strains of Bacillus subtilis (Swinburne, 1973; Swinburne et al., 1975). N.galligena infects apple trees through leaf scar

tissue and it is in the infection court that the antagonistic strains of B.subtilis have been found. Hence this interaction involves both infection court occupation and antagonism. Control of apple canker has been achieved from leaf fall to April of the next season by artificial inoculation of the leaf scar tissue with the antagonistic strains of B.subtilis (Swinburne, 1973).

A successful and well characterised method of control has been developed for Agrobacterium radiobacter var. tumefaciens, the cause of crown gall in stone fruit trees. The non-pathogen A.radiobacter var. radiobacter produces a bacteriocin, known as Agrocin 84, which is active against many strains of A.radiobacter var. tumefaciens (Kerr and Htay, 1974). This observation has been developed and is now in widespread commercial use in Australia (Kerr and Panagopoulous, 1977).

Bacterial antagonism has proved to be potentially effective against a variety of soil-borne fungal plant pathogens. For instance Bacillus subtilis is antagonistic to Fusarium roseum f. sp. dianthi (Aldrich and Baker, 1970). Merriman et al. (1974a; 1974b) found that certain strains of B. subtilis and Streptomyces griseus when used as seed treatments of cereals and carrots were antagonistic to the root pathogen. Rhizoctonia solani, giving increased yields

over plants from untreated seed. This effect presumably is non-specific with respect to the pathogen, but would appear to be a simple method of reducing root disease of crop plants.

Similarly a number of soil-inhabiting saprophytic fungi are antagonistic to soil-borne plant pathogens. Conidia of Trichoderma spp. have been used, as a seed treatment, to control damping-off of pea and radish, caused by Pythium spp. and R.solani. In some cases this type of treatment appears to give better protection than fungicide treatment (Locke et al., 1979; Harman et al., 1981). Almost identical results have been obtained by Hoch and Abawi (1979) and Odvody et al. (1980) for control of Pythium spp. and R.solani damping-off diseases, by use of selected isolates of the soil-inhabiting fungus Corticium sp. These observations have been extended by Al-Hamdani and Cooke (1983) and Al-Hamdani et al. (1983) who have not only shown that these damping-off diseases can also be controlled by seed treatment with the non-pathogen Pythium oligandrum; but that P.oligandrum is effective by forming a protective sheath around the seedling, and through its mycoparasitic life-style is inhibitory to various soil-inhabiting plant pathogenic fungi. Furthermore, Al-Hamdani et al. (1983) report that P.oligandrum is capable of survival in soil up to 18 months in the form of oospores. This suggests that once a crop that was seed-treated with P.oligandrum has

been grown in the soil, then there will be residual inoculum of the control agent present for the next crop.

c. Viral infection.

Control of insect pests of crops has in the past relied heavily on the use of insecticides; however recently the use of Baculoviruses, natural insect pathogens, has become a realistic method of insect pest control (Benz, 1981). Cell culture has allowed the study of these viruses and variants, that may be of use commercially (Vaughn and Dougherty, 1981). As yet economic mass production of a stable, virulent virus that can be easily applied and which would be fast-acting, has not been achieved (Benz, 1981); but if this were possible, it would yield a powerful method of pest control, one that would have to be investigated and used sensibly, so as not to upset the ecosystem; but presumably it would be a control system preferable to the widespread use of insecticides.

The successful biological control of Endothia parasitica, the cause of chestnut blight by infection with ds RNA viruses, (or associated elements), has been discussed in section 1.2.3.

The biological control of plant pathogens has many potential advantages over conventional methods of control, and although the study of this subject does not reveal many examples that have received commercial

acceptance, there are a number of potential control techniques that deserve further study and practical development. As described in section 1.1.4, the soil-borne pathogen, Ggt is unaffected by existing disease control measures. Hence biological control of take-all has many attractions, either on its own or as part of an integrated control programme.

1.4 BACKGROUND AND PERSPECTIVE OF THE PROJECT

The disease take-all of wheat is of serious economic consequence and is not controllable by conventional methods other than rotation. A potential means of control has been identified in the viral infection of the fungus Ggt, (the cause of take-all) by Lapierre et al. (1970). At first sight this claim appears ambitious, as demonstrated by Rawlinson et al. (1973). However, as both Rawlinson (1978) and Rawlinson and Buck (1981) have indicated, more emphasis needs to be placed on the study of particular viruses or virus variants amongst the large and diverse population of Ggt viruses, to assess the potential of controlling take-all through virus infection. As a consequence of this, a series of studies have elucidated much of the biology and biochemistry of the viruses of Ggt (Almond, Buck and Rawlinson, 1977; 1978; Buck et al., 1981a; 1981b; Romanos, Buck and Rawlinson, 1981; McGinty, Buck and Rawlinson, 1981; McFadden, Buck and Rawlinson, 1983; Jamil and Buck, 1984; Jamil, Buck and Carlile, 1984).

In order to assess the effect of the range of different viruses and their variants found in Ggt in relation to the pathogenicity of the fungus it was decided to examine a large population of field isolates, recently isolated from seedlings grown in naturally infested soil of known cropping history, (both decline status and non-decline status soil).

These isolates were to be analysed to assess their pathogenicity, (using a seedling test), and to determine their ds RNA content, i.e. both quantity, (using spectrophotometry), and ds RNA species distributions, (by means of polyacrylamide gel electrophoresis). If a group of ds RNA species are responsible for reduced pathogenicity in Ggt, perhaps qualitatively or quantitatively, (or perhaps it is quantitatively related to all ds RNA found in Ggt), then such a relationship should be indicated by a survey of sufficient size.

More definitive results were to be obtained via two different approaches. The results of McFadden, Buck and Rawlinson (1983), indicating that genetically defined virus-containing isolates could be obtained via isolation of the uninucleate, germinable conidia, (described by Deacon, 1976a); and that these virus-containing isolates can be cured of infection by isolation of single ascospores, provides a method of producing isogenic strains of an isolate of Ggt, with and without virus infection. The second approach was to infect protoplasts from isolates that had been cured from infection or that were naturally ds RNA-free with purified virus preparations from isolates that were believed to be weakly pathogenic due to ds RNA infection. By selecting isolates from the field isolate survey, for the first approach, that were both

weakly pathogenic and ds RNA-containing, cured isolates would be obtained that would be pathogenicity tested. These cured isolates, as well as ds RNA-free, highly pathogenic isolates, (selected from the field isolate survey), would form the basis for the protoplast infection studies, which would be followed by pathogenicity assessment of any newly infected isolates.

Hence direct evidence of the effect of particular ds RNA species on the pathogenicity of certain isolates of Ggt would be obtained. This would determine the potential of ds RNA infected isolates of Ggt as a biological control agent of take-all of wheat, and would also possibly provide a system to study the molecular control of pathogenicity in Ggt.

2. METHODS

2.1 Safety precautions.

Standard safety precautions such as the wearing of a laboratory coat were observed at all times. Gloves, face masks and safety glasses were used in especially hazardous situations as needed. Radioactive manipulations were performed with a perspex shield as added protection. Disposal of radioactive waste was in accordance with Imperial College Safety Regulations and disposal of any cultures or soil contaminated with take-all or any other microorganisms was preceded by autoclaving at 15 psi for 30 minutes.

When working with nucleic acids, especially purified ds RNA, gloves were worn and only autoclaved equipment, (such as eppendorfs and micropipette tips), were used, in an attempt to reduce the risk of nuclease contamination.

2.2 Companies and Suppliers of Reagents and Materials.

The following companies were either regular suppliers or provided an important product for the project:

Alpha Laboratories, 40 Parham Drive, Eastleigh, Hampshire, SO5 4NU.

Amersham International Plc, White Lion Road, Amersham, Buckinghamshire, HP7 9LL.

BDH Chemicals Ltd, Freshwater Road, Dagenham, Essex, RM8 1RZ.

Biorad Laboratories Ltd, Greenhill Crescent, Holywell Industrial Estate, Watford, Herts, WD1 8RP.

Difco Laboratories, P.O. Box 14B, Central Avenue, West Molesey, Surrey, KT8 0SE.

Millipore (U.K.) Ltd, Millipore House, 11-15 Peterborough Road, Harrow, Middlesex, HA1 2YH.

New England Nuclear, 2 New Road, Southampton, SO2 0AA (Ordering telephone number: 06103 803113/4).

Novo Enzyme Products, Windsor, Berkshire.

Oxoid Ltd, Wade Road, Basingstoke, Hampshire, RG24 0PW.

Rose Chemicals Ltd, 83 Darenth Road, London, N16 6EB.

Sigma Chemical Co. Ltd, Fancy Road, Poole, Dorset, BH17 7NH.

Sturge, J & E, Ltd, Selby, Yorkshire.

Whatman Labsales, Springfield Mill, Maidstone, Kent, ME14 2LE.

Worthington Diagnostic Systems Inc., supplied by Flow Labs Ltd, P.O. Box 17, Second Avenue, Industrial Estate, Irvine, Scotland, KA12 8NB.

The majority of chemicals used were supplied by BDH Chemicals Ltd and were of analytical reagent grade, or the equivalent, unless otherwise stated.

2.3 Buffers.

The following buffers were of common use:

GPS: 0.2M Glycine, 0.1M Na₂HPO₄, 0.6 NaCl; adjusted to pH 9.5 with HCl.

PK: 0.03M Na H₂ PO₄, 0.15M KCl; adjusted to pH 7.6

with HCl.

SSC: 0.15M NaCl, 0.015M trisodium citrate; adjusted to pH 7.0 with NaOH.

STE: 0.1M NaCl, 0.05M Tris, 0.001M EDTA; adjusted to pH 7.0 with HCl.

TAE: 40mM Tris, 20mM sodium acetate, 1.6mM EDTA; adjusted to pH 8.0 with acetic acid.

TBE: .09M Tris, 0.09M boric acid, 2.5mM EDTA; adjusted to pH 8.2 with NaOH.

Many of these buffers were made up in concentrated form e.g. 10 x and others were used as concentrations other than 1 x, e.g. 1/20th TAE.

Other buffers used for certain purposes will be described in relation to the method in question.

2.4 Media and Cultural Conditions.

All isolates used were grown on Potato Dextrose Agar (PDA; Oxoid or Difco), using both slopes (12 to 15 ml volume) and Petri dishes. To produce sufficient mycelium isolates were cultured at $22^{\circ}\text{C} \pm 2^{\circ}\text{C}$ for up to 14 days. A stock collection of isolates, (grown on PDA slopes), was maintained at 4°C . Subculturing was kept to a minimum, the whole collection being re-cultured only every twelve months. However for the experimental work it was obviously necessary to subculture more frequently those isolates that were of interest, but once again this was kept to a minimum.

When liquid-grown mycelium was required, isolates were cultured in 100 or 200 ml of glucose-peptone medium or GPM, (2% w/v glucose, 0.5 w/v peptone, 0.25% w.v yeast extract; adjusted to pH 5.8 $\pm$ 0.2) on a rotary shaker set at 140 rpm and 22°C $\pm$ 2°C for up to 14 days unless otherwise stated.

All media, glassware and equipment were sterilized by autoclaving at 15 psi for 20 minutes. All further manipulations such as pouring plates or inoculating media were performed in aseptic conditions using a laminar airflow cabinet.

2.5 Isolate Code Numbers and Their Sources.

A number of isolates of Ggt that have been in culture for some years and the derivatives of one of these isolates have been maintained and used in various experiments. They are as follows:

<u>Isolate</u>	<u>Source</u> (and year of isolation)
3bla	M. Chu-Chou; isolated from third year wheat, Little Knot, Rothamsted, England (1970).
3bla conD	J.J.P. McFadden; single conidial isolate of 3bla (1979).
3bla conDA1, A2 & A3	J.J.P. McFadden, single ascospore isolates of 3bla conD (1980).
3bla ConE*	J.J.P. McFadden, single

	conidial isolate of 3bla (1979).
3bla conEAl*, A2 & A3	J.J.P. McFadden; single ascospore isolates of 3bla ConE (1980).
F911-1*	J.M. Lemaire; single ascospore isolate of isolate from twelfth year wheat from Rennes, France (1969).
F6	C.J. Rawlinson; isolated from second year wheat from Lerheu, near Rennes, France (1974).

*These isolates are also now maintained at the Commonwealth Mycological Institute with the accession numbers; 3bla conE (IMI 286824), 3bla conEAl (IMI 286825) and F911-1 (IMI 286823).

In addition to these isolates an array of isolates was selected from bait wheat seedlings grown in plot soils of known cropping histories. The bait wheat seedlings were kindly donated by Mr D.B. Slope of Rothamsted Experimental Research Station, along with the relevant details of the previous cropping of the soil and the take-all decline status of the soil.

This array of 'field isolates' may be summarized as follows:

<u>Plot</u>	<u>Number of isolates</u>	<u>Identity code range</u>	<u>Sampling Date</u>
02	17	02S2 to 02S19	Autumn 1981
03	15	03S2 to 03S20a	"
06	14	06S2 to 06S18	"
07	18	07S1 to 07S20	"
14	18	14S3 α to 14S19	"
15	21	15S2 to 15S20	"
WW	11	WWR2; WWS2a to WWS12	Spring 1982
0601	22	0601/1 to 0601/22	Autumn 1982
0881	21	0881/1 to 0881/21	"

N.B. 1. Some isolate identity codes have the suffix a or b. This indicates that they arose from the same root fragment as the isolate of the same number, (but the alternate suffix), while appearing distinct.

2. Some isolate identity codes carry the suffix α , β or γ . This indicates that the original isolate, designated α , gave rise to one or two sectors, designated β and γ .

Plots 02 to 15 had been cropped as follows:

Plots 02, 03: spring wheat in 1977

Plots 06, 07: spring oats in 1977

Plots 14, 15: ryegrass in 1977

Following this, all the plots were used to grow winter wheat from 1978 to 1981.

Plot WW was fallow for a couple of years followed by two barley crops and finally one winter wheat crop

in 1981.

Plots 0601 and 0881 had the following cropping history:

1968-1975: winter wheat

1976: fallow

1977-1978: spring barley

1979-1982: winter wheat

With regard to take-all decline status, plots 02, 03, 0601 and 0881 were exhibiting decline at time of sampling; while plots 06 and 07 had just shown peak disease levels and plots 14 and 15 had apparently yet to reach peak take-all incidence and severity. Plot WW was almost certainly not showing decline status, however this was not confirmed.

All the details of the cropping histories and take-all status of the plots were kindly supplied by Mr D.B. Slope.

2.6 Isolation of Ggt from Wheat Roots.

Asher (1978a) achieved isolation of Ggt from wheat roots that had been artificially inoculated and grown in sterile soil by two different methods. In the first, the root fragments were surface-sterilized, and in the second the root fragments were sterilized by repeated washing in sterile distilled water. Both of these methods were used, in essence, on wheat root fragments of bait seedlings that had been kindly donated by Mr D.B. Slope.

For isolation using surface sterilization, root fragments approximately 10 mm long were selected that were showing vascular lesioning. These fragments were sterilized by immersion in 1% silver nitrate for 90 seconds, followed by immersion in 1% sterile sodium chloride for 90 seconds and finally were washed in sterile distilled water; before plating onto PDA containing 100 units/ml streptomycin.

For isolation by root-washing; root fragments were randomly selected and separated into batches of about 20 in number. Each fragment was about 20 mm in length and between 3 to 5 batches were processed as follows per experiment. Each batch was washed fifteen times with about 100 ml of sterile distilled water for at least 5 minutes each and with five changes of sterile flask. After the washing was complete the root fragments were transferred to a sterile mortar and ground in 50 to 100 ml sterile distilled water until their average length was 1 to 2 mm. Of this suspension, 0.5 ml was mixed in a Petri dish with 20 ml of cooled (48°C) PDA plus 100 units/ml streptomycin. For each batch, ten such cultures were set up.

In one experiment, in order to investigate the effect of selecting or not selecting the root fragments for vascular lesions initially, both of these isolation techniques were used on two separate populations; one that had been selected on the presence of lesions, and

the other being a random selection of root fragments.

Fungal colonies developing from either isolation technique were assessed on their growth rate and morphology, (e.g. hyaline hyphae, later followed by dark macrohyphae and back-curving hyphae at the colony edge), to initially identify isolates of Ggt.

2.7 Assessment of Pathogenicity of Ggt Isolates.

Four methods were used to assess the pathogenicity rating of Ggt isolates and these are described as follows:

a) Shoot dry weight assessment.

This test was basically as described by Blanch (1977) and Asher (1980). Inoculum for the test was prepared by inoculating PDA-filled Petri dishes and incubating them for about ten days at 22°C. Discs of 18 mm diameter were cut from the growing edge of the colony and placed on sterile Levington's potting compost used to four fifths fill three inch square seedling pots. For each pot one disc was centrally positioned and for each isolate there were five such replicate pots. Four surface-sterilized and pre-germinated wheat seeds, (c.v. Flanders) were placed on the surface of the potting compost with their radicles orientated towards the centre of the inoculum disc. (The seeds were surface-sterilized in an identical manner to that described in section 2.6 and germinated by placing on moist filter paper for 30 to 40 hours).

Further potting compost was laid over the seeds and the pots were placed in a growth cabinet in a random arrangement. The growth cabinet was maintained at $18^{\circ}\text{C} \pm 1^{\circ}\text{C}$ and an average photosynthetic light energy level of 110 microeinsteins was supplied for 16 hours per day. The seedlings were watered three times a week, starting with day one, so that the soil was kept moist. After 19 days growth the seedlings were severed at soil level, dried at 80°C for 48 hours and weighed. The average shoot dry was multiplied by a factor of 100 for convenience, converted to natural logarithms, (to compensate for the exponential growth rate of seedlings), and then subtracted from the shoot dry weight value of the control plants in each particular test. Hence the larger the value the more pathogenic is the isolate concerned. To measure the accuracy of this test various retests and duplications within tests were performed.

b) Seedling disease index.

This method was adapted from Cook and Naiki (1982) and requires a visual assessment of the condition of the roots and shoots of the seedling following a period of pathogen challenge.

Inoculum discs (6 mm diameter) were cut from the growing edge of isolates cultured on 1/5 strength PDA. Per isolate, two three inch square pots were filled to a depth of 2 to 2.5 inches with sterile vermiculite, onto which surface were placed two inoculum discs per

pot. After a further 1/4 inch layer of vermiculite two surface-sterilized and pre-germinated wheat seeds (c.v Flanders) were placed above the inoculum discs and then covered with further vermiculite. The seedlings were given the same growth conditions as stated in 2.7 a) except that the average temperature was $15^{\circ}\text{C} \pm 1^{\circ}\text{C}$ and, instead of being watered only with tap water, for one of the three weekly waterings 1/5 strength Long Ashtons's nutrient solution was used, (see end of section). After 5 weeks' growth the seedlings were removed, washed and scored for disease as follows:

- 0 = no disease.
- 1 = one or two seminal roots (less than half) with one or more lesions.
- 2 = three, four, or all (more than half) of the roots with one or more lesions each.
- 3 = all roots with one or more lesions and lesions also on coleoptile.
- 4 = lesions uniformly distributed 1-2 cm up the the coleoptile and most extending through the coleoptile into the stem.
- 5 = stem as well as all roots uniformly covered with lesions, seedling dead or nearly so.

Generally all four seedlings subjected to a particular Ggt isolate gave ratings in close agreement. Hence the mean rating gives a reasonable indication. As with the shoot dry weight assessment various

isolates were retested to assess reproducibility.

Long Ashtons's nutrient solution is made up using six stock solutions which are of the following concentrations and diluted as described:

1. $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$	55.0 g/200 ml
2. KNO_3	50.5 g/200 ml
3. $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$	20.0 g/ 200 ml
4. $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	36.0 g/200 ml
5. $\text{FeNa}_2(\text{EDTA})_2$ (adjusted to pH 5.6 with NaOH)	1.22 g/200 ml
6. $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$	0.223 g
$\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$	0.024 g
$\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$	0.029 g in 200 ml
$(\text{NH}_4)_6\text{NO}_7 \cdot 4\text{H}_2\text{O}$	0.035 g
H_3BO_3	0.186 g

For full strength Long Ashtons's solution, take 10 ml of solution 1 and 5 ml of solutions 2 to 6 and dilute to 2.5 litres with distilled water.

c) Seeding/Agar plate disease index.

Wheat seeds (c.v. Flanders) were surface-sterilized in a manner similar to that described in section 2.6 but with an initial 10 minute immersion in 1% silver nitrate. They were allowed to germinate on PDA for 48 hours at 20-22°C and then transferred singly to sterile tap water agar-filled Petri dishes. Each Petri dish was inoculated, close to the seminal roots

of the seedling, with a 6 mm agar disc of inoculum of an isolate of Ggt to be assessed for its pathogenicity. After 5 weeks' growth at 20-22°C the seedlings were scored for disease using the following index:

Mild (1.0); <20% of roots lesioned (one to two lesions only), little stem base discolouration. Shoots healthy.

Fairly Severe (2.0); 20 to 50% of roots lesioned (one to two lesions only), some stem base discolouration. Shoots appear chlorotic.

Severe (3.0); 50 to 75% of roots lesioned; considerable stem base discolouration. Shoots unhealthy.

Very Severe (4.0); >75% of roots heavily lesioned. Shoots dead or nearly so.

d) Mature plant assessment.

In early November 1983 a batch of surface-sterilized wheat seeds (c.v. Flanders) were planted in sterile potting compost, (Levingtons'), and given favourable growth conditions for two weeks. Following this they were moved to the outside environment and left to overwinter. On the 14th March 1984 the seedlings were repotted singly into 5 inch square pots filled with unsterile soil (1:1 mix of Levingtons' and John Innes potting compost). Prior to placing the seedling into the larger pot an inoculum disc (18 mm diameter) was centrally placed on the unsterile soil, so that it and the seedling roots (in their original soil) would be in contact. For each isolate of Ggt

tested there were triplicate pots.

At approximately fortnightly intervals the plants were watered with Long Ashtons' nutrient solution (as described in 2.7 b)) and sprayed with 0.1% Calixin (a general fungicide use to prevent mildew and rust infection). As the weather became drier it became necessary to water the plants more frequently. By the 9th July 1984 the control plants (uninoculated) appeared to be mature and hence at this point the plants were assessed.

The method of assessment was largely based on two keys, including the key used by the Ministry of Agriculture, Fisheries and Food (Clarkson and Polley, 1981) and the key devised by Chambers (1971). The former deals with the degree of root lesioning, while the latter takes into account root length and number, plant height and biomass, and head development and yield.

Five characters were taken into account and averaged across the three plants per isolate. (Generally all three plants from each isolate were very similar in condition). The five characters were; the number of healthy ears of grain, the number of rudimentary or 'whitehead' ears of grain, the percentage of roots with one or more lesions, the size of the root system and, the size of the shoot system. These were judged against the control plants and the

following equally-weighted indices were drawn up:

Number of healthy ears of grain (per 3 plants)	Number of rudimentary/ 'Whitehead' ears of grain (per 3 plants)

12 + 11 = 0

3 = 0

10 + 9 = 1

4 + 5 = 1

8 + 7 = 2

5 + 6 = 2

6 + 5 = 3

7 + 8 = 3

4 + 3 = 4

9 + 10 = 4

% Roots lesioned

Root system

0% = 0

Healthy = 0

<10% = 1

Largely healthy = 1

10-25% = 2

Slightly stunted = 2

25-50% = 3

Badly stunted = 3

50-75% = 4

Severely stunted = 4

>75% = 5

Shoot system

Normal = 0

Reduced Shoot height = 1

To produce a disease rating for an isolate the scores from each of these indices were added together, the higher the total being the more pathogenic the isolate.

e) Statistical assessment.

Where populations of figures were collected as results, and it was felt that these numerical populations should be analysed statistically, the

computer programme 'Minitab' (Ryan, Joiner and Ryan, 1981) was used via the mainframe computer of Imperial College to produce means and standard deviations of these populations; and also to test the differences between means of populations, allowing for their variances, (which were not assumed to be equal). This permitted the populations to be assessed to 95% confidence limits on the null hypothesis of one mean being equal to another, if this is not the case then the populations are presumed to be significantly different with a maximum of $p = 0.05$.

2.8 Passaging and Re-isolation of Ggt Isolates.

The isolates to be passaged were given the opportunity to infect wheat seedling roots using the same experimental design as described in section 2.7 a), except that only three pre-germinated seeds were placed in each pot. After six weeks' growth, (to allow establishment of infection), the seedling roots were harvested, washed and then surface-sterilized as in section 2.6. Root fragments exhibiting vascular lesions were selected and plated out on PDA and streptomycin (100 units/ml). Subsequently Ggt isolates were observed growing out from these root fragments and were subcultured thus producing a passaged isolate from the original isolate.

2.9 Production of Germinable Conidia and Isolation of Single Conidia from Isolates of Ggt.

These techniques were performed as described by Deacon (1976a) and McFadden (1982).

Conical flasks (250 ml) each received a bottom layer of 30 ml agar, composed of glucose: 20 g; asparagine: 2 g; KH_2PO_4 : 1 g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$: 0.5 g; $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$: 1 mg; $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$: 0.9 mg; $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$: 0.4 mg; thiamine: 100 μg ; biotin 10 μg ; agar (Difco): 15 g; distilled water: 1 l. The agar, (Deacon Conidial Agar or DCA), was made up and adjusted to pH 6.8 ± 0.1 (McFadden, 1982) before aliquoting into the conical flasks for autoclaving.

Each flask was centrally inoculated with a 6 mm plug of inoculum from a PDA Petri dish or an isolate to be induced to form conidia. Flasks were incubated on a sunlit window-sill (east-facing), at room temperature $20^\circ\text{C} \pm 5^\circ\text{C}$ for about 7 days until mycelium covered about two-thirds of the agar surface. The mycelium was now flooded with 70 ml of sterile distilled water and then returned to the window-sill. Samples of water were aseptically taken from the flask^s every day, following a short period of agitation of the flasks. Each sample was examined under the microscope for the presence of conidia. After 7 to 14 days most cultures, if they were going to produce conidia, were doing so abundantly. Furthermore these conidia were of the type described by Deacon (1976a) i.e. $6 \mu\text{m} \times 3 \mu\text{m}$,

elliptical and not strongly curved. The sooner the conidia were isolated, the more likely they were to germinate.

Prior to isolation the flask was shaken fairly vigorously for about five minutes and about 5 ml of spore suspension was extracted and then filtered through a sintered glass filter, porosity 3, (maximum pore diameter $25\mu\text{m} \pm 5\mu\text{m}$). This permitted the passage of the spores but not mycelial fragments, into the filtrate. About 0.5 ml of the filtrate was spread on 1% PDA and allowed to dry onto the agar in sterile air. If the spore suspension was dense it was often found to be helpful if the PDA had been poured into the lid of the Petri-dish; this allowed use of a greater surface area without a protruding vertical edge. The agar surface was examined using the x40 objective of a microscope fitted with a Meopta Monosporic Isolator, (type no. 5960, kindly loaned by Dr C.J. Rawlinson, Rothamsted Experimental Station). When a conidium was observed under the x40 objective to be suitable for isolation, i.e. it appeared to be healthy and sufficiently distant from any other conidium that it could be singly isolated, the objective was changed to the monosporic isolator, (a x10 objective with a protruding, tapering cylinder), which was used to cut a 1 mm cylinder around the conidium, once it had been centrally located. The isolator was once more replaced

by the x40 objective and the plug observed to check the conidium was intact and single. The 1 mm plug was then carefully removed using a fine glass needle and placed on PDA, (adjusted to pH7.0). Colonies were usually seen about 5 days later, though in some cases this could be as much as 10 to 12 days later.

2.10 Production of Perithecia, Asci and Ascospores from Ggt isolates.

Isolates that were to be investigated with regard to their ability to produce ascospores were inoculated onto crushed wheat agar. Cunningham (1981) has reviewed the use of this type of agar for the production of perithecia. The crushed wheat agar consisted of: 6 to 7% crushed wheat (Mature plants were used and ground into a fine powder), 0.2% yeast extract, 1.5% agar (Difco), (N. Jamil, personal communication).

Petri dishes containing about 20 ml of this agar were centrally inoculated with a 6 mm inoculum plug of the isolate to be investigated. The cultures were incubated in a controlled environment growth cabinet at 18 to 20°C with a 16/8 hour, light/dark cycle. After 3 to 4 weeks perithecia were seen to develop and become mature.

2.11 Growth Rate Assessment.

Growth rate assessments were made, (largely according to McFadden, 1982), on PDA filled Petri

dishes, (each filled with 25 ml), at $\text{pH} 5.6 \pm 0.1$, (the natural pH of Difco or Oxoid PDA), and $\text{pH} 7.0 \pm 0.1$. The latter pH was achieved by mixing equal volumes of twice-normal concentration PDA and citrate-phosphate buffer, pH 7.0 (Gomori, 1955). In order to prepare the citrate-phosphate buffer pH 7.0, 6.4 ml of 0.2M citric acid was titrated with 43.6 ml of $\text{Na}_2 \text{HPO}_4$ and diluted to 100 ml with distilled water.

Each Petri dish was centrally inoculated with a 6 mm plug of inoculum and incubated at $22\text{--}24^\circ\text{C}$. After 5 and 10 days growth the diameter of the colony was measured taking an average of two readings taken at right angles to each other. These readings were converted to give an average/day growth assessment; the first being for 5 days, the second for the whole 10 days.

2.12 Extraction and Purification of ds RNA from Isolates of Ggt.

The method was an adaptation of that described by Morris and Dodds (1979). Between 8 to 10 g of fungal mycelium was prepared by liquid culture in GPM for approximately 14 days, (i.e. one 500 ml conical flask containing 200 ml of GPM), and then harvested by filter-drying. (At this stage the weight of mycelium was assessed). The dry fungal tissue was frozen in liquid nitrogen until it was highly brittle and then ground into a very fine powder. (From microscope

examination it was estimated that this caused up to 90% cell breakage). The ground mycelium, still in the mortar, was brought up to 0° to 4°C, placed on ice and suspended in the following mixture of solutions: 10 ml of GPS buffer, 1 ml of 10% SDS, 10 ml of chloroform-isoamylalcohol (24:1), 10 ml of water-saturated phenol (containing 0.1% 8-hydroxiquinoline) and 0.1 ml of mercaptoethanol. (Later on in the work it was found that these volumes could be halved). This mixture was stirred occasionally to maintain it in an emulsion while being incubated at 4°C for 30 minutes. Following this the emulsion was centrifuged at (8,000 g for 20 minutes at 4°C, and the resultant aqueous phase containing the fungal nucleic acids, was retained.

The ds RNA was selectively purified from the crude supernatant using a slightly modified version of 'Method 2' of Morris and Dodds (1979). From the aqueous supernatant 4.25 ml was taken and mixed with 0.75 ml of absolute ethanol, hence adjusting it to 15% ethanol. Cellulose powder (Biorad Cellex N-1) was added to the 15% ethanol/supernatant mixture in the proportion of 0.0625 g cellulose per 5 ml of solution. This cellulose/15% ethanol/supernatant suspension was gently shaken occasionally and kept on ice for 10 minutes. Following this, a column was formed by pouring the suspension into a 5 ml syringe, (with a column support at the bottom), and by allowing the

supernatant to slowly run through. The column was washed with 10 ml of STE/15% ethanol to remove ss RNA and ds DNA. Remaining ds RNA was eluted in 2.5 ml of ethanol-free STE and ethanol precipitated using 2 volumes 96% ethanol, (at -20°C), 0.1 volume of 2.5M sodium acetate (pH5); followed by thorough mixing and storing overnight at -20°C. The precipitated ds RNA was spun down at 23,000 rpm in a Beckman SW28.1 rotor (72,000 g), or the equivalent, for 45 to 60 minutes. The supernatant was decanted and residual ethanol removed by vacuum. The precipitated ds RNA was re-dissolved in 1/20 TAE, normally using a volume of 250µl.

2.13 Extraction of Virus from Ggt Isolates.

Isolates were cultured for 10 to 14 days at 22°C in GPM in a shaking incubator to yield approximately 20 g of mycelium after harvesting by vacuum filtration, (i.e. two 500 ml concial flasks were required, each containing 200 ml of GPM). The mycelium was resuspended in 60 ml of ice-cold PK buffer and disrupted by shaking with 10 g of glass beads (0.45 to 0.5 mm diameter) at 4,000 rpm for 60 seconds in a Braun, model MSK, cell homogeniser. The homogenate was diluted with 140 ml of ice-cold PK buffer and stirred for 4 h at 4°C. The virus was isolated by two cycles of differential centrifugation, each cycle involving a low speed spin, (15,000 g for 30 mins), and a high

speed spin, (27,000 rpm in a Beckman SW28 rotor, i.e. 96,000 g for 3 h), followed by a brief microfuge spin, (approximately one minute in a Beckman model B microfuge), leaving a final volume of 1.5 ml in PK buffer. This virus suspension was purified by centrifugation through linear sucrose density gradients (10 to 45% w/w in PK buffer) at 40,000 rpm in a Beckman SW50.1 rotor (149,000 g) for 1 hour. The gradients were fractionated into 0.2 ml fractions and assessed by agarose gel electrophoresis (see section 2.20) for virus content. Fractions that did contain virus were diluted with two volumes of PK buffer, and the virus was repelleted by centrifugation at 45,000 rpm in a Beckman SW50.1 rotor (189,000^g) for 2 hours. The purified virus was resuspended in either 1 or 1.5 ml of PK buffer.

2.14 Extraction of ds RNA from Viral Preparations.

Virus particles were disrupted by addition of SDS to a final concentration of 1% and by heating to 60°C for 20 minutes. The ds RNA was extracted by two phenol extractions and three 'sevag' (chloroform: isoamylalcohol = 24:1) extractions, followed by ethanol precipitation, (as in section 2.12). If only a small volume was being dealt with, short microfuge spins were found to be sufficient to separate the liquid phases in the extractions, and for the ethanol precipitation, a microfuge spin of 15 minutes at 4°C was sufficient to

pellet the ds RNA.

2.15 Spectrophotometry.

Spectrophotometric measurements were made using a Beckman 35 model spectrophotometer. In buffers near pH7.0, it was assumed that a 1 mg/ml solution of ds RNA had an E_{260} reading of 20, and that a 1 mg/ml solution of virus particles had an E_{260} reading of 4 (Romanos, 1981). These are only approximations, but do give a reasonable assessment.

Samples were scanned from 310 nm to 220 nm, taking readings at 280, 260 and 230 nm. A reasonably pure sample should have an $E_{260}:E_{280}$ in excess of 2.0, along with an $E_{280}:E_{230} = 1$. If this was so, then the E_{260} reading was used to calculate the concentration of ds RNA present.

2.16 Polyacrylamide Gel Electrophoresis of ds RNA (PAGE).

Electrophoresis of ds RNA was carried out in 4% polyacrylamide vertical slab gels, (20 x 16.5 x 0.2 cm), (Dingman and Peacock, 1968; Shatkin, Sipe and Loh, 1969; Maniatis, Fritsch and Sambrook, 1982) comprising: 5 ml 40% acrylamide/0.4% N N'-methylenebisacrylamide, 2 ml 25 x TAE buffer, 0.5 ml 5% TEMED and 40 ml distilled water. This solution, after mixing, was degassed for 20 minutes and following this 2.5 ml of 1.5% ammonium persulphate was added to the solution. The gel was then poured, the slot-former

inserted, and left to set for between 5 and 24 hours. Up to 60 μ l of sample, including loading buffer was applied per slot and the gel was eletrophoresed for 20 to 22 hours at 60 V, (28MA approximately), or the equivalent, with 1 x TAE as the running buffer.

The loading buffer used, commonly named 'Reaction Stop' or 'RX' consists of 5 ml 10 x TAE, 2 ml 0.5M EDTA, 100 μ l 10% SDS, 2 g Ficoll 400 and 0.02 g Bromophenol Blue. One-tenth volume of RX was added to the sample volume prior to applying the sample to the gel slot.

2.17 Gel Staining and Photography.

Polyacrylamide gels were stained after running by immersion of the gel in 0.5 μ g/ml ethidium bromide in distilled water for twenty minutes at room temperature with occasional agitation. Ethidium bromide bound to nucleic acid was visualised on an ultraviolet transilluminator, (Chromato-Vue C-63). All gels (i.e. including those in sections 2.18 and 2.20) were photographed using an Exa 1 camera with two yellow filters and one U.V. filter fitted, on an aperture setting of f5.6, and using exposure times of 3 to 12 seconds. Both Ilford HP5 and Pan F film was used.

2.18 Denaturation of ds RNA and Electrophoresis of Denatured ds RNA.

Two methods of denaturation of ds RNA and

electrophoresis of denatured ds RNA were used and these will be described separately as follows:

a) Urea - SDS PAGE.

This was performed as described by Buck and Ratti (1977). Samples for electrophoresis were buffered to 0.25 x TAE and also made up to 0.1% SDS and 8M Urea (Aristar, BDH). They were then heated to $98^{\circ}\text{C} \pm 2^{\circ}\text{C}$ for 5 minutes before being cooled rapidly on ice. 4% polyacrylamide vertical slab gels were used for electrophoresis of the samples that were identical to those described in 2.16 except that instead of 40 ml of distilled water, 40 ml of 10M Urea/0.1% SDS was included in the gel mix prior to degassing. For electrophoresis (with a duration of 20 h at 60 V), the top running buffer was 1x TAE/0.1 SDS/8M Urea. Samples were loaded in 0.1 volume RX buffer. Prior to staining with ethidium bromide gels were washed for 1 to 2 hours, with at least four changes of distilled water to remove all traces of SDS from the gel, because it precipitates ethidium bromide. The RNA in the gel was observed using a U.V. transilluminator.

b) Glyoxalation and PAGE.

Samples of ds RNA were denatured by glyoxalation using a slightly different set of conditions to those reported by McMaster and Carmichael (1977). Samples were heated to 50°C for 1 hour with 1M purified glyoxal, 60% DMSO and 10mM sodium phosphate, pH7.0, (final concentrations). Reaction mixtures contained

15 μ l DMSO, 4 μ l 40% glyoxal, 1 μ l 250mM sodium phosphate, pH7.0 and 5 μ l/ds RNA in 0.05 x TAE. These were applied to the gel following the addition of 5 μ l of loading buffer (100mM sodium phosphate, pH7.0, 0.4% bromophenol blue and 50% glycerol). The gels used were 2.4% polyacrylamide/0.12% bisacrylamide slab gels (see 2.16) and contained 8M urea/0.1 SDS, (as in Urea-SDS PAGE). Gels were electrophoresed and washed as described for Urea-SDS PAGE and then stained with acridine orange (20 μ g/ml) in sodium phosphate (10mM), pH7.0 for 15 minutes at room temperature in the dark with agitation. The gel was destained in an enamel dish for about 2 hours using at least four changes of 10mM sodium phosphate pH7.0, with conditions the same as for staining. Visualisation of RNA was performed using a U.V. transilluminator, (the acridine orange complexes of ds RNA/DNA appear green in U.V. light, while those of ss RNA/DNA appear red).

2.19 Nuclease Digestions.

Nucleic acid extracts purified selectively for ds RNA by cellulose affinity chromatography, (see 2.12), were in certain cases digested with DNase or RNase and RNase at high salt concentration, prior to PAGE, to ascertain whether all the nucleic acid present was ds RNA.

Digestions were conducted using a universal buffer, (effective for RNase, DNase and S1 nuclease),

of 10mM sodium acetate and 5mM magnesium chloride, (operating concentrations), and digestions were incubated at 30°C for 20 minutes, (Morris and Dodds, 1979; DNase 1 RNase-free supplied by Worthington Diagnostic Systems Inc.), was used at a final concentration between 50 and 75µg/ml, while RNase A and T₁ (Sigma) was used at an operating concentration of 0.2µg/ml. (A stock solution of RNase was boiled to destroy any DNase contamination). Ralph (1969) reports that ds RNA can be distinguished from ss RNA by its resistance to RNase digestion at high salt concentrations. For this purpose the same RNase digestion conditions were used but at a concentration of 0.33M sodium chloride.

2.20 Agarose Gel Electrophoresis of Intact Virions.

Crude and purified virus preparations and fractions from sucrose density gradients were electrophoresed using agarose gels (Buck et al., 1981). These gels were generally 'half-size' in dimensions, (11.5 cm x 15.5 cm x 0.4 cm); although 'full sized' gels, (23 cm x 15.5 cm x 0.4 cm), divided into half by having two rows of slots, and sometimes 'mini-gels' (8.0 cm x 6.7 cm x 0.3 cm) were also used. Gel concentrations used ranged from 0.5% to 0.75% agarose. To prepare 75 ml of gel mix, (sufficient for a 'half gel'), of 0.5% agarose, 0.375 g of agarose (Sigma) was dissolved in 7.5 ml of 10x TBE and 67.5 ml of distilled

water by boiling. This solution was cooled to 60°C and adjusted to a concentration of 0.5µg/ml ethidium bromide. The gel mix was poured into a horizontal slab gel former, with a slot former positioned near one end of the gel. About one hour was allowed for the gel to set. Samples were applied to the gel, after dilution with 0.1 volume of RX buffer, and electrophoresed for 2 to 3 hours at 60 volts. The ds RNA in the gel was visualised on the U.V. transilluminator due to their fluorescence from bound ethidium bromide. Bands thus observed were confirmed as being nucleoprotein in nature by subsequent staining in Coomassie Blue, (0.25% Coomassie Blue stain, 45% methanol, 9% glacial acetic acid), for 1 hour. This was followed by destaining to 45% methanol and 9% glacial acetic acid. Bands were visualised by observing the gel on a light box. Viral bands stain with both ethidium bromide and Coomassie Blue since they are nucleoprotein in composition.

2.21 Polyacrylamide Gel Electrophoresis of Polypeptides.

This technique was adapted from Laemmli (1970) and Maizel (1971). These gels are divided into two sections, and as with other polyacrylamide gels systems described, they are set in vertical slabs (see 2.16). the lower gel, the separating gel, in this case was 8% acrylamide/0.2% bisacrylamide, while the upper gel, the

stacking gel, was 3% acrylamide/0.75% bisacrylamide. The separating gel mixture consisted of 8 ml acrylamide stock solution (40% acrylamide/1% bisacrylamide), 21.2 ml 0.75M tris-HCl, pH8.8, 0.4 ml 10% SDS, 0.4 ml 5% TEMED, 8.0 ml distilled water. This mixture was degassed for 20 minutes, mixed with 2.0 ml 1.5% ml ammonium persulphate and poured into the gel plates to give a gel depth of about 12 cm. During the time taken for the separating gel to set, (a minimum of one hour), it was overlaid with 0.1% SDS; this ensured a level top edge to the gel and prevents non-polymerization of the top of the gel through contact with air. The stacking gel mixture consisted of 1.2 ml acrylamide stock solution (40% acrylamide/1% bisacrylamide), 0.16 ml 5% TEMED and 13.68 ml 0.15 M tris-HCl, pH6.8. This mixture was also degassed for 20 minutes, then mixed with 0.8 ml 1.5% ammonium persulphate and poured on top of the separating gel, once the 0.1% SDS overlay had been poured off. The slot-former was inserted and the gel was left to set for 5 hours. The electrophoresed buffer comprised 0.02M tris, 0.192M glycine and 0.1% SDS, adjusted to pH8.3 with HCl. Prior to electrophoresis, samples were made up to 1% SDS, 1% 2-mercaptoethanol and 0.001 EDTA and then boiled for 3 minutes, followed by rapid cooling on ice. Samples were mixed with 0.1 volume of RX buffer, then applied to the gel and electrophoresed for 16 hours at 55

volts. At the completion of electrophoresis the gel was washed for one hour in four changes of distilled water, stained at room temperature in Coomassie Blue (0.25% Coomassie Blue, 45% methanol and 9% glacial acetic acid), with constant agitation, and then destained for 4 hours in 45% methanol and 9% glacial acetic acid. Polypeptide bands were observed by transilluminating the gel on a light box and, if required, photographed using an Exa 1 camera loaded with 35 mm film set to f11 with exposure times of 1/2, 1/30 and 1/60 of a second.

2.22 Electron Microscopy of Virus Particles.

Purified virus preparations were observed by transmission electron microscopy, using a Phillips 301 model, in the following manner. The volume of 5 μ l of a virus sample was applied to a formvar-coated grid and dried. The virus particles were stained by applying 5 μ l of methylamine tungstate to the grid, leaving the stain to dry for a few minutes. The virus particles were thus negatively stained. Grids were inserted into the electron microscope and once a field of view containing many particles was located a photograph was taken. Final magnification ranges from 66,000x to 84,000x. Taking this into account virus particle diameters were measured by comparison with a standard virus particle of known diameter, i.e. virus Tl-A (Buck et al., 1981a).

2.23 Isolation and Regeneration of Protoplasts of Ggt.

The strains 3bla, 3bla conD, 3bla conE and some of the ascospore progeny of the latter two were selected for isolating and regenerating protoplasts from Ggt mycelium, since they represent a collection of strains that are genetically related, (identically so, in the case of the single conidial isolates and their ascospore progeny), but are either virus-containing or completely virus-free (McFadden, Buck and Rawlinson, 1983).

It was decided to attempt protoplast isolation and regeneration largely according to the protocols for Penicillium spp. and Aspergillus spp. since Ggt is also a filamentous Ascomycete. Mycelium of Ggt was prepared by shaking in liquid culture at 24°C for seven days using the minimal medium of Pontecorvo et al. (1953):

sodium nitrate	6.0 g/l
magnesium sulphate .7H ₂ O	0.52 g/l
potassium chloride	0.52 g/l
iron (Fe ²⁺) sulphate .7H ₂ O	0.005 g/l
zinc sulphate 7H ₂ O	0.005 g/l
glucose	20.0 g/l
yeast extract	5.0 g/l
casamino acids	2.0 g/l

The medium was adjusted, if necessary to pH6.5 prior to autoclaving. The glucose was autoclaved

separately and added to the remainder of the medium as a sterile solution. The medium was supplemented with yeast extract and casamino acids as suggested by Peberdy and Kevei (1979). Growth of fungal mycelium in minimal medium is believed to give higher yields of protoplasts than mycelium grown in complete medium, (J.F. Peberdy, personal communication), although this was not investigated for Ggt.

The mycelium was harvested by vacuum filtration and approximately 5 g (wet weight) was resuspended in 25 ml of buffer/osmotic stabilizer; 0.2M phosphate, pH5.8 and 0.6M potassium chloride (Hamlyn et al., 1981) and centrifuged at 730 g for 5 minutes. The pellet was resuspended in 75 ml of buffer/stabilizer containing the lytic enzymes Novozym 234 (Novo Enzyme Products) and Cellulose CP (John and E. Sturge Ltd) both at a concentration of 5 mg/ml as suggested by Hamlyn et al., 1981. (N.B. This enzyme solution was sterilized by filtration). The mycelium was incubated in the lytic enzyme solution for 4 hours at 24°C while being agitated using an orbital shaker set up at 140 rpm. The released protoplasts were separated from the mycelial fragments by filtration; first through glass wool and secondly through a sintered glass filter, (maximum pore size $25\mu\text{m} \pm 5\mu\text{m}$), and were then collected by centrifugation, (550 g for 5 minutes). The protoplasts were washed twice in 30 to 40 ml of buffer/stabilizer and collected, (550 g for 5 minutes)

and finally resuspended in 3 to 5 ml of buffer/stabilizer (Hamlyn et al., 1981; Peberdy and Kevei, 1979).

A dilution series from 10^{-1} , to 10^{-6} was made and 0.1 ml aliquots from each dilution were mixed with molten complete medium, (at 48°C) of Pontecorvo et al. (1953) with certain supplements; salts and glucose (as in the liquid minimal growth medium of Pontecorvo et al., 1953), yeast extract 1 g/l, peptone 1 g/l, casamino acids 1 g/l, bovine serum albumin 1 g/l, biotin 1 mg/l, thiamine 1 mg/l, agar 15 g/l, adjusted to pH6.0 (Peberdy and Kevei, 1979). A set of 3 replicate Petri dishes was made for each step in the dilution series. Cultures were incubated at 20°C to 22°C; colonies became visible normally 5 to 7 days later.

In an attempt to improve regeneration frequencies 0.1 ml aliquots were spread on pre-set Petri dishes of the regeneration complete medium described above, and then cultured as previously described.

2.24 Inoculation of Ggt Protoplasts with Virus or ds RNA.

Various methods of infecting protoplasts with viruses and nucleic acids have been developed for other host phyla e.g. higher plants (Takebe, 1979). In the case of fungi, Lhoas (1971b) and Pallett (1976) have achieved infection through simply incubating purified

virus with isolated protoplasts. However, these infections did not appear to be stable or lasting (Pallett, 1976), and other workers could not repeat the results (e.g. Bozarth, 1975). The use of polyethylene glycol in association with fungal protoplasts has been shown to stimulate high-frequency protoplast fusion (Ferenczy et al., 1975a; 1975b; 1976), and this has been utilized by Ghabrial (1980) and Ghabrial and Menaugh (1983) to achieve infection of fungal protoplasts; although with only a very low success rate.

Protoplasts of Ggt were inoculated following a protocol adapted from those of Cassells and Barlass (1978) and Maule et al. (1980), both of which were used for virus and virus RNA infection of higher plant protoplasts. To 0.2 ml of protoplast suspension (ca. 5×10^7 protoplasts) in buffer/stabilizer (see 2.25), were added 2 ml of 40% w/v polyethylene glycol 6,000 containing 3mM CaCl_2 and 0.5 ml of filter-sterilized virus suspension (130-180 $\mu\text{g/ml}$), or 0.5 ml of filter-sterilized ds RNA (extracted from virus, 20 $\mu\text{g/ml}$; see 2.14). After 10 to 20 seconds, 20 ml of buffer/stabilizer was added and the mixture was incubated for 1 hour at 20 to 22°C with occasional gentle agitation. Following this a dilution series (10^{-1} to 10^{-6}) was created from the inoculation suspension and protoplasts were regenerated according

to the protocol described in the previous section.

2.25 Assessment of Regenerated Protoplasts for Infection.

After 7 to 10 days growth the regenerating colonies were on average 3 mm in diameter. Colonies that were at least 1 cm distant from all other colonies were presumed to have arisen from single protoplasts and were picked off and shake cultured in 200 ml of GPM at 22 to 24°C. Most of the mycelium produced thus was extracted for ds RNA (section 2.12) and analysed by PAGE (section 2.16) and spectrophotometry (section 2.15). The remaining mycelium (ca. 10 mg) was used to inoculate a PDA slope. If a regenerated colony was demonstrated to contain ds RNA then a further culture was grown up in GPM and extracted for virus (section 2.13). The resultant virus preparation was examined by electron microscopy (section 2.23) and by PAGE for polypeptides (section 2.22). If a culture from a regenerated protoplast was shown to be virally-infected then it was put through a further two cycles of growth in GPM and extraction and analysis for ds RNA content, to examine the level and stability of the infection.

3. RESULTS

3.1 ANALYSIS OF THE FIELD ISOLATES OF Ggt.

A collection of field isolates of Ggt was obtained from infected wheat roots. These isolates were examined in terms of their pathogenicity ratings, (in three separate tests), and ds RNA content. The ds RNA species distribution in each isolate was assessed by PAGE and the quantity of ds RNA in each isolate was estimated by spectrophotometry, (and confirmed by PAGE). Nuclease digestions were carried out to confirm that the nucleic acid in the 'cellulose-purified' preparations (Morris and Dodds, 1979) was ds RNA. Denaturing gel electrophoresis systems were used to verify the molecular weights of the ds RNA species, estimated from non-denaturing PAGE. Certain isolates were also examined for their viral content, and for the ds RNA species that could be extracted from virus preparations.

The results of the separate pathogenicity tests were examined in conjunction with the results of the ds RNA analysis of the respective field isolates of Ggt. The intention of this combined examination was the determination of any relationship between pathogenicity and ds RNA content, (both qualitative and quantitative), in Ggt.

3.1.1 Isolation of Ggt From Infected Wheat Roots.

In total 157 isolates of Ggt were obtained from

nine field plots of varying cropping history, (see section 2.5 and Table 4), using bait seedling plants. To maintain the identity of, and possible effect of, the plot, (i.e. its experimental history), isolations were made in batches from each separate plot. Growth of potential Ggt isolates was generally observed to begin from the tips of the root fragments, 3 to 5 days from the start of culturing. Colonies arising thus were observed for characteristic Ggt morphology. If an isolate appeared to sector readily then subcultures were taken from these sectors.

All of the 157 isolates recorded in Table 4 were obtained using the surface sterilization technique, (described in section 2.6). A success rate of between 55 and 80% was observed for isolations from root fragments selected for vascular lesions. The ratio of isolation success rate from randomly selected root fragments and root fragments selected for vascular lesions was 1:10.

The root washing technique (section 2.6) was not successful. When the method was applied as described, then no growth of any description was observed. If the roots were washed less stringently, then they were not sufficiently sterile in their outer layers, and various saprophytes were observed, e.g. Fusarium spp., Penicillium spp., and a variety of bacteria. Certain colonies did appear to be Ggt, however they were too

Table 4. Isolates of Ggt obtained and examined for pathogenicity and ds RNA content.

<u>Plot number</u> ¹	<u>Number of isolates</u>		
	Original	Sectors	Total
02	13	4	17
03	15	0	15
06	14	0	14
07	18	0	18
14	13	5	18
15	18	3	21
WW	11	0	11
0601	22	0 ²	22
0881	21	0 ²	21
TOTAL	145	12	157

¹ These relate to the plots of agricultural soil in which the bait wheat seedlings were grown. The plot numbers identify varying cropping histories used in experiments performed at Rothamsted Experimental Station by Mr D.B. Slope, while investigating take-all decline. (See section 2.5 for details).

² No attempt to isolate sectors made.

contaminated to isolate into axenic culture. It is perhaps possible that if a greater range of antibiotics had been included in the PDA, (only streptomycin was in fact used), then many of the bacterial contaminants could have been eliminated. In order to separate the potential Ggt colonies from other fungi present, infection of further bait wheat seedlings, followed by isolation through surface sterilization, might^{have} proved successful.

3.1.2 Examination of Pathogenicity of the Field Isolates of Ggt.

All 157 field isolates were tested using the shoot dry weight assessment technique (section 2.7a) to determine their pathogenicities. A selection of isolates was re-tested, using the same technique to assess fluctuation in pathogenicity levels and variation between tests.

When twenty four sets of seedlings, (i.e. 5 pots per set, each pot containing four seedlings), were grown without inoculation with Ggt, a range of shoot dry weight values was observed. These values, (after multiplication by 100 and conversion to natural logarithms; Asher, 1980), ranged from 2.25 to 2.00 with a mean of 2.12 and a standard deviation of 0.07. Hence it was decided that all shoot dry weight values from inoculated sets of seedlings should be subtracted from the control value, (i.e. uninoculated), in each

particular test. Therefore all subsequent shoot dry weight values are 'control-adjusted', and hence the greater the value is, the higher the pathogenicity rating of a particular isolate.

The pathogenicity ratings of all of the field isolates are recorded in Appendix I. The variation in pathogenicity levels observed is represented in table 5. These results are presented according to the three separate seedling shoot dry weight pathogenicity tests conducted to assess pathogenicity in the population of Ggt field isolates. The results from the three tests have not been integrated since both Blanch (1977) and Asher (1980) have noted that comparisons between tests of this kind are not valid since, although relative pathogenicities are generally maintained, absolute values of pathogenicities vary significantly between tests. It is interesting to note that some of the Ggt isolates caused no decrease in seedling shoot dry weight, (compared with the uninoculated seedlings), during the 19 day experimental period, while the majority of isolates caused shoot dry weight loss to varying degrees, the maximum reduction caused being in the region of 30% of healthy shoot dry weight. The isolates in Test 1 show a normal distribution, perhaps slightly skewed towards low pathogenicity; this distribution is also borne out when the isolates in the table are subdivided into plots. The pathogenicities of the isolates in Tests 2 and 3 are, however, notably

more skewed towards low values, especially in the case of Test 3. When the results of Tests 2 and 3 are subdivided into plots then mostly the same pattern is observed, (i.e. skewness towards low pathogenicity), however plots 15 and 0881 demonstrate pathogenicity distributions of a more balanced nature. It is interesting that in Test 2 there appear to be two separate peaks to the pathogenicity distribution. This is the result of the different pattern observed in plot 15 compared with plots 07, 14 and WW; and also to a lesser extent, the dual distribution peak seen in plot 14. It is noteworthy, however, that the number of isolates per plot is relatively small and the observed distribution may not hold up if larger numbers were tested.

The differences in distributions of pathogenicities observed plot by plot do not directly relate to the decline status of the respective plots. Plots 02, 03 and 0881 were in decline at the time of isolation but yielded isolate populations with normal pathogenicity distributions, as did the non-decline plots 06 and 15. The non-decline plots WW and 14, on the other hand yielded isolate populations that are skewed towards low pathogenicity, along with the decline plot 0601. Plot 07, (a non-decline plot), is difficult to analyse, (largely because it is split between tests); however its isolate population would

TABLE 5 Analysis of Pathogenicity of Ggt Field Isolates.

(a) Test 1

<u>Pathogenicity class</u>	<u>Isolate source</u>				<u>Total</u>
	<u>02</u>	<u>03</u>	<u>06</u>	<u>07 (part)</u>	
0.00 - 0.09	2	0	1	0	3
0.10 - 0.19	6	3	3	0	12
0.20 - 0.29	3	3	4	5	15
0.30 - 0.39	5	3	5	3	16
0.40 - 0.49	0	4	1	1	6
0.50 - 0.59	1	2	0	0	3
0.60 - 0.69	0	0	0	0	0

(b) Test 2

<u>Pathogenicity class</u>	<u>Isolate source</u>				<u>Total</u>
	<u>07 (part)</u>	<u>14</u>	<u>15</u>	<u>WW (part)</u>	
0.00 - 0.09	5	7	0	1	13
0.10 - 0.19	3	2	3	1	9
0.20 - 0.29	1	0	2	2	5
0.30 - 0.39	0	2	2	0	4
0.40 - 0.49	0	3	6	0	9
0.50 - 0.59	0	2	5	0	7
0.60 - 0.69	0	2	2	0	4

(C) Test 3

<u>Pathogenicity</u> <u>class</u>	Isolate source			
	WW (part)	0601	0881	Total
0.00 - 0.09	7	15	3	25
0.10 - 0.19	0	6	6	12
0.20 - 0.29	0	1	7	8
0.30 - 0.39	0	0	3	3
0.40 - 0.49	0	0	2	2
0.50 - 0.59	0	0	0	0
0.60 - 0.69	0	0	0	0

TABLE 6. Repetition of pathogenicity testing of a selection of field isolates.

Isolate Identity Code	Initial Pathogenicity Rating*	Repeat Pathogenicity Rating*
02S7	0.06 (10)	0.20 (10)
06S3	0.35 (2)	0.39 (3)
06S12	0.32 (3)	0.42 (1)
06S14	0.19 (5)	0.26 (8)
07S5b	0.38 (1)	0.36 (5)
07S15	0.24 (4)	0.42 (1)
15S20	0.11 (7)	0.30 (6)
WWS2a	0.07 (9)	0.30 (6)
WWS10	0.00 (11)	0.13 (11)
0601/4	0.00 (11)	0.10 (12)
0601/12	0.16 (6)	0.38 (4)
0881/14	0.08 (8)	0.24 (9)

* Ranking of isolate with respect to the other eleven isolates indicated in brackets.

also appear to be skewed towards low pathogenicity.

On re-testing a selection of isolates, (see Table 6), a certain amount of variation in pathogenicity ratings was observed for each isolate. However, as Blanch (1977) has noted the relative ranking of isolates with respect to each other is largely conserved. On these grounds isolates could be placed on a scale of pathogenicity with reasonable accuracy.

In a subsequent test of the same isolates the values for most of the isolates were noticeably lower. This could be the result of storing the isolates. It is well-known that Ggt isolates can lose their pathogenicity when stored in axenic culture (Naiki and Cook, 1983a; 1983b). For this reason the pathogenicities of all 157 isolates (Tests 1, 2 and 3, Table 5) were measured as soon as possible after their isolation and multiple retesting of all the isolates was avoided.

3.1.3 Examination of the ds RNA Content of the Ggt Field Isolates.

The ds RNA content of each field isolate was examined, after extraction and purification, (as described in section 2.12), by PAGE, (section 2.16), and spectrophotometry, (section 2.15). A selection of purified samples, from a variety of the field isolates, was subjected to a series of nuclease digestions to demonstrate that ds RNA is preferentially selected by

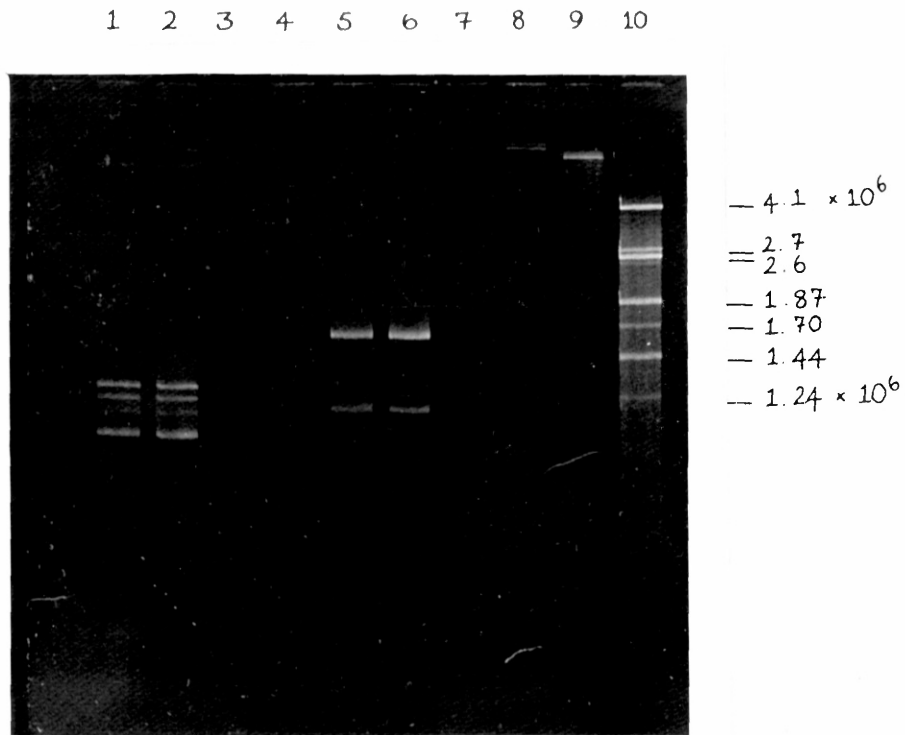
the purification technique.

Molecular weights of ds RNA species were determined by PAGE using a number of standard ds RNA species. The molecular weights of these standards, which had previously been determined in non-denaturing gels were verified using denaturing gels.

a) Nuclease digestions of purified extracts from Ggt field isolates.

Purified nucleic acid samples from various Ggt field isolates were subjected to nuclease digestion prior to electrophoresis, (section 2.19). Figure 3 shows the effect of DNase digestion on a number of isolates from the WW plot. Evidently, the majority of nucleic acid bands observed are not susceptible to DNase; only a few higher molecular weight bands being digested. However, when two of the purified samples observed in figure 3, (i.e. those from isolates WWS6 and WWS7b), were subjected to RNase at low salt concentrations, no nucleic acid bands remained visible; while RNase incubation at 'high salt' did not permit digestion (figure 4). Hence it seems that the ds RNA extraction and purification technique outlined in section 2.12 is effective in selecting for ds RNA. Furthermore, under these conditions of electrophoresis, ss RNA components do not form discrete bands and hence cannot be confused with ds RNA. Consequently a large range of isolate samples was incubated with DNase prior to electrophoresis, to eliminate any traces of

Figure 3

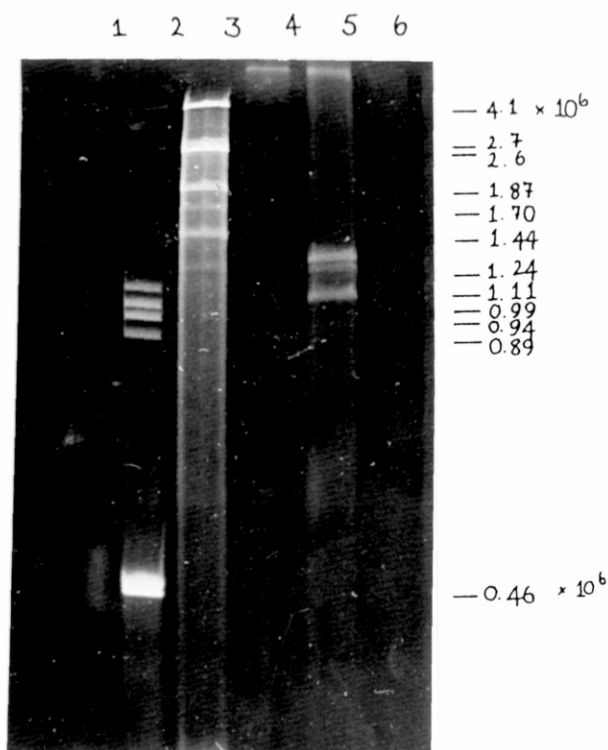


4% polyacrylamide gel, as in section 2.16

Lanes,

- 1: ds RNA fraction from isolate WWS7a, DNase digested.
 - 2: ds RNA fraction from isolate WWS7a.
 - 3: ds RNA fraction from isolate WWS6, DNase digested.
 - 4: ds RNA fraction from isolate WWS6.
 - 5: ds RNA fraction from isolate WWS2a, DNase digested.
 - 6: ds RNA fraction from isolate WWS2a.
 - 7: ds RNA fraction from isolate WWR2, DNase digested.
 - 8: ds RNA fraction from isolate WWR2.
 - 9: HmV ds RNA
 - 10: AfV ds RNA
- } Standards

Figure 4.



4% polyacrylamide gel, (as in section 2.16).

Lanes,

- 1: PsV ds RNA (standard).
- 2: AfV ds RNA, 'high salt' RNase digested (standard)
- 3: WWS6 sample, 'high salt' RNase digested.
- 4: WWS7b sample 'high salt' RNase digested.
- 5: WWS6 sample, 'low salt' RNase digested.
- 6: WWS7b sample, 'low salt' RNase digested.

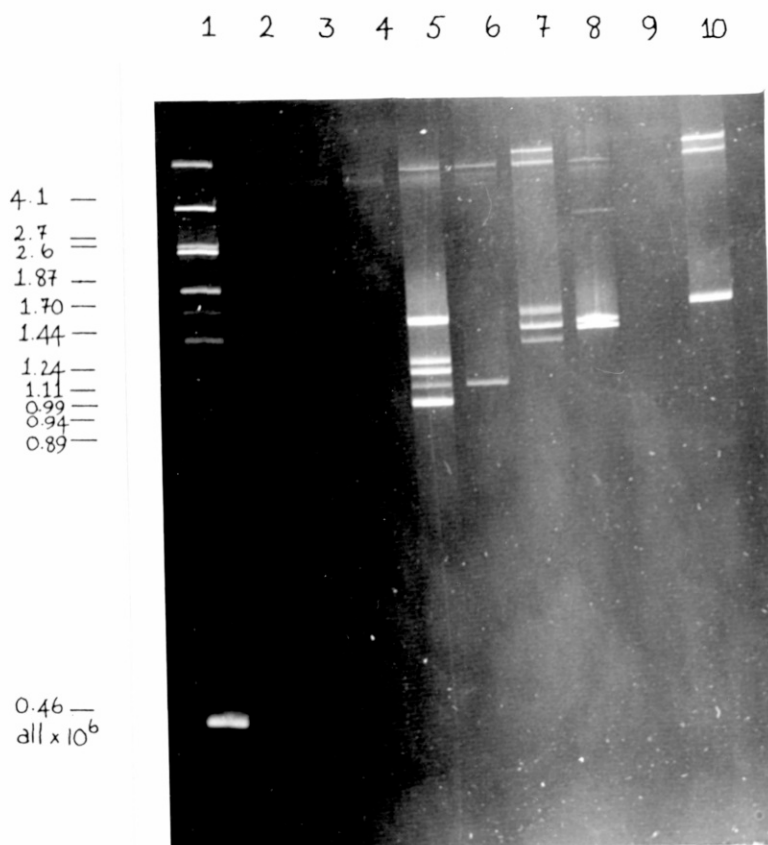
DNA, and therefore the remaining discrete bands were presumed to be ds RNA.

b) PAGE of purified ds RNA extracted from the Ggt field isolates.

It has been demonstrated that when electrophoretic mobilities of ds RNA species, (with molecular weights from 5.7×10^6 to 0.26×10^6), in PAGE are plotted against their respective logarithms of molecular weight, a smooth curve is obtained (Bozarth and Harley, 1976; Buck and Ratti, 1977; Harley et al., 1977; Bozarth, 1977).

In this study of the ds RNA species found in a population of Ggt isolates, a selection of ds RNA standards were used including: HmV ds RNA (5.7×10^6 ; Bozarth, 1977; Bozarth et al., 1981), Ggt 3blaV ds RNA (4.1 to 1.11×10^6 ; Buck et al., 1981), PsV ds RNA (1.11 to 0.46×10^6 ; Bozarth et al., 1971; Bozarth and Harley, 1976) and AfV ds RNA (4.1 to 0.27×10^6 ; Ratti and Buck, 1972; Buck and Ratti, 1977). From these standards, for each gel, a graph was drawn which gave a standard curve which permitted the estimation of the molecular weights of the ds RNA species observed in the Ggt field isolates from their relative gel mobilities. A typical polyacrylamide gel is shown in Figure 5; while Figure 6 represents the standard curve from the gel in Figure 5. In appendix II a complete record of the polyacrylamide gels used for these molecular weight estimations may be found, (including a number of gels,

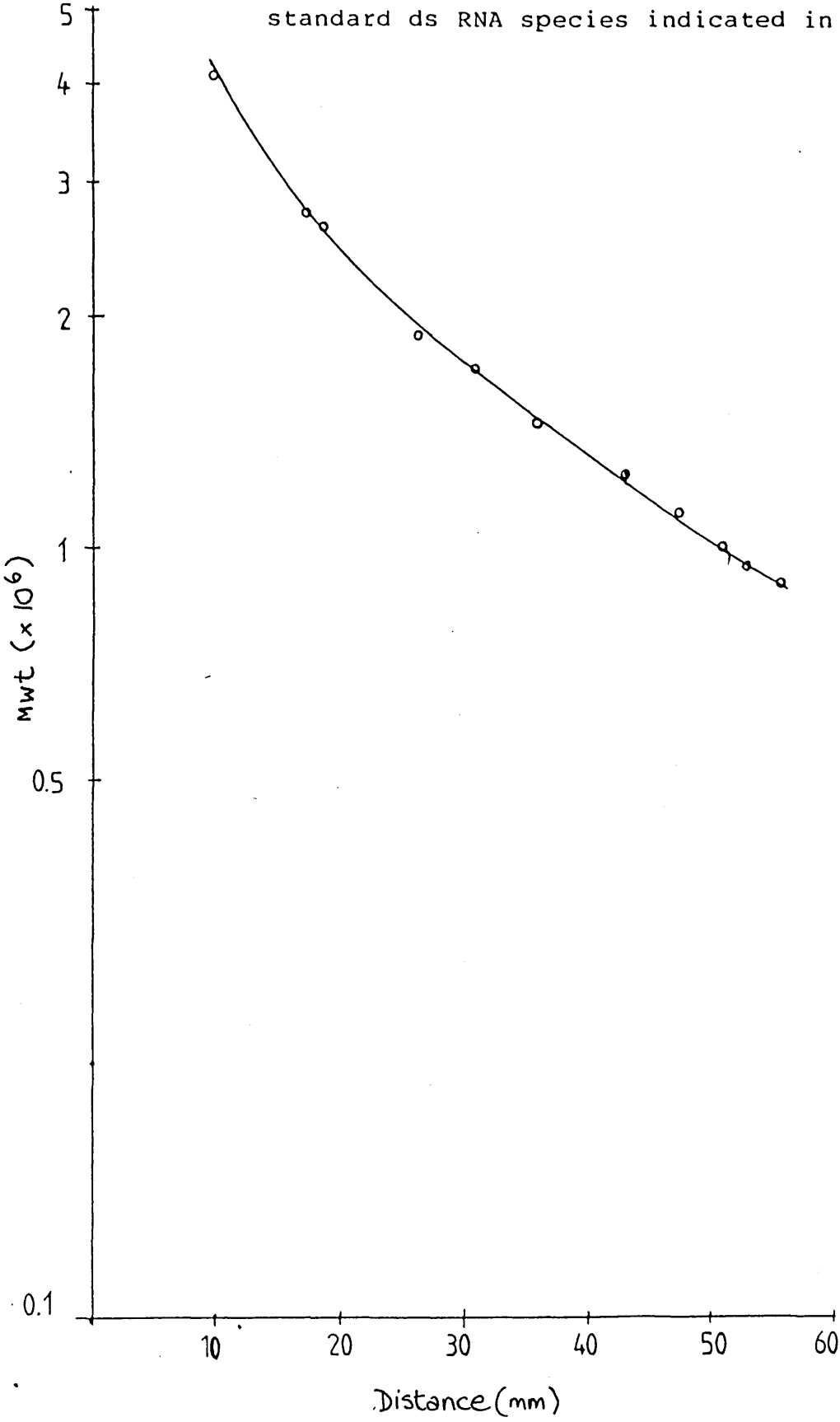
Figure 5.



4% polyacrylamide gel, (as in section 2.16)

Lanes, 1: Standards (HmV, AfV and PsV ds RNA); 2: 0.601/1;
3: 0601/2; 4: 0601/3; 5: 0601/4; 6: 0601/5; 7: 0601/6;
8: 0601/7; 9: 0601/8; 10: 0601/9.

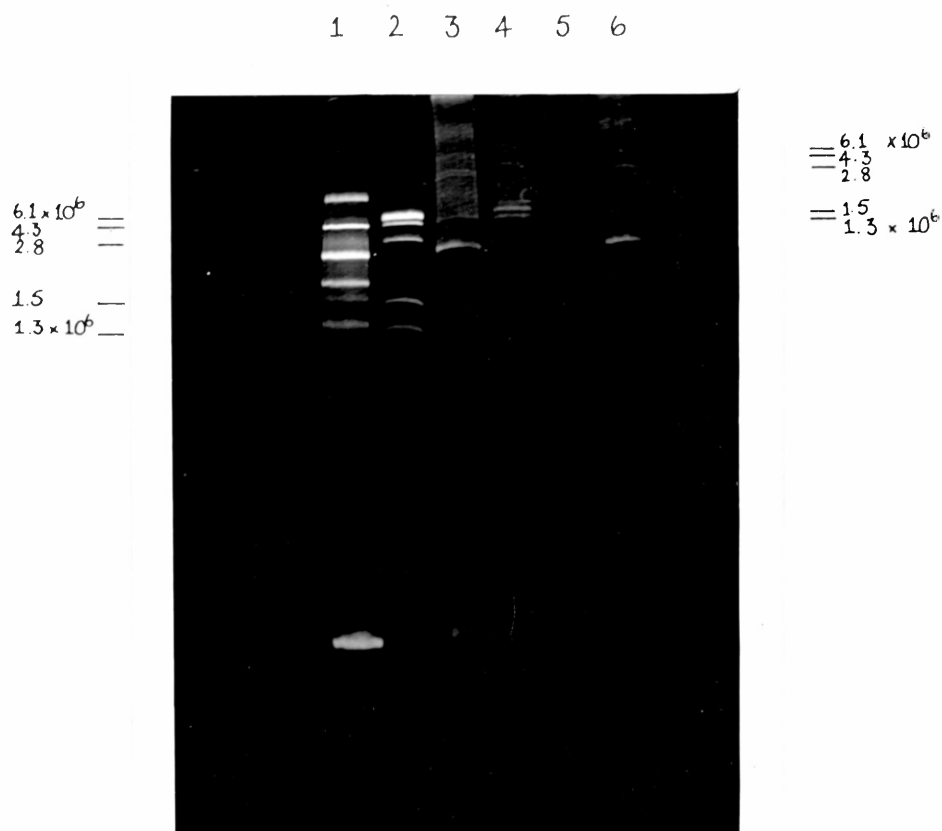
Figure 6. Standard curve plotted from the migration of the standard ds RNA species indicated in Figure 5.



i.e. gels $\overset{XX}{\lambda}^{II}$ to $\overset{XX}{\lambda}^{VI}$, that were used to confirm the results from the original gels).

The standards AfV, PsV and Ggt 3blaV ds RNA all appeared from assessment on denaturing gels to have been accurately estimated, e.g. AfV, (Figures 7 and 8). McMaster and Carmichael (1977) have noted that the use of glyoxalated DNA molecules of known molecular weights as standards, (e.g. lambda bacteriophage DNA digested with HindIII restriction endonuclease, see Figure 8), yields accurate molecular weight estimates of RNA molecules. Hence the ds RNA standards of AfV, PsV and Ggt 3blaV were used as accurate guides for the estimation of molecular weights of the ds RNA species observed in the purified extracts of the Ggt field isolates. However HmV ds RNA, (reported to be 5.7×10^6 ; Bozarth, 1977; Bozarth et al., 1981), did not appear to be an accurate standard, since from its relative mobility in PAGE, it would have had a molecular weight in excess of 7.0×10^6 , whilst under denaturing conditions it was not visualised after electrophoresis, (Figures 7 and 8). (It is possible that the anomalous behaviour of the HmV ds RNA was the result of the ds RNA molecules being nicked). Hence it was decided that HmV ds RNA should be disregarded as a reference point, and therefore the molecular weights of all of the ds RNA bands in excess of 4.1×10^6 , (i.e. equivalent to the largest standard ds RNA species), observed in purified extracts of the Ggt field isolates

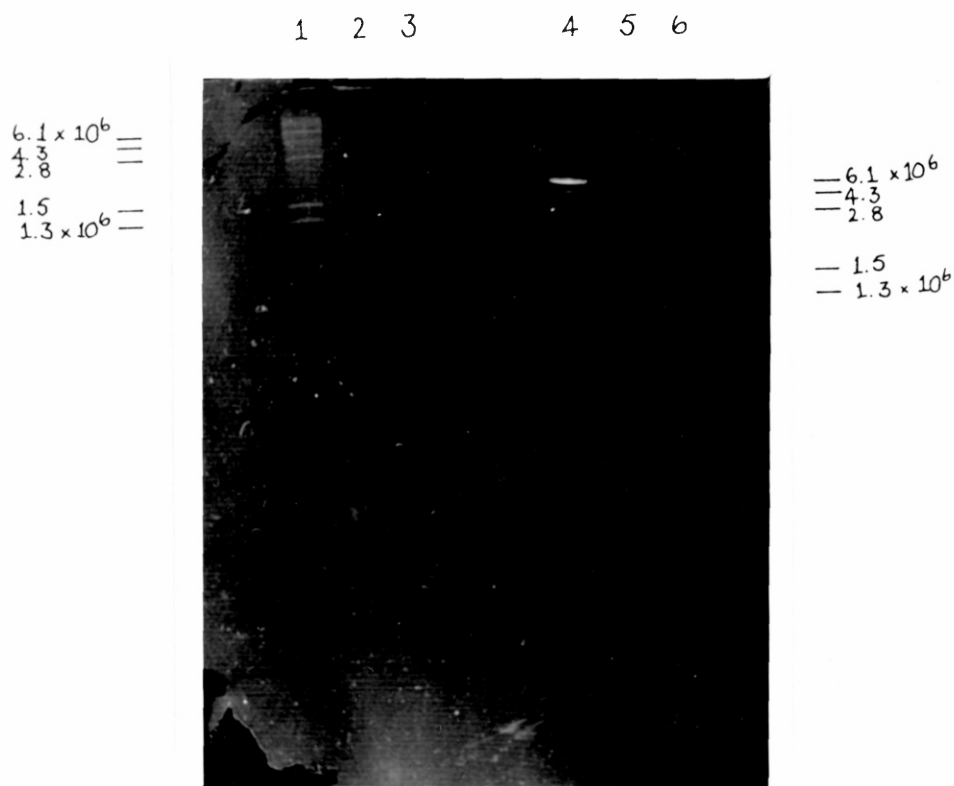
Figure 7. Urea-SDS 4% polyacrylamide gel (see section 2.18a).



Lanes (numbering from the left)

1:	HmV, AfV and PsV ds RNA	}	non-denatured prior to electrophoresis
2:	λ HindIII		
3:	HmV, AfV, PsV ds RNA		
4:	λ HindIII		
5:	HmV ds RNA	}	Denatured prior to electrophoresis
6:	AfV ds RNA		

Figure 8. 4% polyacrylamide gel with glyoxalated and non-glyoxalated samples.



Lanes

- 1: λ HindIII
- 2: HmV ds RNA} glyoxalated
- 3: AfV ds RNA
- 4: λ HindIII
- 5: HmV ds RNA} non-glyoxalated
- 6: AfV ds RNA

N.B. Denatured nucleic acid stained with acridine appears red, while non-denatured nucleic acid appears green when viewed on a U.V. transilluminator.

TABLE 7. Estimated molecular weights of the ds RNA species found in the 157 isolates of Ggt isolated from plots.

<u>Isolate</u>	<u>ds RNA Species (x10⁶)</u>	<u>ds RNA Class(es)</u>	<u>*Gel Number</u>
02S2	1.54, 1.24, 1.19, 1.14, 1.11	C, D	i), xxii)
02S3	5.4, 5.25	A	ii), xxii)
02S5	1.11	D	i), xxii), xxvi)
02S6	1.11	D	i), xxii)
02S7	1.24, 1.19, 1.14, 1.11, 1.02, 0.96	D	i), xxii)
02S8 α, β, γ	7.0, 5.4, 4.4, 1.58, 1.54, 1.27, 1.21, 1.19, 1.11, 0.98	A, B, C, D	i), xxii)
02S9 α, β, γ	7.0, 5.4, 4.4, 1.58, 1.54, 1.27, 1.21, 1.19, 1.11, 0.98	A, B, C, D	i), xxii), xxiv)
02S11	1.21, 1.14, 1.11	D	ii), xxii), xxvi)
02S14	1.60, 1.54, 1.42, 1.24, 1.19, 1.11	C, D	ii), xxii)
02S15	1.60, 1.54, 1.24, 1.19	C, D	ii)
02S16	1.19	D	ii)
02S18	6.7, 1.60, 1.54, 1.44, 1.27, 1.21	A, C, D	iii), xxiii)
02S19	1.21, 1.14	D	iii), xxiii)

continued over

TABLE 7 continued

<u>Isolate</u>	<u>ds RNA Species (x10⁶)</u>	<u>ds RNA Class(es)</u>	<u>*Gel Number</u>
03S2	1.58, 1.54, 1.27, 1.21, 1.11, 1.04	C, D	iii), xxiii), xxxvi)
03S3a			iii)
03S3b	6.1, 1.58, 1.54, 1.27, 1.21, 1.11, 1.02	A, C, D	iii), xxiii), xxvi)
03S5	1.27	D	iii), xxiii)
03S6	6.7, 1.21	A, D	iv)
03S8a	1.19	D	iv)
03S8b			iv)
03S10	1.24, 1.19, 1.14	D	iv)
03S13	6.1, 5.4, 1.27, 1.19	A, D	iv), xxiii), xxvi)
03S14			iv)
03S15	6.1, 5.4, 1.45, 1.24, 1.19, 1.14	A, C, D	iv)
03S17	6.1, 5.4, 1.19	A, D	iv)
03S18	6.1, 5.4, 1.27, 1.14	A, D	iv)
03S20a	6.0, 5.6, 4.7	A	v), xxvi)
03S20b	1.27, 1.19	D	v)

continued over

TABLE 7 continued

<u>Isolate</u>	<u>ds RNA Species (x 10⁶)</u>	<u>ds RNA Class(es)</u>	<u>*Gel Number</u>
06S2	6.9	A	v)
06S3	6.45, 6.05, 1.58, 1.52, 1.21, 1.14	A, C, D	v)
06S4	6.05, 1.21, 1.14	A, D	v)
06S7	6.45, 6.05	A	v)
06S8	6.4, 1.68, 1.60, 1.54, 1.27, 1.21	A, C, D	vi), xxiv)
06S9	6.0, 5.4	A	vi)
06S10	6.7, 6.0, 5.4, 1.60, 1.54, 1.21, 1.14	A, C, D	vi)
06S11	6.0	A	vi)
06S12	6.0, 5.4	A	vi)
06S14	6.7, 6.0, 5.4, 1.60, 1.54, 1.21, 1.14, 0.75, 0.69	A, C, D	vi), xxiv), xxvi)
06S15	6.0, 5.4, 1.60, 1.58, 1.54, 1.27, 1.14	A, C, D	vi)
06S16	6.0, 5.4, 1.60, 1.54, 1.21, 1.14	A, C, D	vi)
06S17	6.7, 6.0, 5.4, 1.60, 1.54, 1.42, 1.21, 1.14	A, C, D	vii)
06S18	-		vii)

continued over

TABLE 7 continued

<u>Isolate</u>	<u>ds RNA Species (x10⁶)</u>	<u>ds RNA class(es)</u>	<u>*Gel Number</u>
07S1	6.0, 5.4, 1.58, 1.50, 1.27, 1.14, 1.12	A, C, D	vii)
07S2	6.0, 5.4, 1.19, 1.13	A, D	vii)
07S4	-	-	vii)
07S5a	-	-	vii), xxiv)
07S5b	-	-	vii)
07S6	1.14	D	vii), xxiv)
07S7	6.0, 5.4, 1.54, 1.50, 1.27, 1.14	A, C, D	viii)
07S8	-	-	viii)
07S9	1.54, 1.50, 1.27	C, D	viii)
07S10	6.0, 5.4, 1.58, 1.54, 1.50, 1.27, 1.19, 1.14, 1.04, 1.00	A, C, D	viii)
07S11	1.54, 1.50, 1.48, 1.27, 1.25, 1.14, 1.11	C, D	viii)
07S12	-	-	viii)
07S14	1.54, 1.50, 1.48, 1.42, 1.38, 1.30, 1.27, 1.12, 1.08	C, D	viii)
07S15	6.0, 5.4	A	viii)

continued over

TABLE 7 continued

<u>Isolate</u>	<u>ds RNA Species (x10⁶)</u>	<u>ds RNA Class(es)</u>	<u>*Gel Number</u>
07S16	6.0, 5.4, 1.19, 1.14	A, D	ix)
07S17	6.0, 1.54, 1.50, 1.48, 1.34, 1.30, 1.27, 1.24, 1.14	A, C, D	ix)
07S18	6.0	A	ix)
07S20	6.0, 5.4, 1.50, 1.48, 1.27, 1.19, 1.14, 1.00	A, C, D	ix)
14S3α, β	6.0, 5.4, 1.14	A, D	ix)
14S4	6.0, 5.4, 1.14	A, D	ix)
14S7	6.0, 5.4, 1.14	A, D	x)
14S8	6.0, 5.4, 1.14	A, D	x), xxvi)
14S10	1.27	D	x)
14S11α, β	6.0, 5.4, 1.14	A, D	x)
14S13	1.11, 0.99	D	x)
14S15α, γ	6.0, 5.4, 1.14	A, D	xi)
14S15	1.21	D	xi), xxiv)
14S17a	1.21, 1.14	D	xi)

continued over

TABLE 7 continued

<u>Isolate</u>	<u>ds RNA Species (x10⁶)</u>	<u>ds RNA Class(es)</u>	<u>*Gel Number</u>
14S17b	6.0, 5.4, 1.40, 1.21, 1.14	A, C, D	xi), xxiv)
14S19	1.60, 1.56, 1.27, 1.21	C, D	xi)
15S2	-	-	xxi)
15S3	1.60, 1.21, 1.14	C, D	xxi)
15S4 α , β	-	-	xii)
15S5 α , β	1.34, 1.21	D	xii), xxiv)
15S6 α , β	-	-	xxi), xxvi)
15S7	-	-	xiii)
15S8	-	-	xiii)
15S9	-	-	xiii)
15S10	1.58, 1.27, 1.21	C, D	xiii)
15S11	6.0, 5.4, 1.21, 1.14	A, D	xiii)
15S12a, b	1.58, 1.27, 1.21	C, D	xiii), xiv)
15S14	-	-	xiv)
15S15	6.0, 5.4	A	xiv)

continued over

TABLE 7 continued

<u>Isolate</u>	<u>ds RNA Species (x10⁶)</u>	<u>ds RNA Class(es)</u>	<u>*Gel Number</u>
15S16	5.4	A	xiv)
15S18	6.0, 5.4	A	xiv)
15S19	-	-	xiv)
15S20	6.0, 5.4, 1.27, 1.14	A, D	xv)
WWR2	7.0, 6.7, 6.0, 5.4	A	xv)
WWS2a	6.7, 6.0, 1.60, 1.14	A, C, D	xv)
WWS2b	1.70, 1.24	C, D	xv)
WWS4	-	-	xv)
WWS6	6.0	A	xv)
WWS7a, b	6.5, 5.8, 1.27, 1.21, 1.14, 1.02	A, D	xvi)
WWS8	5.8, 1.60, 1.58, 1.34, 1.19, 1.14, 1.04	A, C, D	xvi)
WWS10	5.8, 1.70, 1.58, 1.14	A, C, D	xvi)
WWS11	-	-	xvi)
WWS12	-	-	xvi)

continued over

TABLE 7 continued

<u>Isolate</u>	<u>ds RNA Species (x10⁶)</u>	<u>ds RNA Class(es)</u>	<u>*Gel Number</u>
0601/1	-	-	xvii)
0601/2	-	-	xvii)
0601/3	-	-	xvii)
0601/4	5.5, 1.50, 1.27, 1.21, 1.11, 1.02	A, C, D	xvii), xxv)
0601/5	5.5, 5.1, 4.6, 1.11	A, D	xvii)
0601/6	6.2, 5.5, 1.56, 1.46, 1.36	A, C	xvii)
0601/7	5.5, 5.1, 4.6, 3.3, 1.48, 1.44	A, B, C	xvii)
0601/8	-	-	xvii)
0601/9	6.2, 5.5, 1.58, 1.56	A, C	xvii), xxv)
0601/10	1.58, 1.48, 1.44, 1.14	C, D	xviii)
0601/12	6.2, 5.5, 1.52	A, C	xviii)
0601/13	5.5, 5.1, 3.3, 1.48, 1.44	A, B, C	xviii)
0601/14	-	-	xviii)
0601/15	5.5, 5.1, 4.1, 1.52, 1.30, 1.27, 1.21, 1.11, 1.02	A, B, C, D	xviii) xxv)
0601/16	5.5, 5.1	A	xviii)

continued over

TABLE 7 continued

<u>Isolate</u>	<u>ds RNA Species (x10⁶)</u>	<u>ds RNA Class(es)</u>	<u>*Gel Number</u>
0601/17	5.5, 5.1	A	xviii)
0601/18	1.30	D	xviii)
0601/19	1.48, 1.42, 1.27, 1.21, 0.99, 0.82	C, D	xix)
0601/20	3.33, 1.56, 1.48, 1.42, 1.21, 1.11	B, C, D	xix)
0601/21	1.11	D	xix)
0601/22	1.48, 1.42, 1.27, 1.21, 0.99	C, D	xix)
0881/1	1.58, 1.56	C	xix), xxv)
0881/2	1.52, 1.27, 1.21	C, D	xix)
0881/3	-	-	xix)
0881/4	5.4, 1.11, 1.08	A, D	xix), xxi)
0881/5	6.50, 1.56, 1.21, 1.19	A, C, D	xix)
0881/6	1.58, 1.50, 1.46, 1.27, 1.21, 1.19	C, D	xx)
0881/7	1.58	C	xx)
0881/8	5.8, 5.2, 1.52, 1.40, 1.30	A, C, D	xx), xxv)
0881/9	1.50, 1.27, 1.19	C, D	xx)

continued over

TABLE 7 continued

<u>Isolate</u>	<u>ds RNA Species (x10⁶)</u>	<u>ds RNA Class(es)</u>	<u>*Gel Number</u>
0881/10	5.8, 5.2	A	xx)
0881/11	1.58, 1.56	C	xx)
0881/12	1.30, 1.21, 1.02	D	xx)
0881/13	1.58, 1.56	C	xx)
0881/14	5.8, 5.2, 1.58	A, C	xx), xxv)
0881/15	1.58, 1.56	C	xxi), xxv)
0881/16	1.30, 1.21, 1.02	D	xxi)
0881/17	1.17	D	xxi)
0881/18	-	-	xxi)
0881/19	1.17	D	xxi)
0881/20	1.17, 1.08	D	xxi)
0881/21	6.5, 5.4, 3.3, 1.56, 1.50, 1.30, 1.21, 1.19, 1.11, 1.02	A, B, C, D	xxi)

* Gel number in Appendix II.

were estimated by linear extrapolation of the standard curve; as a result these estimated molecular weights are almost certainly underestimated.

The ds RNA species detected in each of the field isolates of Ggt ^{are} ~~is~~ detailed in Table 7. Of the 157 isolates, 126, (i.e. 80.3%) were found to contain at least one ds RNA species. Four classes of ds RNA species were selected, (based on molecular weight range), these being; 7.0 to 4.6×10^6 (class A), 4.4 to 3.0×10^6 (class B), 1.7 to 1.35×10^6 (class C) and 1.35 to 10^6 (class D). In Table 7 the ds RNA class content of each isolate is also indicated, as well as the number of the gel, (relating to Appendix II), in which the ds RNA fraction of each isolate was examined.

The distribution of the ds RNA species in these class sizes is represented in Table 8, the Ggt field isolates being divided into plots. From Table 8 a number of salient points emerge. Firstly, the frequency of the four ds RNA classes in descending order is D, A, C, B. Secondly, this order is preserved in the majority of the plots, the exceptions being 06, WW, 0601 and 0881. [N.B. Plot 06, $A > D > C > B$; plot WW, $A > D > C > B$; plot 0601, $C > A > D > B$; plot 0881, $D = C > A > B$]. Thirdly, only isolates from plots 02, 0601 and 0881 were found to contain ds RNA species from size class B. Fourthly, certain plots, especially 15 and also 07, WW and 0601 have a greater percentage of

TABLE 8. Distribution of ds RNA Species Classes Amongst the Field Isolates of Ggt.

Ds RNA Class	Number of Isolates in Plots										% of total no. field isolates
	02	03	06	07	14	15	WW	0601	0881	Total	
A	1	1	5	2	0	3	2	2	1	17	10.8
A + C	0	0	0	0	0	0	0	3	1	4	2.5
A + D	1	4	1	2	11	2	2	1	1	25	15.9
A + B + C	0	0	0	0	0	0	0	2	0	2	1.3
A + C + D	1	2	7	5	2	0	3	1	2	23	14.6
A + B + C + D	6	0	0	0	0	0	0	1	1	8	5.1
B + C + D	0	0	0	0	0	0	0	1	0	1	0.6
C	0	0	0	0	0	0	0	0	5	5	3.2
C + D	3	1	0	3	0	4	1	3	3	18	11.5
D	5	4	0	1	4	2	0	2	5	23	14.6
O*	0	3	1	5	1	10	3	6	2	31	19.7
										<u>157</u>	

continued over/...

TABLE 8 continued

ds RNA Class	Number of Isolates in Plots										% of Total No. Field Isolates
	02	03	06	07	14	15	WW	0601	0881	Total	
Total A	9	7	13	9	12	5	7	10	6	77	49.0
Total B	6	0	0	0	0	0	0	4	1	11	7.0
Total C	10	3	7	8	3	4	4	11	12	62	39.5
Total D	16	11	8	11	17	8	6	9	12	98	62.4

* 0 = no ds RNA detected.

isolates that are lacking ds RNA than the average for the whole population; while the converse is noticeably true for plots 02, 06, 14 and 0881.

c) Assessment of ds RNA concentration in the Ggt field isolates.

A selection of the Ggt field isolates, (approximately 20% of the total number), were assessed spectrophotometrically as described in section 2.15. The values obtained thus were converted into μg ds RNA/g fresh weight mycelium, (i.e. after filter-drying). These values were confirmed by examination of the appearance of each of the isolates in ethidium bromide stained polyacrylamide gels. Generally there was a good correlation between the value obtained by spectrophotometry and the number and intensity of ds RNA species observed by PAGE. From the range of values obtained for this selection of isolates, the ds RNA content ($\mu\text{g/g}$) for the remaining isolates in the population studied, was estimated using PAGE and the spectrophotometrically assessed isolates as reference points.

All of the isolates were observed to contain between 0 and 5 μg ds RNA/g fresh weight mycelium, which is similar to previous observations on yields of ds RNA from mycelium of other Ggt isolates (McFadden, 1982). The distribution of ds RNA concentration in the Ggt field isolates, (divided into plots), is shown in Table 9; whilst the detailed isolate composition of

TABLE 9. Distribution of ds RNA concentration in the Ggt field isolates.

ds RNA ($\mu\text{g/g}$)	Number of Isolates in Plots									Total
	02	03	06	07	14	15	WW	0601	0881	
0	0	3	1	5	1	10	3	6	2	31
0.01 - 1.00	4	0	2	3	5	3	2	3	6	28
1.01 - 2.00	3	5	4	1	12	8	2	4	10	49
2.01 - 3.00	5	4	1	3	0	0	1	8	1	23
3.01 - 4.00	0	3	6	3	0	0	0	1	2	15
4.01 - 5.00	5	0	0	3	0	0	3	0	0	11

each of the ds RNA concentration classes is recorded in Appendix III.

From Table 9 it is clear that when the Ggt field isolates are examined for ds RNA content, a major class of isolates is actually composed of those isolates which are lacking ds RNA altogether. When only the ds RNA-containing isolates are considered, then the distribution is seen to peak in the 1.01-2.00 $\mu\text{g/g}$ category. If the isolates are analysed plot by plot, then mostly this pattern is reproduced. It is noteworthy that plots 14 and 15 both show a strong bias towards low ds RNA content; whilst other plots, especially 0601, and also 02 and 06 appear to be rather biased towards high ds RNA content.

3.1.4. Virus Content of a Selection of the Ggt Field Isolates.

A selection of the field isolates that were considered to be of interest, since they had been observed to be both ds RNA containing and weak pathogens, were investigated further. It was considered important to determine if the ds RNA species observed in these weakly pathogenic isolates were encapsidated as virus or not. This would be a significant consideration for later infectivity experiments, where virus as opposed to ds RNA would be required as the inoculum. Hence virus preparations were made from each of the following isolates: 02S7,

06S14, WWS2a, WWS10, 0601/4 and 0881/14, (see section 2.13). These viral preparations were analysed by agarose gel electrophoresis of intact virions, (both crude and purified viral preparations, see section 2.20), and the ds RNA extracted from purified virus preparations, (see section 2.14), was analysed by PAGE.

The putative viruses identified by agarose gel electrophoresis are indicated in Figure 9. The Ggt isolate 3bla was used as a control since the number of viruses occurring in 3bla was already known (Buck et al., 1981). The ds RNA content of these viral preparations was extracted, (see section 2.14), and examined by PAGE, (see Figure 10).

All of the field isolates examined were observed to be virus containing, (except 0881/14), with 02S7, WWS2a, WWS10 and 0601/4 exhibiting one nucleoprotein band each; while 06S14 exhibited three nucleoprotein bands. The lack of virus observed in 0881/14 could have been due to virus instability, so that it was degraded in preparation, or it may have been because the virus was lost on storage of the isolate, since on re-examination at a later date, ds RNA could not be found either.

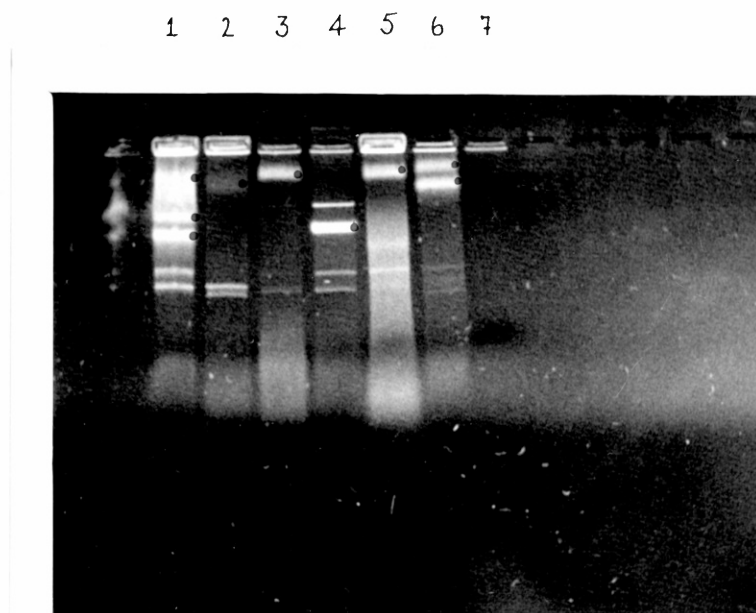
Similarly ds RNA patterns were observed originating from these viral preparations, i.e. both crude and purified preparations, (except in the case of 0881/14, of course). Largely these patterns were

identical to those seen by PAGE of ds RNA extracts of mycelium, (i.e. section 2.12). Two points of interest though should be noticed; 06S14 V ds RNA lacked the 'heavyweight' triplet of ds RNA species, (mwts 6.7, 6.0 and 5.4×10^6), previously observed, (whilst 0601/4 V ds RNA also lacked the 5.5×10^6 species previously seen), and secondly, 0601/4 V ds RNA exhibited a new ds RNA species, (mwt 2.55×10^6), of a size only recently observed in Ggt, (i.e. between size classes 1.35 to 1.70×10^6 and 3.0 to 4.4×10^6). Prior to the discovery of a 2.3×10^6 ds RNA species in four isolates of Ggt by Jamil et al. (1984), no Ggt isolate had been found to contain a ds RNA species with a molecular weight between 3.0 and 1.7×10^6 . Interestingly Jamil et al. (1984) could only find this 2.3×10^6 ds RNA species in ds RNA, but not virus, extractions, implying that it is not encapsidated. In the case of the 2.55×10^6 ds RNA of 0601/4, the converse appears to be true. This could be further investigated by examination of virus particle sizes in 0601/4 using electron microscopy and by separation of the viruses of 0601/4 by sucrose density centrifugation.

Hence it would seem reasonable to presume that the vast majority, if not all, of ds RNA species observed and recorded in section 3.1.3, (and Appendix II), are viral in origin. Presumably the viruses containing the

Figure 9

Agarose gel electrophoresis of intact crude virus preparations.



The gel was stained with ethidium bromide

Lanes

1: 3bla V

2: 02S7 V

3: 06S14 V

4: WWS2a V

5: WWS10 V

6: 0601/4 V

7: 0881/14 V

N.B. Indicated bands stained

with both ethidium bromide

and Coomassie blue, demonstrating

their nucleoprotein nature.

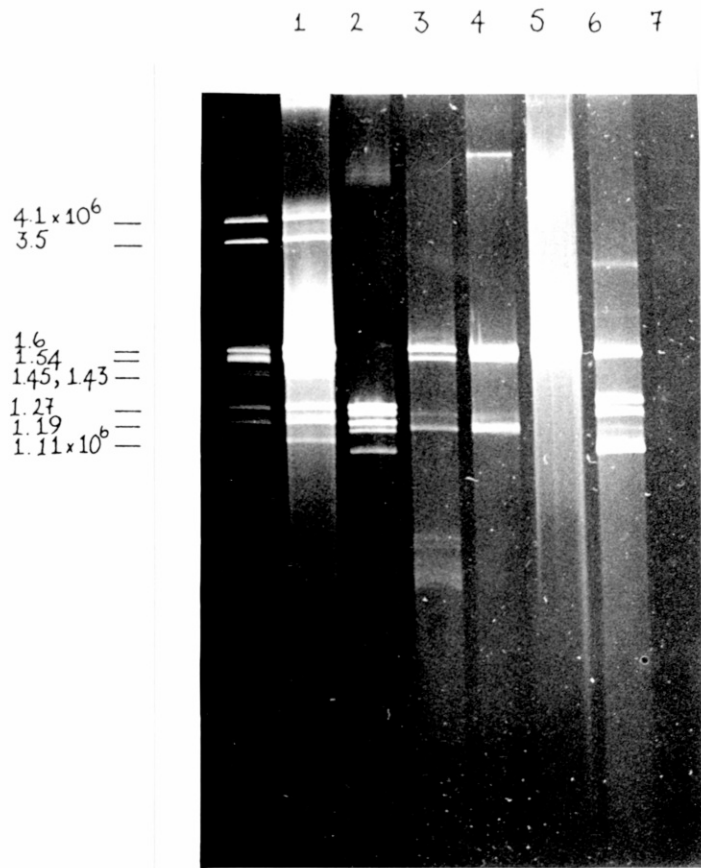
Other bands which stained only

with ethidium bromide were

probably free ds RNA released

from disrupted virions.

Figure 10



Lanes

- 1: 3bla V ds RNA
- 2: 02S7 V ds RNA
- 3: 06S14 V ds RNA
- 4: WWS2a V ds RNA
- 5: WWS10 V ds RNA
- 6: 0601/4 V ds RNA
- 7: 0881/14 V ds RNA

'heavy' ds RNA species of 06S14 and 0601/4 are unstable in purification, however the 'heavy' ds RNA species of WWS2a appear to survive this method of analysis. The appearance of three viruses in 06S14, even without the large ds RNA species may be the result of separate encapsidation of the two unusually small, ds RNA species, (i.e. for Ggt).

3.1.5 Interaction of the Pathogenicity Ratings with ds RNA Content of the Field Isolates of Ggt.

In order to assess the possible effect, that would be seen as a general trend, of ds RNA on pathogenicity, either as a quantitative effect, or as an effect of a particular class or classes of ds RNA elements, the pathogenicity ratings of the Ggt field isolates, (divided according to the three separate seedling shoot dry weight pathogenicity tests), have been analysed in the light of the ds RNA content of the respective isolates. Firstly, Tables 10a, 10b, 10c, 10d, 10e and 10f demonstrate the interaction of different ds RNA size classes (see Tables 7 and 8), with pathogenicity. Secondly, Tables 11a, 11b and 11c demonstrate the interaction of different ds RNA content classes (see Appendix III and Table 9), with pathogenicity. In both cases the distribution patterns observed for each class, either examined as a whole, or divided into plots, were examined in conjunction with the

TABLE 10a. Interaction of ds RNA species class with pathogenicity in Ggt isolates assessed by seedling shoot dry weight (test 1).

ds RNA Species Class	Isolate Source (i.e. Plot)	Pathogenicity Rating						
		0.00 to 0.09	0.10 to 0.19	0.20 to 0.29	0.30 to 0.39	0.40 to 0.49	0.50 to 0.59	0.60 to 0.69
A	02	1	2	2	2	0	1	0
	03	0	2	1	1	2	1	0
	06	1	3	3	5	1	0	0
	07	0	0	1	1	1	0	0
	TOTAL	2	7	7	9	4	2	0
B	02	1	1	1	2	0	1	0
	03	0	0	0	0	0	0	0
	06	0	0	0	0	0	0	0
	07	0	0	0	0	0	0	0
	TOTAL	1	1	1	2	0	1	0

continued over

TABLE 10a continued

ds RNA Species Class	Isolate Source (i.e. Plot)	Pathogenicity Rating						
		0.00 to 0.09	0.10 to 0.19	0.20 to 0.29	0.30 to 0.39	0.40 to 0.49	0.50 to 0.59	0.60 to 0.69
C	02	1	2	2	4	0	1	0
	03	0	1	2	0	0	0	0
	06	1	3	1	2	0	0	0
	07	0	0	2	1	0	0	0
	TOTAL	2	6	7	7	0	1	0
D	02	2	6	2	5	0	1	0
	03	0	2	2	2	3	2	0
	06	1	3	1	3	0	0	0
	07	0	0	3	1	1	0	0
	TOTAL	3	11	8	11	4	3	0

continued over

TABLE 10a continued

ds RNA Species Class	Isolate Source (i.e. Plot)	Pathogenicity Rating						
		0.00 to 0.09	0.10 to 0.19	0.20 to 0.29	0.30 to 0.29	0.40 to 0.49	0.50 to 0.59	0.60 to 0.69
None	02	0	0	0	0	0	0	0
	03	0	1	1	0	1	0	0
	06	0	0	1	0	0	0	0
	07	0	0	2	2	0	0	0
	TOTAL	0	1	4	2	1	0	0

TABLE 10b. Interaction of ds RNA species class with pathogenicity in Ggt isolates assessed by shoot dry weight (test 2).

DS RNA Species Class	Isolate Source (i.e. Plot)	Pathogenicity Rating						
		0.00 to 0.09	0.10 to 0.19	0.20 to 0.29	0.30 to 0.39	0.40 to 0.49	0.50 to 0.59	0.60 to 0.69
A	07	3	2	1	0	0	0	0
	14	6	2	0	1	1	2	0
	15	0	3	0	0	2	0	0
	WW	1	0	1	0	0	0	0
	TOTAL	10	7	2	1	3	2	0
B	07	0	0	0	0	0	0	0
	14	0	0	0	0	0	0	0
	15	0	0	0	0	0	0	0
	WW	0	0	0	0	0	0	0
	TOTAL	0	0	0	0	0	0	0

continued over

TABLE 10 b continued

ds RNA Species Class	Isolate Source (i.e. Plot)	Pathogenicity Rating						
		0.00 to 0.09	0.10 to 0.19	0.20 to 0.29	0.30 to 0.39	0.40 to 0.49	0.50 to 0.59	0.60 to 0.69
C	07	3	2	0	0	0	0	0
	14	0	1	0	0	1	1	0
	15	0	0	1	1	0	2	0
	WW	1	0	1	0	0	0	0
	TOTAL	4	3	2	1	1	3	0
D	07	4	2	0	0	0	0	0
	14	7	2	0	2	3	2	1
	15	0	1	1	2	1	3	0
	WW	1	0	1	0	0	0	0
	TOTAL	12	5	2	4	4	5	1

continued over

TABLE 10b continued

ds RNA Species Class	Isolate Source (i.e. Plot)	Pathogenicity Rating						
		0.00 to 0.09	0.10 to 0.19	0.20 to 0.29	0.30 to 0.39	0.40 to 0.49	0.50 to 0.59	0.60 to 0.69
None	07	1	0	0	0	0	0	0
	14	0	0	0	0	0	0	1
	15	0	0	1	0	5	2	2
	WW	0	1	0	0	0	0	0
	TOTAL	1	1	1	0	5	2	3

TABLE 10c. Interactions of ds RNA species class with pathogenicity in Ggt isolates assessed by shoot dry weight (test 3).

ds RNA Species Class	Isolate Source (i.e. Plot)	Pathogenicity Rating						
		0.00 to 0.09	0.10 to 0.19	0.20 to 0.29	0.30 to 0.39	0.40 to 0.49	0.50 to 0.59	0.60 to 0.69
A	WW	5	0	0	0	0	0	0
	0601	8	2	0	0	0	0	0
	0881	1	2	2	0	1	0	0
	TOTAL	14	4	2	0	1	0	0
B	WW	0	0	0	0	0	0	0
	0601	2	2	0	0	0	0	0
	0881	0	0	0	0	1	0	0
	TOTAL	2	2	0	0	1	0	0

continued over

TABLE 10c continued

ds RNA Species Class	Isolate Source (i.e. Plot)	Pathogenicity Rating						
		0.00 to 0.09	0.10 to 0.19	0.20 to 0.29	0.30 to 0.39	0.40 to 0.49	0.50 to 0.59	0.60 to 0.69
C	WW	2	0	0	0	0	0	0
	0601	6	4	1	0	0	0	0
	0881	2	4	4	1	1	0	0
	TOTAL	10	8	5	1	1	0	0
D	WW	4	0	0	0	0	0	0
	0601	4	4	1	0	0	0	0
	0881	1	1	7	2	1	0	0
	TOTAL	9	5	8	2	1	0	0

continued over

TABLE 10 c continued

ds RNA Species Class	Isolate Source (i.e. Plot)	Pathogenicity Rating						
		0.00 to 0.09	0.10 to 0.19	0.20 to 0.29	0.30 to 0.39	0.40 to 0.49	0.50 to 0.59	0.60 to 0.69
None	WW	2	0	0	0	0	0	0
	0601	5	1	0	0	0	0	0
	0881	0	1	0	0	1	0	0
	TOTAL	7	2	0	0	1	0	0

TABLE 10d. Interaction of ds RNA class combinations and pathogenicity rating, (test 1).

ds RNA Class Combination	Pathogenicity Rating Class						
	0.00 to 0.09	0.10 to 0.19	0.20 to 0.29	0.30 to 0.39	0.40 to 0.49	0.50 to 0.59	0.60 to 0.69
A, C	0	0	0	0	0	0	0
A, D	0	1	0	1	2	0	0
A, B, C	0	0	0	0	0	0	0
A, B, C, D	1	1	1	2	0	0	0
B, C, D	0	0	0	0	0	0	0
C, D	0	0	3	2	0	0	0

TABLE 10e. Interaction of ds RNA class combination and pathogenicity rating, (test 2).

ds RNA Class Combination	Pathogenicity Rating Class						
	0.00 to 0.09	0.10 to 0.19	0.20 to 0.29	0.30 to 0.39	0.40 to 0.49	0.50 to 0.59	0.60 to 0.69
A, C	0	0	0	0	0	0	0
A, D	7	2	0	1	2	1	0
A, B, C	0	0	0	0	0	0	0
A, C, D	4	1	0	0	0	1	0
A, B, C, D	0	0	0	0	0	0	0
C, D	1	1	2	1	1	2	0

TABLE 10f. Interaction of ds RNA class combination and pathogenicity rating, (test 3).

ds RNA Class Combination	Pathogenicity Rating Class						
	0.00 to 0.09,	0.10 to 0.19	0.20 to 0.29	0.30 to 0.39	0.40 to 0.49	0.50 to 0.59	0.60 to 0.69
A, C	3	1	0	0	0	0	0
A, D	3	0	1	0	0	0	0
A, B, C	2	0	0	0	0	0	0
A, C, D	3	1	1	0	0	0	0
A, B, C, D	0	1	0	0	1	0	0
B, C, D	0	1	0	0	0	0	0
C, D	1	1	4	0	0	0	0

TABLE 11a. Interaction of ds RNA content ($\mu\text{g/g}$) with pathogenicity in Ggt isolates assessed by seedling shoot dry weight, (test 1).

ds RNA Content ($\mu\text{g/g}$)	Isolate Source (i.e. Plot)	Pathogenicity Rating						
		0.00 to 0.09	0.10 to 0.19	0.20 to 0.29	0.30 to 0.39	0.40 to 0.49	0.50 to 0.59	0.60 to 0.69
0	02	0	0	0	0	0	0	0
	03	0	1	1	0	1	0	0
	06	0	0	1	0	0	0	0
	07	0	0	2	2	0	0	0
	TOTAL	0	1	4	2	1	0	0
0.01 to 1.00	02	0	3	1	0	0	0	0
	03	0	0	0	0	0	0	0
	06	0	0	1	1	0	0	0
	07	0	0	1	0	0	0	0
	TOTAL	0	3	3	1	0	0	0

continued over

TABLE 11a continued

ds RNA Content ($\mu\text{g/g}$)	Isolate Source (i.e. Plot)	Pathogenicity Rating						
		0.00 to 0.09	0.10 to 0.19	0.20 to 0.29	0.30 to 0.39	0.40 to 0.49	0.50 to 0.59	0.60 to 0.69
1.01 to 2.00	02	0	1	0	2	0	0	0
	03	0	1	0	2	1	1	0
	06	0	0	1	2	1	0	0
	07	0	0	1	0	0	0	0
	TOTAL	0	2	2	6	2	1	0
2.01 to 3.00	02	1	1	1	2	0	0	0
	03	0	1	1	1	0	1	0
	06	0	0	1	0	0	0	0
	07	0	0	0	0	1	0	0
	TOTAL	1	2	3	3	1	1	0

continued over

TABLE 11a continued

ds RNA Content ($\mu\text{g/g}$)	Isolate Source (i.e. Plot)	Pathogenicity Rating						
		0.00 to 0.09	0.10 to 0.19	0.20 to 0.29	0.30 to 0.39	0.40 to 0.49	0.50 to 0.59	0.60 to 0.69
3.01 to 4.00	02	0	0	0	0	0	0	0
	03	0	0	1	0	2	0	0
	06	1	3	0	2	0	0	0
	07	0	0	1	1	0	0	0
	TOTAL	1	3	2	3	2	0	0
4.01 to 5.00	02	1	1	1	1	0	1	0
	03	0	0	0	0	0	0	0
	06	0	0	0	0	0	0	0
	07	0	0	0	0	0	0	0
	TOTAL	1	1	1	1	0	1	0

TABLE 11b. Interaction of ds RNA content (ug/g) with pathogenicity in Ggt isolates assessed by seedling shoot dry weight, (test 2).

ds RNA Content ($\mu\text{g/g}$)	Isolate Source (i.e. Plot)	Pathogenicity Rating						
		0.00 to 0.09	0.10 to 0.19	0.20 to 0.29	0.30 to 0.39	0.40 to 0.49	0.50 to 0.59	0.60 to 0.69
0	07	1	0	0	0	0	0	0
	14	0	0	0	0	0	0	1
	15	0	0	1	0	5	2	2
	WW	0	1	0	0	0	0	0
	TOTAL	1	1	1	0	5	2	3
0.01 to 1.00	07	0	1	1	0	0	0	0
	14	2	0	0	0	1	1	1
	15	0	2	0	0	1	0	0
	WW	0	0	1	0	0	0	0
	TOTAL	2	3	2	0	2	1	1

continued over

TABLE 11b continued

ds RNA Content (µg/g)	Isolate Source (i.e. Plot)	Pathogenicity Rating						
		0.00 to 0.09	0.10 to 0.19	0.20 to 0.29	0.30 to 0.39	0.40 to 0.49	0.50 to 0.59	0.60 to 0.69
1.01 to 2.00	07	0	0	0	0	0	0	0
	14	5	2	0	2	2	1	0
	15	0	1	1	2	1	3	0
	WW	1	0	0	0	0	0	0
	Total	6	3	1	4	3	4	0
2.01 to 3.00	07	2	0	0	0	0	0	0
	14	0	0	0	0	0	0	0
	15	0	0	0	0	0	0	0
	WW	0	0	1	0	0	0	0
	Total	2	0	1	0	0	0	0

TABLE 11b continued

ds RNA Content ($\mu\text{g/g}$)	Isolate Source (i.e. Plot)	Pathogenicity Rating						
		0.00 to 0.09	0.10 to 0.19	0.20 to 0.29	0.30 to 0.39	0.40 to 0.49	0.50 to 0.59	0.60 to 0.69
3.01 to 4.00	07	0	1	0	0	0	0	0
	14	0	0	0	0	0	0	0
	15	0	0	0	0	0	0	0
	WW	0	0	0	0	0	0	0
	Total	0	1	0	0	0	0	0
4.01 to 5.00	07	2	1	0	0	0	0	0
	14	0	0	0	0	0	0	0
	15	0	0	0	0	0	0	0
	WW	0	0	0	0	0	0	0
	Total	2	1	0	0	0	0	0

TABLE 11c Interaction of ds RNA content (ug/g) with pathogenicity in Ggt isolates assessed by seedling shoot dry weight, (test three).

ds RNA Content ($\mu\text{g/g}$)	Isolate Source (i.e. Plot)	Pathogenicity Rating						
		0.00 to 0.09	0.10 to 0.19	0.20 to 0.29	0.30 to 0.39	0.40 to 0.49	0.50 to 0.59	0.60 to 0.69
0	WW	2	0	0	0	0	0	0
	0601	5	1	0	0	0	0	0
	0881	0	1	0	0	1	0	0
	TOTAL	7	2	0	0	1	0	0
0.01 to 1.00	WW	1	0	0	0	0	0	0
	0601	1	1	1	0	0	0	0
	0881	0	1	2	3	0	0	0
	TOTAL	2	2	3	3	0	0	0

continued over

TABLE 11c continued

ds RNA Content ($\mu\text{g/g}$)	Isolate Source (i.e. Plot)	Pathogenicity Rating						
		0.00 to 0.09	0.10 to 0.19	0.20 to 0.29	0.30 to 0.39	0.40 to 0.49	0.50 to 0.59	0.60 to 0.69
1.01 to 2.00	WW	1	0	0	0	0	0	0
	0601	4	0	0	0	0	0	0
	0881	3	4	3	0	0	0	0
	TOTAL	8	4	3	0	0	0	0
2.01 to 3.00	WW	0	0	0	0	0	0	0
	0601	5	3	0	0	0	0	0
	0881	0	0	1	0	0	0	0
	TOTAL	5	3	1	0	0	0	0

continued over

TABLE 11c continued

ds RNA Content ($\mu\text{g/g}$)	Isolate Source (i.e. Plot)	Pathogenicity Rating						
		0.00 to 0.09	0.10 to 0.19	0.20 to 0.29	0.30 to 0.39	0.40 to 0.49	0.50 to 0.59	0.60 to 0.69
3.01 to 4.00	WW	0	0	0	0	0	0	0
	0601	0	1	0	0	0	0	0
	0881	0	0	1	0	1	0	0
	TOTAL	0	1	1	0	1	0	0
4.01 to 5.00	WW	3	0	0	0	0	0	0
	0601	0	0	0	0	0	0	0
	0881	0	0	0	0	0	0	0
	TOTAL	3	0	0	0	0	0	0

distribution patterns recorded for the relevant pathogenicity test, (see Table 5). From these analyses the following salient points are apparent; firstly, no particular ds RNA species class or combination of classes appears to be associated with biasing pathogenicity, (except possibly the combination of ds RNA classes A,^{B,}_λ C, D in Test 1, Table 10d); secondly, there is no effect on pathogenicity associated with increasing ds RNA concentration; thirdly, there is an association between ds RNA-free isolates and increased pathogenicity; demonstrated in Tables 10b and 11b, (N.B. plot 15); whilst in other plots, (e.g. 0601), this does not appear to be true.

Hence on the basis of these results, there does not appear to be any relationship between pathogenicity of Ggt isolates and their ds RNA content, either qualitative or quantitative, except perhaps for the observation that from non-decline soils, (e.g. plot 15), ds RNA-free isolates tend to be more pathogenic than ds RNA-containing isolates. However it should be borne in mind that the skewness observed towards low pathogenicity in two of the three seedling shoot dry weight pathogenicity tests may have masked any association between ds RNA and reduced pathogenicity.

3.1.6 Discussion.

The field isolates of the take-all fungus, Ggt, were isolated from infected wheat roots of seedlings

grown in naturally infested soil. A total of 157 isolates was considered to be sufficiently large to represent a random sample for the analysis of the ds RNA content and pathogenicity of field isolates of Ggt.

All isolates were isolated using a surface sterilization technique; root-washing proved to be unsuccessful. Asher (1978a) achieved isolation of Ggt isolates from infected wheat seedling roots by a washing technique, however these seedlings were grown in artificially infested soil. Hence it would not seem to be possible to compare differences between natural populations isolated by these two distinct techniques. This would have been of interest since Asher (1978a) suggested that Ggt isolates of high pathogenicity may be preferentially selected by surface sterilization of lesioned root fragments. On the other hand it is important to note that Phialophora sp. can be isolated from naturally infected wheat roots by surface sterilization (Slope et al., 1978), indicating that there may not be any preferential selection with regard to pathogenicity when using surface sterilization for isolation. Examination of the effect of isolating Ggt from lesioned root fragments and random root fragments, both by surface sterilization, demonstrated that a far higher success rate is achieved when lesioned fragments are selected.

The pathogenicity results, (Tables 5a, 5b and 5c), from the seedling shoot dry weight tests appear to show

an overall bias towards low pathogenicity. However it is important to note that when each of the three tests are individually examined this is not the case; in test 1, (Table 5a), the pathogenicity distribution is far less biased towards low pathogenicity than in tests 2 and 3, (Tables 5b and 5c). Furthermore two distribution peaks are recorded in test 2, the greater peak occurring in pathogenicity class 0.00 to 0.09, the lesser peak occurring in pathogenicity class 0.40 to 0.49. Also certain plots, e.g. 15 and 0881, do not show pathogenicity distributions typical of the tests in which they were assessed, (i.e. tests 2 and 3, respectively). Asher (1980) also found a general trend towards low pathogenicity in Ggt isolates using the same seedling pathogenicity test. Conversely, both Rawlinson et al. (1973) and Cook and Naiki (1982) using disease indexes to assess Ggt pathogenicity in a large sample of isolates found the majority of Ggt isolates were moderately to highly pathogenic.

Asher (1980) suggests that there are two possible explanations for the bias in pathogenicity levels observed. Firstly, selection pressure on a polygenic system, (i.e. the control of pathogenicity), may result in directional selection producing a skewed distribution (Mather, 1967; 1973; Mather and Jinks, 1977). Secondly, the bias towards low pathogenicity may be an artefact of the test. An explanation for this could be that in the 19 days of growth allowed for

in the shoot dry weight assessment, the fungal isolate may not always have sufficient time to become established in the host, and exert its effect fully on the biomass of the seedlings. Furthermore, the use of a rich potting compost to support the growth of the seedlings during the test may have led to the seedlings being more resistant to infection; alternatively the slightly acidic nature of Levingtons' potting compost may have induced self-inhibition of the Ggt isolates (Romanos et al., 1980; McGinty et al., 1984), again causing failure to infect the seedlings. It is also interesting to note that Naiki and Cook (1983b) have suggested that increased inhibitor production potential in Ggt isolates that have been in culture may lead to decreased pathogenicity, and therefore the possibility that inhibitor production, (for whichever reason), is influencing Ggt pathogenicity results should be considered.

The study of Deacon and Henry (1978a) indicated that due to the damage inflicted on the vascular system by Ggt infection (Asher, 1972b; Clarkson et al., 1975; Fitt and Hornby, 1978) the use of shoot dry weight data to assess intensity of infection is more accurate than measurement of vascular discolouration i.e. root lesions. Deacon and Henry (1978a) suggest that eosin uptake past infection sites would be more accurate still, since it assesses the plant's capacity to

function. However, the measurement of seedling biomass is directly related to the degree to which the vascular system of the seedling is disrupted, and therefore should be an accurate assessment of the pathogenicity of the infecting Ggt isolate, provided the timescale of pathogenicity test permits full expression of vascular disruption and decreased shoot biomass. On these grounds Asher (1980) proposed that field isolates of Ggt are predisposed towards low pathogenicity possibly as a result of directional selection acting on the polygenic control of pathogenicity (Blanch, Asher and Burnett, 1981). However from the surprising overall bias seen in two of the three tests and from the variation in pathogenicity ratings observed where isolates were retested, (see Table 6, and Blanch, 1977) it would seem unwise to believe that the pathogenicity ratings provided by the seedling shoot dry weight tests are accurate; it is more likely that this type of pathogenicity test often provides an underestimated value.

Although there seems to be reasonable doubt about the accuracy of the seedling shoot dry weight pathogenicity test, there are certain points of interest arising from the results provided by the test. Firstly, it was possible on repetition of testing certain Ggt isolates to identify an order of ranking amongst these isolates, (see Table 6). Similarly Blanch (1977), using the same assay, found variation in

shoot dry weight values, but concluded that, on repetition of the test with a selection of isolates, the ranking pathogenicities of the isolates did not change significantly. Hence, secondly it is apparent pathogenic variation in Ggt is a natural phenomenon, (not simply the product of isolate culture), as observed by Rawlinson et al. (1973), Asher (1980) and Cook and Naiki (1982), even though these reports did show differing biases in pathogenicity distribution. Thirdly, when the pathogenicity distributions found in each plot are examined, (taking into account the overall distribution of the test itself), it becomes obvious that these do not always correlate with the decline status of the plot. Notable examples being provided by the decline plots 03 and 0881 and conversely the non-decline plot WW. Similar results were reported by Asher (1980). Therefore it appears that take-all decline is not the result of pathogenic variation, and is more likely to be the result of specific antagonism, as discussed in section 1.3.3.

Analysis of the ds RNA content and distribution of ds RNA species amongst the population of Ggt isolates emphasized the frequency of infection previously observed (Rawlinson et al., 1973) and also the diversity in the nature of the infection (Almond, Buck and Rawlinson, 1977; Buck et al., 1981; Buck, 1984). In this survey marginally over 80% of all the isolates

were seen to be ds RNA containing, and very few of these isolates were seen to have identical ds RNA species distributions, although many ds RNA species, (identified by molecular weight), were seen in a wide variety of isolates, (Tables 7 and 8). The ds RNA species were divided into four groups, based on their molecular weights, three of these groups were chosen to correspond to the molecular weight ranges of previously described Ggt virus groups, i.e. group B ($4.4-3.0 \times 10^6$) corresponds to the group III range, group C ($1.70-1.35 \times 10^6$) to the group II range and group D ($<1.35 \times 10^6$) to the groups I and IV ranges which overlap (Buck et al. 1981; Buck, 1984). Ggt viruses with molecular weights in the range of the fourth group, group A ($7.00-4.60 \times 10^6$) have been described e.g. F3-A (6.3 and 4.9×10^6) and F10-A (6.3×10^6), but the diverse properties of those viruses, e.g. capsid polypeptide molecular weights ranging from 94 000 to 125 000 suggest that more than one group will be needed to accommodate them (Rawlinson and Buck, 1981).

It should be noted that there are also a few isolates in this survey that contain ds RNA species that are not of a size previously seen in Ggt. Isolate of 0601/4 revealed a band of 2.55×10^6 from a virus preparation, (but not from direct ds RNA extraction), that does not fit into any of the previously described ds RNA classes. Jamil et al. (1984) have also recently discovered a 2.3×10^6 ds RNA species in four isolates

of Ggt, however this species was only associated with direct ds RNA extraction fractions, it could not be found associated with viral preparations. Ds RNA of this size would represent a new class, or, since it has only been detected in association with ds RNAs of higher molecular weight, it might be a defective or satellite RNA.

A number of isolates, (e.g. 06S14, 0601/19), contain ds RNA species considerably smaller, (i.e. $<1.00 \times 10^6$), than previously observed (Buck et al., 1981; Buck and Rawlinson, 1981). It is conceivable that these 'unusual' ds RNA species may be satellite RNAs as found in GgV 38-4-A (Romanos, Buck and Rawlinson, 1981; see section 1.2.2). Furthermore, as satellite RNAs they might be capable of altering the phenotype of their host, as seen in the study of ScV (Bostian, Sturgeon and Tipper, 1980; Lolle et al., 1984; see section 1.2.3b), UmV (Koltin et al., 1978; Bozarth et al., 1981), or CARNA5 of cucumber mosaic virus (Waterworth et al., 1979).

The divisions or classes of ds RNA species observed in this study appear to be maintained in Phialophora sp. (lobed hyphopodia), Phialophora graminicola and Ggg; all of which are closely related to Ggt and are also inhabitants of the graminaceous rhizosphere (McGinty, Buck and Rawlinson, 1981; Buck, McGinty and Rawlinson, 1981). Jamil and Buck (1984)

have demonstrated that Phialophora virus 2-2A and Ggt virus 87-1-L are indistinguishable on the grounds of virus particle size and migration in agarose electrophoresis, migration of virus polypeptide and ds RNA species in PAGE, nucleotide sequence homology and antigenic reaction. However virus 87-1-L did not react with antisera to any of the Ggt group I, II or III viruses, suggesting that 87-1-L virus could have been originally a Phialophora virus, while appearing very similar to a group I Ggt virus. It is plausible, as Jamil and Buck (1984) suggest, that virus transfer through anastomosis, (even partial fusion), may occur between Gaeumannomyces spp. and Phialophora spp., and therefore explain their observations regarding viruses 2-2A and 87-1-L.

A survey of ds RNA occurrence in Rhizoctonia solani, another soil-inhabiting plant pathogen, revealed that 49 out of 50 isolates examined, contained at least one ds RNA species (Zanzinger et al., 1984). Furthermore the ds RNA species identified in R.solani fall into the same molecular weight ranges as observed for Ggt and closely-related fungi, (i.e. 3.0 to 5.0×10^6 and 1.0 to 1.6×10^6 , with some species less than 1.0×10^6). Presumably the occurrence of such a similar distribution in molecular weight sizes relates to genome organisation, i.e. divided and undivided genomes and coding capacity, indicating the division between the Totiviridae and the Partitiviridae, the two

families of ds RNA mycoviruses (Brown, 1985). No other soil-borne ds RNA-containing fungi have been surveyed on a large scale to examine their ds RNA species distribution.

From Tables 7 and 8 it is evident that the majority of infected isolates contain at least one ds RNA species from group D, whilst ds RNA species from groups A and C are also widespread. However ds RNA species from group B appear to be rare. In terms of plots, group A ds RNA species are most common in plots 06, 14 and 0881, while group D ds RNA species are most frequent in plots 02 and 14. Isolates from plot 15 are the most often ds RNA-free isolates. Therefore it appears that isolates from non-decline plots, e.g. 14 are frequently ds RNA-containing, as well as isolates from decline plots. Rawlinson et al. (1973) also found that VLP occurred in Ggt isolates from both types of soil.

When the ds RNA species distribution patterns of isolates within each plot are examined, certain salient points arise. Firstly, it is worthy of note that certain isolates that were selected from the same root fragment have the same ds RNA pattern, e.g. 15S12a and 15S12b, while other isolates, e.g. 14S17a and 14S17b, show considerably different patterns. Similarly certain isolates selected from sectoring isolates have identical ds RNA patterns between the parental and

offspring isolates, e.g. 02S8 α and 02S8 β or 02S8 γ , however certain offspring isolates show markedly different patterns from their parents, e.g. 14S8 α and 14S8 β ; 14S15 α and 14S15 β , (although 14S15 α and 14S15 γ have identical ds RNA species patterns). Secondly, it is clear that although few isolates contain exactly the same ds RNA species pattern, there are, especially within plots, certain ds RNA species sub-patterns. Listed below, plot by plot are a series of examples where isolates appear to share a number of ds RNA species:

PLOT	ISOLATES	DS RNA SPECIES (mol. wt., x 10 ⁶)
02	2/14/15	[1.54, 1.24, 1.19]
03	3b/13/15/17/18	[6.1, 5.4, 1.27, 1.19]
06	9/10/14/15/16/17	[6.0, 5.4, 1.60, 1.54, 1.21, 1.14]
07	1/7/10/17/20	[6.0, 5.4, 1.58, 1.54, 1.27, 1.14]
14	3 α /3 β /4/7/8 α /11 α /11 β / 15 α /15 γ /17b	[6.0, 5.4, 1.14]
15	3/11/15/18/20	[6.0, 5.4, 1.21, 1.14]
WW	7a/7b/8/10	[5.8, 1.58, 1.14]
0601	1/6/8/9/10/11/13/14/15	[5.8, 1.58, 1.56]
0881	1/6/8/9/10/11/13/14/15	[5.8, 1.58, 1.56]

Thirdly, it appears that certain ds RNA species, (and quite possibly many others), are capable of replicating in their host isolate in the absence of other ds RNA species, e.g. 6.9 x 10⁶ (06S2), 6.0 x 10⁶ (06S11, 07S18, WWS6), 1.30 x 10⁶ (0601/18), 1.27 x 10⁶

(03S5, 14S10), and 1.19×10^6 (02S16, 03S8a). Hence these ds RNA species must be capable of providing all that they require in order to replicate either as a virus or perhaps as a form of unencapsidated ds RNA. It is quite feasible that ds RNA species of these sizes can replicate as viruses without additional species, since there are identified Ggt viruses with single ds RNA species sizes (Buck et al., 1981; Rawlinson and Buck, 1981; Buck, 1984). There is no evidence of unencapsidated ds RNA replication in Ggt, although this is believed to be the case in some strains of Endothia parasitica (Van Alfen, 1982).

From these three observations certain deductions do seem to be plausible. Firstly, the diversity of ds RNA patterns generally, and also in the cases of isolates that originated from the same root fragment or were separated in culture through sectoring, implies that isolates of Ggt do not readily anastomose, hence maintaining their individual nuclear and cytoplasmic composition. However on the other hand the existence of sub-patterns of ds RNA distribution that appear to have identities confined within plots, strongly suggests that transfer of ds RNA species, (presumably encapsidated), does occur. This latter argument is strengthened by the report of Jamil et al. (1984) who demonstrated that ds RNA species from various isolates of Ggt in several v-c groups show sequence homology,

which suggests that virus transmission between isolates of different v-c groups could have occurred.

The surprisingly large number of v-c groups, (18), observed by Jamil et al. (1984) from the study of 31 isolates of Ggt from a single field, presumably would also be the case in each of the plots studied here. Provided this is the case, ds RNA species transfer must be limited by the number of v-c groups in Ggt, which probably results in any one isolate being relatively stable in its nuclear and cytoplasmic composition after an initial period of susceptibility to full or partial anastomosis. Furthermore the findings of Jamil and Buck (1984) implying that virus transfer may occur between Phialophora spp. and Ggt, suggest that the observations of Rawlinson and Muthyalu (1975; 1976), regarding the appearance of virus in Ggt isolates in newly cereal-cultivated soil result from virus transfer between Ggt isolates, possibly initiated by virus transfer between isolates of Phialophora sp. and Ggt.

Analysis of the concentration of ds RNA found in each of the field isolates of Ggt indicated a range of 0 to 5 µg/g, (ds RNA/g fresh weight mycelium), which is similar to previous observations on ds RNA yeilds from Ggt (McFadden, 1982), and less than the range observed for Phialophora spp. and Ggg, i.e. up to 20 µg ds RNA/g fresh weight mycelium (McGinty, Buck and Rawlinson, 1981). The overall distribution pattern of

ds RNA-containing isolates in this survey is slightly skewed towards low concentration, the distribution curve peaking in the 1.01-2.00 $\mu\text{g/g}$ category (see Table 9). It is noticeable that for plots 0601, 02 and 06 the distribution peak is in the 2.01-3.00 $\mu\text{g/g}$ category, (or greater), whilst both plots 14 and 15 appear to be biased towards low or zero ds RNA concentration, (especially in the case of the latter plot). Although it could be said that the differing biases in ds RNA concentration distribution seem to correlate with decline status of the plot, (i.e. plots 02 and 0601, in decline; plots 14 and 15, non-decline), it should be noted that plot 06, which has ds RNA concentration distribution similar to plots 02 and 0601, is a non-decline plot, having been under wheat cultivation only as long as plots 14 and 15. On the other hand the non-decline plots WW and 07 appear to be mostly biased towards low ds RNA content, although a number of their isolates do have high ds RNA levels. This implies that the overall level of virus infection found in isolates from a particular plot therefore does not necessarily correlate with either the decline status or the duration of continuous cereal cultivation of the plot.

It is interesting to note that those isolates that have high ds RNA concentrations, (i.e. in excess of 3.01 $\mu\text{g/g}$), have both high molecular weight ds RNA

species and also a considerable number of ds RNA species. However not all isolates with such ds RNA species compositions fall into this category, (i.e. they have lower ds RNA concentrations); indeed isolate 3bla, when studied previously, was shown to contain nine ds RNA species, (the largest being 4.1×10^6) and yet the ds RNA yield from it did not exceed $3\mu\text{g/g}$ (Buck et al., 1981; McFadden, 1982). It should be noted though that Ggt isolates studied here, which only contained a few ds RNA species, very rarely had ds RNA concentrations greater than $2\mu\text{g/g}$. Hence it would seem that ds RNA concentration in isolates of Ggt is largely dependent on the number and size of the ds RNA species present, although in certain examples it is plausible to suggest that the host isolate is suppressing the replication of the ds RNA species, which would explain the low ds RNA levels seen in certain isolates that contain many ds RNA species, (cf. 02S9 β and 02S9 α).

Hence through PAGE and spectrophotometry a detailed picture of the ds RNA composition of a field isolate population of Ggt was obtained. The examination of a selection of isolates for virus and viral ds RNA shows that the ds RNA patterns observed by direct nucleic acid extraction are almost certainly of viral origin. This observation could be of importance for later protoplast infectivity experiments using virus as inoculum.

The interaction of the pathogenicity ratings and

ds RNA composition does not demonstrate any obvious trend that permits the conclusion that a specific group or groups of ds RNAs are reducing the pathogenicity of Ggt, (see Tables 10a to f, 11a, b and c). This is in agreement with the investigation of Rawlinson et al. (1973) where the presence or absence of VLP in an isolate was examined in relation to the pathogenicity of an isolate. It is interesting to note that the study of Rawlinson et al. (1973) only observed 35 and 27 nm virus particles, the ds RNA of which would correspond to the ds RNA classes C and D of this study, (i.e. $1.70-1.35 \times 10^6$ and $<1.35 \times 10^6$, respectively). Therefore the inclusion in this study of the ds RNA classes A and B, (i.e. $7.0-4.60 \times 10^6$ and $4.40-3.0 \times 10^6$, respectively), which are presumably the genomic components of the 40 nm virus particles (Buck et al., 1981; Rawlinson and Buck, 1981), expands as well as confirms the report of Rawlinson et al. (1973).

The bias to the seedling shoot dry weight pathogenicity results may tend to underestimate pathogenicity levels in Ggt, (as previously discussed). If this is accepted as being the case, then it should be noted that this confirms the overall conclusion that ds RNA either qualitatively or quantitatively does not appear to reduce pathogenicity generally. Indeed if the pathogenicity distributions examined were actually higher than recorded then this conclusion would be

strengthened.

This overall conclusion does not eliminate the possibility that a small proportion of ds RNAs might cause hypovirulence, or at least severely reduce pathogenicity. These would presumably be mutant or satellite viruses, (discussed earlier in this section), and therefore they would not be evident in an overall survey, particularly if the skewness towards low pathogenicity is an artefact of the seedling shoot dry weight assay.

The observation that isolates from plot 15 are most frequently ds RNA-free and highly pathogenic is intriguing. Taken along with the considerations discussed above it would appear that the factors affecting pathogenicity in Ggt could be quite complex. The possibilities include: a general adverse effect on the fitness of the pathogen due to virus infection, specific effects caused by mutant or satellite viruses, and the ability of some isolates of Ggt to suppress the replication of its ds RNA viruses.

It should be borne in mind that the assertion that hypovirulence occurs in Ggt and is virus-associated (Lapierre et al., 1970; Lemaire et al., 1970) has not been confirmed or discounted by this survey, (unlike the situation in Endothia parasitica, where this association appears to be well-founded; Day et al., 1977; Anagnostakis and Day, 1979). As has been demonstrated by the results of Finkler et al. (1983)

and Zanzinger et al. (1984), ds RNA is widespread in Rhizoctonia solani and is apparently not generally associated with reduced virulence; which would appear to be in contrast with the report of Castanho et al. (1978). Therefore to identify such an association between ds RNA and reduced pathogenicity, (if there is any), genetically-related isolates of Ggt with and without ds RNA must be investigated, (as explained in section 1.4). This survey of ds RNA composition and pathogenicity in field isolates of Ggt provided suitable isolates for further study, (i.e. ds RNA-containing, weakly pathogenic isolates), as described in section 3.2.

3.2 ANALYSIS OF SINGLE CONIDIAL AND SINGLE ASCOSPORE ISOLATES OF GGT.

A number of single conidial isolates were made from a selection of field isolates. The field isolates were of two types; weakly pathogenic and ds RNA-containing, (with varied ds RNA species patterns), or strongly pathogenic and ds RNA-free; the latter being the control isolates. These single conidial isolates, along with a number of isolates discussed by McFadden, Buck and Rawlinson (1983), were assessed for their ds RNA content and pathogenicity. The single conidial isolates of the field isolates, prior to being pathogenicity-tested, were passaged through wheat seedlings in order to maintain their pathogenicity levels. A selection of these single conidial isolates were growth-rate tested at different pHs and were also induced to form perithecia.

The field isolates selected for further study are listed below:

Weakly pathogenic and ds RNA-containing

02S7, 06S14

WWS2a, WWS10,

0601/4, (0881/14)*

Isolates discussed by McFadden, Buck and Rawlinson (1983), used in this study:

3blA (original isolate).

3bla con D and 3bla con E (two conidial isolates of

Highly pathogenic and ds RNA-free

14S12, 15S8,

0881/18.

3bla with the same ds RNA species as 3bla).

3bla con DA3 (Ascospore isolate of 3bal con D; ds RNA-free).

3bla con EA1 (Ascospore isolate of 3bla con E; ds RNA-free).

* This isolate was believed to be ds RNA-containing at the beginning of this study.

3.2.1 Production and Isolation of Conidia from Field Isolates of Ggt.

Conidia of Ggt, (i.e. of the elliptical type; $6 \times 3 \mu\text{m}$), were produced and isolated by the method described in section 2.9. In Figure 11, a number of conidia, (of the type formed and isolated), are indicated. A culture of isolate 15S8 producing abundant conidia was estimated, to have 5.4×10^5 conidia/ml; which is similar to the figure of 2.6×10^5 conidia/ml reported by Deacon (1976). (Most cultures produced 10^4 - 10^5 conidia/ml).

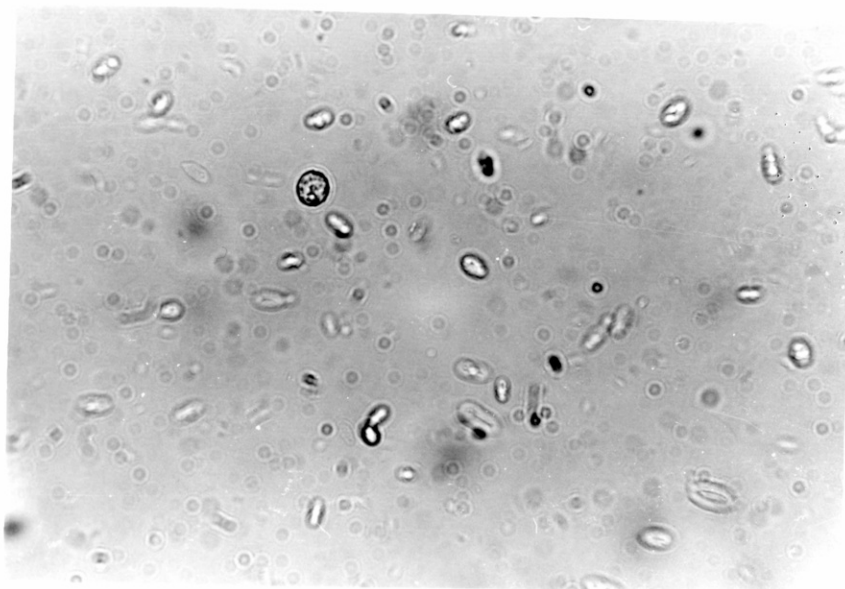
Listed below are the number of single conidial isolates obtained from the field isolates:

<u>Field Isolate</u>	<u>Number of Progeny</u>
02S7	4; 02S7 A, 02S7 B, 02S7 C, 02S7 D
06S14	3; 06S14 A, 06S14 B, 06S14 C
WWS2a	3; WWS2a A, WWS2a B, WWS2a C
WWS10	2; WWS10 A, WWS10 B
0601/4	None
0881/14	3; 0881/14 A, 0881/14 B, 0881/14 C

14S12	4; 14S12 A, 14S12 B, 14S12 C, 14S12 D
15S8	4; 15S8 A, 15S8 B, 15S8 C, 15S8 D
0881/18	2; 0881/18 A, 0881/18 B

A total of 25 conidial isolates.

Figure 11



A suspension of conidia from isolate WWS10 (Magnification =
x 400).

3.2.2. Production of Perithecia, Asci and Ascospores from Isolates of Ggt.

This was achieved using the method described in section 2.10. A selection of the field isolates and conidial isolates were examined in this respect, the results being represented in Table 12.

TABLE 12

Isolate	Number of perithicia/ petri dish
O2S7 A	None
O6S14 A	None
O6S14 C	2 to 5
WWS2a A	50 to 100
14S12	50 to 100
O881/14	25 to 50
O881/18	None
3bla conD	2 to 5

Figures 12 and 13 show a perithecium with asci, and asci with ascospores, respectively, from isolate WWS2aA. Ascospores from WWS2aA were observed to be on average $84.5 \times 3 \mu\text{m}$, with a range in length of 62.7 to $101.2 \mu\text{m}$. Ascospores from other isolates appeared to be of the same dimensions.

3.2.3. Passaging of Single Conidial Isolates of Ggt.

The single conidial isolates of the Ggt field isolates were passaged through wheat seedlings, (see

Figure 12



Peritheciium of WWS2a A, broken open demonstrating asci
(Magnification = x250).

Figure 13



Asci and ascospores of isolate WWS2a A. (Magnification =
x 625).

section 2.8), in an attempt to avoid loss of pathogenicity in the isolates, due to maintenance in culture.

The following isolates were successfully re-isolated, (these isolates are designated 'p', to indicate that they have been passaged):

02S7 A p

06S14 B p, 06S14 C p

WWS2a A p, WWS2a B p, WWS2a C p

14S12 A p, 14S12 D p

15S8 A p

0881/14 A p, 0881/14 B p, 0881/14 C p

Hence in addition to the 25 conidial isolates, 12 passaged conidial isolates were obtained.

3.2.4. Growth Rate Assessment.

A number of the conidial isolates, including one of the passaged conidial isolates, were observed to be slow-growing on normal PDA (pH5.6). Hence it was postulated that these cultures may have become inhibitor-producing (Romanos et al., 1980; McGinty et al., 1984). To investigate this possibility, the slow-growing isolates, along with a selection of normal isolates, were grown on PDA at pH5.6 and pH7.0 and their growth rates measured, (section 2.11). McGinty et al. (1984) observed that inhibitor-producers are most active in this respect at pHs between 3.5 to 5.0;

while at pH7.0 no inhibitor production was ever observed. Hence it was reasoned if these conidial isolates had switched on their capacity to produce inhibitor, then they should return to normal growth on PDA buffered to pH7.0.

The slow-growing isolates were: 15S8A, 15S8B, 15S8D, 0881/14B and 06S14 B p. The control isolates used were: 15S8, 0881/14, 0881/14 A, 02S7 A p, 14S12 A p and 06S14 B. The results are summarised in Table 13. From these results it appears that of the slow-growing isolates only 0881/14 B responds to the pH shift from pH5.6 to pH7.0, implying that 0881/14 B was inhibiting its own growth at pH5.6. The other slow-growing isolates are presumably not self-inhibiting since at pH7.0 their growth rates were not increased. Hence some other cause, possibly linked with morphogenesis is responsible.

3.2.5 Assessment of Pathogenicity of the Conidial Isolates of Ggt.

The pathogenicity ratings of the conidial isolates of Ggt were primarily determined using the seedling disease index assessment derived from Cook and Naiki (1982), (see section 2.7 b). Although this test takes considerably longer than the shoot dry weight assessment it was found to be easy to use and to give good agreement on repetition of the test. The pathogenicity ratings of these isolates were also

TABLE 13 Comparison of growth rates of conidial isolates of Ggt on PDA (pH5.6) and PDA (pH7.09).

<u>Isolate</u>	<u>pH</u>	<u>Growth rate)</u> <u>(mm/day</u>
02S7 A p	5.6	7.70
	7.0	5.15
06S14 B	5.6	7.75
	7.0	8.25
14S12 A p	5.6	7.05
	7.0	9.55
15S8	5.6	7.25
	7.0	9.10
0881/14	5.6	7.65
	7.0	10.30
0881/14 A	5.6	8.75
	7.0	10.50
06S14 B p	5.6	3.4
	7.0	2.4
15S8 A	5.6	3.65
	7.0	3.85
15S8 B	5.6	3.65
	7.0	3.20
15S8 D	5.6	3.45
	7.0	3.65
0881/14 B	5.6	2.90
	7.0	8.60

assessed using the seedling/agar plate disease index, (section 2.7c) and the mature plant assessment, (section 2.7d).

As Cook and Naiki (1982) also found, close agreement was noted between the condition of the four seedlings used to assess each isolate. Table 14 represents a compilation of the results of two separate seedling disease index assessments. It is noticeable that there is little difference between the average disease index values for any isolate in the two assessments. Furthermore, the conidial isolate, (including passaged conidial isolates), mostly gave index values close to those values observed for the field isolate from which they were derived. Instances where this was not so, with the average disease index values differing by 1.50 or more were observed between: 06S14 and 06S14 C; 06S14 and 06S14 C p; WWS2a and all the WWS2a conidial isolates; 0881/14 and 0881/14 B; 0881/14 and 0881/14 B p; 3bla conD and both 3bla and 3bla conDA3.

Using the scores that produced the average disease index values of these isolates, the differences between the means of the populations were tested, (section 2.7e). In each of the cases quoted above the populations were found to be significantly different ($p < 0.05$); while a test of 02S7:02S7 A or WWS2a A:WWS2a C gave a result of $p > 0.05$, indicating similar or identical populations.

TABLE 14 Seedling disease index pathogenicity ratings of a selection of Ggt isolates and their conidial progeny isolates.

Isolate	Average disease index value*	
	a)†	b)†
02S7	0.25 (0,1,0,1)	0.75 (1,1,0,1)
02S7 A	1.00 (1,1,1,1)	0.50 (1,0,0,1)
02S7 B	0.50 (1,0,1,0)	0.75 (0,1,1,1)
02S7 C	0.75 (1,1,1,0)	0.75 (1,0,1,1)
02S7 D	0.25 (1,0,0,0)	0.75 (0,1,1,1)
02S7 A p	1.00 (1,1,1,1)	-
06S14	0.25 (0,1,0,0)	1.00 (1,1,1,1)
06S14 A	1.75 (2,1,2,2)	1.00 (1,1,1,1)
06S14 B	1.25 (1,2,1,1)	1.25 (1,1,2,1)
06S14 C	4.25 (4,5,4,4)	3.75 (4,4,3,4)
06S14 B p	0.75 (1,0,1,1)	-
06S14 C p	2.75 (3,4,2,2)	-
14S12	4.25 (4,5,4,4)	4.25 (4,4,4,5)
14S12 A	4.00 (4,4,4,4)	4.00 (4,4,4,4)
14S12 B	3.50 (3,4,3,4)	-
14S12 C	3.25 (3,4,3,3)	-
14S12 D	3.75 (3,4,4,4)	-
14S12 A p	4.00 (4,4,4,4)	-
14S12 D p	4.25 (4,5,4,4)	-
15S8	1.25 (1,1,1,2)	-
15S8 A	0.25 (1,0,0,0)	-
15S8 B	0.25 (0,0,0,1)	-

continued over

TABLE 14 continued

Isolate	Average disease index value*	
	a)†	b)†
15S8 C	0.75 (1,1,0,1)	-
15S8 D	0.25 (0,0,1,0)	-
15S8 A p	0.50 (1,0,1,0)	-
WWS2a	1.25 (1,0,2,2)	1.75 (1,1,2,3)
WWS2a A	3.25 (3,3,3,4)	3.75 (4,3,4,4)
WWS2a B	3.50 (4,4,3,3)	3.25 (3,4,3,3)
WWS2a C	4.00 (4,5,3,4)	3.50 (3,3,4,4)
WWS2a A p	3.75 (4,4,4,3)	-
WWS2a B p	3.00 (3,3,4,2)	-
WWS2a C p	4.25 (4,4,4,5)	-
WWS10	2.25 (2,3,2,2)	-
WWS10 A	3.00 (2,3,3,4)	-
WWS10 B	3.00 (3,2,4,3)	-
0881/14	4.00 (4,3,14,5)	3.75 (3,4,4,4)
0881/14 A	3.50 (3,3,4,4)	3.25 (3,3,4,3)
0881/14 B	0.75 (1,0,1,1)	-
0881/14 C	4.00 (4,4,4,4)	-
0881/14 A p	4.50 (5,4,4,5)	-
0881/14 B p	0.75 (1,0,1,1)	-
0881/14 C p	3.50 (3,3,4,4)	-
0881/18	3.25 (2,5,4,2)	-
0881/18A	3.75 (5,3,3,4)	-

continued over

TABLE 14 continued

Isolate	Average disease index value*	
	a) †	b) †
0881/18 B	3.00 (3,4,3,2)	-
3bla	2.25 (2,2,3,2)	2.25 (2,3,2,1)
3bla conD	0.75 (1,1,0,1)	1.25 (1,1,2,1)
3bla conDA3	3.75 (4,4,3,4)	3.00 (3,3,3,3)
3bla conE	1.25 (1,2,1,1,)	1.50 (2,1,2,1)
3bla conEA1	2.00 (2,2,2,2)	2.25 (2,3,2,2,)

* Actual scores in brackets

† two separate assessments

TABLE 15 Results of the seedling/agar plate disease index assesement.

Isolate	Disease Rating	
02S7 B	Fairly severe	2.0
02S7 C	Severe/v. severe	3.5
02S7 D	Fairly severe	2.0
02S7 A p	Mild	1.0
06S14 A	Fairly severe/mild	1.5
06S14 B	Fairly severe/mild	1.5
06S14 C p	Severe/v. severe	3.5
14S12 B	V. severe	4.0
14S12 C	V. severe	4.0
14S12 A p	Severe	3.0
14S12 D p	Severe	3.0
15S8 B	Mild/fairly severe	1.5
15S8 C	Mild/fairly severe	1.5
15S8 D	Fairly severe	2.0
WWS2a B p	Severe	3.0
WWS2a C p	Severe	3.0
WWS10 B	V. severe	4.0
WWS10 A p	Mild/healthy	0.5
0881/14 A p	V. severe	4.0
0881/14 B p	Mild	1.0
0881/18 A	V. severe/severe	3.5

A number of the conidial isolates were assessed using the seedling/agar plate disease test, (section 2.7c), and these results are represented in Table 15. Overall the same pattern is observed from Table 15 as from Table 14, i.e. most isolates appear in the same relative position, in terms of pathogenicity. One or two isolates, (e.g 02S7 C, WWS10 A p), appear to have a considerably different pathogenicity value from that observed in the seedling disease index assessment; this may be due to the artificiality of the test. However, in general the seedling/agar plate disease assessment does seem to give good agreement with the seedling disease index assessment.

The pathogenic effect of a selection of the conidial isolates, along with the field isolates from which they were derived, on fully grown wheat plants was determined, (see section 2.7d). Table 16 summarizes these results. From the disease index ratings, the pattern observed using the seedling disease index assessment appears to be largely confirmed. For instance isolates 02S7, 06S14 and their progeny, (except 06S14 C), appear to be weakly pathogenic; (isolate 06S14 C is confirmed as strongly pathogenic). Conversely isolates 14S12, 0881/14 and their progeny, (except 0881/14 B), are confirmed as highly pathogenic isolates. (It is worthy of note that 0881/14 B, though is evidently much less pathogenic than its parent). Certain isolates, however appear to

TABLE 16 Pathogenic effect of certain Ggt isolates on fully-grown wheat plants.

Isolate	Ears of Grain		Observations	Disease Index
	Healthy*	Rudimentary*		
Control plants (uninoculated)	12	3	Healthy	0
02S7	9	5	Roots slightly stunted; 10-25% lesioned NSH ¹	7
02S7 A	9	5	Roots mostly healthy; <10% lesioned NSH	4
06S14	11	3	Roots slightly stunted; < 10% lesioned NSH	3
06S14 B	8	4	Roots largely healthy; <10% lesioned NSH	5
06S14 C	5	6	Roots severely stunted; 50-75% lesioned RSH ²	14
14S12	5	7	Roots severely stunted; 50-75% lesioned RSH	15

TABLE 16 continued

Isolate	Ears of Grain		Observations	Disease Index
	Healthy*	Rudimentary*		
14S12 B	6	5	Roots severely stunted; 50-75% lesioned RSH	14
15S8	8	5	Roots slightly stunted; <10% lesioned NSH	6
15S8 A	9	1	Roots slightly stunted; <10% lesioned NSH	4
15S8 C	9	8	Roots largely healthy; <10% lesioned NSH	7
WWS2a	8	5	Roots slightly stunted; 25-50% lesioned RSH	10
WWS2a C	7	10	Roots badly stunted; 50-75% lesioned RSH	14

continued over

TABLE 16 continued

Isolate	Ears of Grain		Observations	Disease Index
	Healthy*	Rudimentary*		
WWS10	6	6	Roots slightly stunted; 25-50% lesioned RSH	11
WWS10 B	6	10	Roots badly stunted; 50-75% lesioned RSH	15
0881/14	5	8	Roots severely stunted; >75% lesioned RSH	16
0881/14 A	6	7	Roots severely stunted; >75% lesioned RSH	16
0881/14 B	8	7	Roots slightly stunted; 25-50% lesioned NSH	9
0881/18	8	6	Roots slightly stunted; 10-25% lesioned NSH	8
0881/18 A	8	6	Roots slightly stunted; 10-25% lesioned NSH	8

TABLE 16 continued

Isolate	Ears of Grain		Observations	Disease Index
	Healthy*	Rudimentary*		
0881/18 B	3	6	Roots slightly stunted; 10-25% lesioned RSH	11
3b1a	7	5	Roots largely healthy; <10% lesioned NSH	6
3b1a conD	6	4	Roots largely healthy; <10% lesioned NSH	6
3b1a conDA3	9	4	Roots slightly stunted; 50-75% lesioned NSH	8
3b1a conE	8	7	Roots slightly stunted; <10% lesioned NSH	7
3b1a conEA1	8	8	Roots slightly stunted; 10-25% lesioned NSH	9

* Number of ears of grain per 3 mature wheat plants.

¹ NSH = Normal shoot height. ² RSH = Reduced shoot height.

behave slightly differently than as observed in the seedling tests. Isolate 0881/18 and its progeny appear to be slightly less pathogenic, while isolate WWS2a appears to be slightly more pathogenic. These apparent changes in behaviour may well be true reflections of the relative pathogenic activity of these isolates at different stages in the development of wheat; or it may be an artifact of the method of assessment; perhaps greater emphasis should be placed on the numbers of healthy and rudimentary ears of grain produced by the plants.

In general, the two seedling tests and the mature plant test appear to have provided a consistent assessment of the pathogenicity levels of the isolates examined.

3.2.6 Analysis of ds RNA Content of the Conidial Isolates of Ggt.

All of the conidial isolates, including those that had been passaged were cultured and subjected to the extraction technique described in section 2.12. Both crude and cellulose-purified, (i.e. ds RNA selective purification), samples were analysed by PAGE, (section 2.16). Figures 14 and 15 are representative of the polyacrylamide gels of the crude and purified samples, respectively. As a result it was observed that many of the isolates were identical in ds RNA species content to the field isolates, from which they were derived;

however certain isolates appeared to have lost the ds RNA species observed in their parental isolates, (see Table 17).

Apart from the 0881/14 derived isolates, isolates in which no ds RNA species could be observed can be separated into two classes, i.e. those that appear to have lost their ds RNA on conidiation and those that appear to have lost their ds RNA on passaging. Into the former category fall isolates 06S14 C, WWS2a A, WWS2a B and WWS2a C, and into the latter category fall isolates 02S7 A p and 06S14 B p. The isolates derived from 0881/14 appear to be ds RNA-free, while 0881/14 was initially seen to be ds RNA-containing. However on re-examination of 0881/14, after isolation of the 0881/14 conidial isolates, it was found to be ds RNA-free. Hence it is presumed that 0881/14 lost its ds RNA infection during culture, prior to isolation of the conidial isolates, 0881/14 A, 0881/14 B and 0881/14 C.

The level of sensitivity of analysis by PAGE was assessed by electrophoresing a dilution series of a sample of ds RNA of known concentration from a variety of isolates e.g. WWS2a, (see Figure 16). The limit of sensitivity in each case was found to be the 10^{-2} dilution, which in the case of isolate WWS2a was equivalent to ca. 10 ng/ds RNA band.

3.2.7. Discussion.

Conidia of the uninucleate elliptical type,

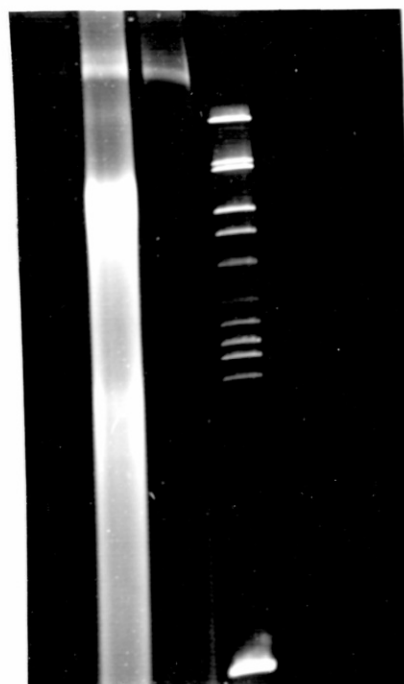
Figure 14. Polyacrylamide gel electrophoresis of crude extracts of conidial isolates of Ggt.

a) 1 2 3 4 5



-4.1×10^6
 -3.5
 -1.6
 -1.54
 $-1.43, 1.43$
 -1.19
 -1.11×10^6

b) 1 2 3 4



-4.1×10^6
 -2.7
 -2.6
 -1.87
 -1.70
 -1.44
 -1.24
 -1.11
 -0.99
 -0.94
 -0.89

-0.46×10^6

a) 1: O6S14 C (crude extract)

2: O6S14 B p (crude extract)

3: O6S14 B (crude extract)

4: O6S14 A (crude extract)

5: 3bla ds RNA (standard)

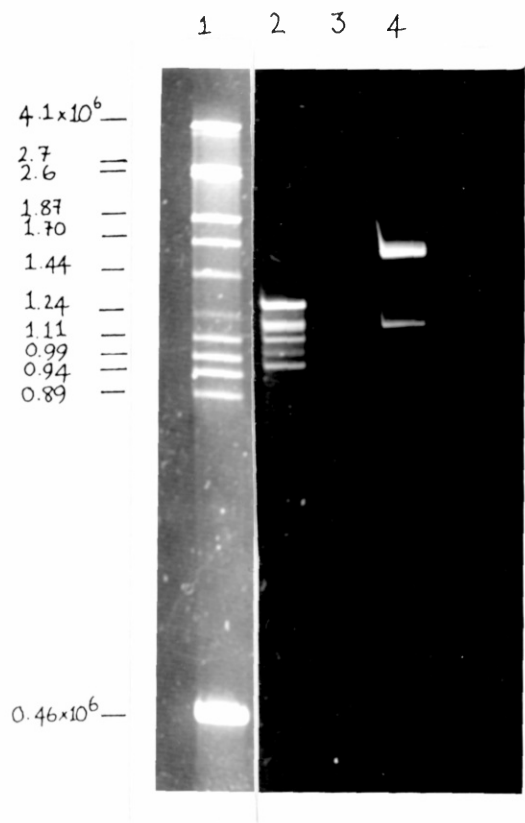
b) 1: WWS2a C (crude extract)

2: WWS2a B (crude extract)

3: WWS2a A (crude extract)

4: AfV, PsV, ds RNA (standard)

Figure 15. PAGE of purified extracts of conidial isolates of Ggt.



- 1: AfV, PsV (standard)
- 2: O2S7 A ds RNA
- 3: O6S14 C ds RNA
- 4: WWS10 B ds RNA

TABLE 17 Summary of ds RNA species distributions in the conidial isolates of Ggt.

Isolate	ds RNA	Isolate	ds RNA
02S7 A	Ft ¹	15S8 C	N/p
02S7 B	Ft	15S8 D	N/p
02S7 C	Ft	15S8 A p	N/p
02S7 D	Ft	WWS2a A	N
02S7 A p	N ²	WWS2a B	N
06S14 A	Ft	WWS2a C	N
06S14 B	Ft	WWS2a A p	N
06S14 C	N	WWS2a B p	N
06S14 B p	N	WWS2a C p	N
06S14 C p	N	WWS10 A	Ft
14S12 A	N/p ³	WWS10 B	Ft
14S12 B	N/p	0881/14 A	N/p
14S12 C	N/p	0881/14 B	N/p
14S12 D	N/p	0881/14 C	N/p
14S12 A p	N/p	0881/14 A p	N/p
14S12 D p	N/p	0881/14 B p	N/p
15S8 A	N/p	0881/14 C p	N/p
15S8 B	N/p	0881/18 A	N/p
		0881/18 B	N/p

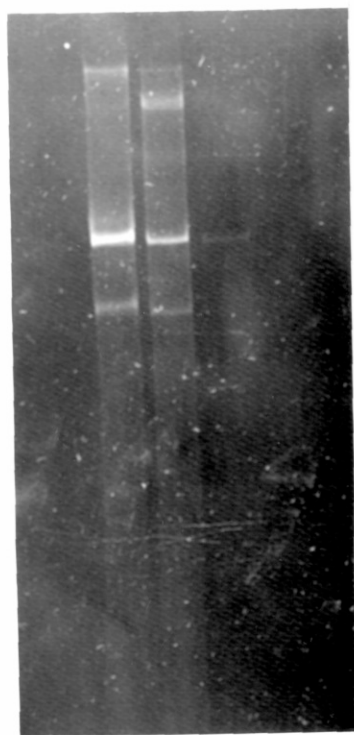
¹Ft = faithful transmission of ds RNA species distribution.

²N = No ds RNA observed.

³N/p = No ds RNA observed, as seen in parental field isolate.

Figure 16 PAGE of a dilution series of a ds RNA sample from isolate WWS2a.

1 2 3 4



- 1: WWS2a ds RNA 10^0
- 2: WWS2a ds RNA 10^{-1}
- 3: WWS2a ds RNA 10^{-1}
- 4: WWS2a ds RNA 10^{-3}

(average dimensions, $6 \times 3 \mu\text{m}$), were produced using the method described by Deacon (1976a) and McFadden (1982), from all of the field isolates that were selected for study. Single conidia were successfully isolated from all of these isolates, (except isolate 0601/4). These conidia were found to be germinable, especially if they were from a culture that had just begun producing conidia. (The conidia produced by isolate 0601/4 did not appear to be germinable). Yields of conidia produced did vary to some extent, according to the isolate, a maximum in the order of 5×10^5 conidia/ml being observed, which is in agreement with the observation of Deacon (1976a).

Certain of the isolates, (both field isolates and conidial isolates), were shown to be capable of producing perithecia, while certain other isolates appeared to be incapable of this. It is noticeable that highly pathogenic isolates, e.g. WWS2a A and 14S12 produced abundant perithecia while weakly pathogenic isolates, such as 02S7 A and 06S14 A produced no perithecia. However these characteristics are not necessarily linked, contrary to the suggestion of Chambers and Flentje (1967a) and Lapierre et al. (1970), since isolate 0881/18 (a highly pathogenic isolate), produced no perithecia. Moreover, the variation in numbers of perithecia produced by each isolate does not show any relationship with their respective pathogenicities. The ascospores of isolate

WWS2a had dimensions typical of Ggt ascospores (Walker, 1981) and furthermore the ascospores from the other producing isolates appeared to be similar in shape and dimensions.

The observations on the growth rates, (Table 13), of certain of the conidial isolates illustrate that the majority grow at a rate of approximately 6-10 mm/day under the conditions described. This is in agreement with the growth rate normally observed for Ggt (Walker, 1981). However, isolates such as 15S8 A and 06S14 B p, (but not 06S14 B), have a much reduced growth rate, at either pH5.6 or pH7.0. Isolate 0881/14 B is similarly very slow growing at pH5.6, but when grown at pH7.0, it grows at a normal rate. Hence isolate 0881/14 B is probably inhibiting its own growth at pH5.6 (Romanos et al., 1980) but at pH7.0 it cannot express and/or secrete the inhibitor, as has been seen with all other inhibitor producing Ggt isolates (McGinty et al., 1984). Presumably the process of conidiation (and, in the case of 06S14 B p, the process of passaging), has activated a control mechanism which retards the growth of the isolate. In the case of 0881/14 B, the mechanism has been apparently identified as inhibitor production, however for the remaining isolates the control mechanism is unidentified. Provided 0881/14 B is capable of inhibitor production then this observation is in agreement with the reports of Romanos

et al. (1980) and McGinty et al. (1984) that inhibitor production by Ggt isolates is independent of virus infection, since 0881/14 B appeared to be virus-free. Moreover, Naiki and Cook (1983a; 1983b) have suggested that loss of pathogenicity in Ggt, (during culture), is not associated with mycovirus infection since single ascospore isolates produced from three parental isolates, (that had decreased in pathogenicity during culture), were less frequently pathogenic than single ascospore isolates, (i.e. whilst the latter were still highly pathogenic). Hence it would seem more likely that these results are due to a shift in nuclear genetic determination, either through a change in nuclear ratio, (implying that many Ggt isolates are heterokaryotic), or through a change in gene expression. This may result in part from an increased potential of Ggt isolates to produce self-inhibitor after prolonged culture (Naiki and Cook, 1983b).

The pathogenicity ratings of all the conidial isolates, their parents and isolate 3bla and its progeny, were determined using two seedling tests, (Tables 14 and 15), ^{and} an assessment of infected mature wheat plants, (Table 16). These tests were in close agreement and generally speaking confirmed the results obtained by the shoot dry weight assessment, (allowing for the limitations of the latter test, as discussed in section 3.1.6). The seedling disease index assessment of Cook and Naiki (1982) was found to give easily

reproducible results, which made identification of isolates, that had pathogenicities noticeably different from their parent isolates, straightforward. Hence isolates 06S14 C, 06S14 C p, WWS2a A, WWS2a B, WWS2a C and 3bla conDA3 were found to have significantly increased pathogenicity levels when compared with their parent isolates. Conversely isolates 0881/14 B and 0881/14 B p had significantly decreased pathogenicity levels compared with 0881/14, possibly because 0881/14 B inhibits its own growth rate. Apart from slight variations, the same pattern in terms of pathogenicity was observed in both the seedling/agar plate assessment and the mature wheat plant assessment. Notably none of the passaged isolates appeared to behave any differently from its non-passaged counterpart, implying that either passaging does not provide Ggt with the chance to regain its pathogenicity, or none of the passaged isolates had the potential to be more pathogenic. Since 15S8 was originally observed by shoot dry weight assessment to be highly pathogenic, but in all subsequent tests both 15S8 and its progeny appeared to be weak pathogens, it must be presumed that loss of pathogenicity due to culture occurred, and passaging did not restore the isolate to its former status, (see results for 15S8 A p).

The close agreement between the pathogenicity results of the seedling tests and the mature wheat

plant assessment indicates that such seedling disease indexes can be used as an alternative, rapid test. This is in contrast with the assertion made by Deacon and Henry (1978a) that disease indexes, (especially those largely based on root lesions), are inaccurate in assessing pathogenicity in Ggt. In this investigation it appeared that the disease index results were more accurate than the shoot dry weight assessment results when compared with the mature plant assessment results. Furthermore, this agreement, (between seedling and mature plant disease index results), indicates that some of the conidial and ascospore isolates studied here, differ considerably from their parental isolates in their pathogenic effect on fully-grown wheat.

Most of the conidial isolates formed in this study were seen to contain the same ds RNA species that were found in their respective parental isolates, (see Table 17), i.e. transmission of the ds RNA pattern was observed into the conidial isolates of 02S7, 06S14, WWS2a and WWS10 at a frequency of approximately 66%. The isolates 06S14 C, WWS2a A, WWS2a B and WWS2a C appeared to have lost the ds RNA found in their respective parental isolates, presumably due to an exclusion mechanism acting in the process of conidiation. Previously McFadden (1982) observed that of 14 conidial isolates of Ggt, (from 3 parental isolates; 3bla, F6 and 45/10), 12 had identical ds RNA species patterns to their respective parents, while two

conidial isolates of F6 (from a total of 10) had lost the same single ds RNA species, (n.b. isolate F6 contains 7 ds RNA species). These two sets of data concerning ds RNA in conidial isolates of Ggt are rather contrasting, but are presumably the result of only a small number of parent isolates being examined. As discussed in section 1.2.1, transmission of ds RNA into conidia of other fungi e.g. Penicillium spp., is generally seen to occur at a high frequency (ca. 90%, De Marini et al., 1977), which is close to the figure for Ggt, if the data of McFadden and those reported here are combined, (i.e. ca 80% faithful transmission). Isolate 0881/14 is believed to have lost its ds RNA during culture, probably prior to production and isolation of conidia. Hence the conidial isolates of 0881/14, are ds RNA-free, but are not considered examples of unsuccessful transmission of ds RNA into conidia. The two isolates 02S7 A p and 06S14 B p appear to lack ds RNA, although their counterparts, 02S7 A and 06S14 B possess the ds RNA species pattern observed in their respective parents. Hence it would appear that the process of passaging has caused the suppression of ds RNA replication in these isolates either totally, or to the extent that PAGE was not sufficiently sensitive to detect the amount of ds RNA extracted from the mycelia of these isolates. In contrast to this apparent loss of ds RNA on passaging,

Rawlinson et al. (1973) reported that of a number of isolates that were passaged through wheat seedlings, none were seen to either lose or gain virus particles. It is therefore possible that isolates 02S7 A p and 06S14 B p still contain the ds RNA species patterns found in 02S7 A and 06S14 B respectively, although at a much lower concentration. This could be explained by suppression of ds RNA replication as discussed in section 3.1.6.

The exclusion of ds RNA from the conidia of Ggt apparently observed here, (e.g. isolate 06S14 C), may not be absolute. McFadden, Buck and Rawlinson (1983) estimate that there are approximately 400 ds RNA molecules per hyphal cell in isolate 3bla. Since WWS2a produces 1-2 μ g ds RNA/g mycelium, which is the same yield as has been observed for 3bla (McFadden, 1982), it is reasonable to assume that WWS2a contains an approximately similar number of ds RNA molecules per hyphal cell. Hence the dilution series of ds RNA from WWS2a analyzed by PAGE (Figure 26), indicates that ds RNA could still be present in the isolate at the level of 4 ds RNA molecules per hyphal cell. However it is evident that such isolates as 06S14 C or the conidial isolates of WWS2a have at least greatly reduced levels of ds RNA infection. McFadden (1982) found that hyphal tip isolates from Ggt isolate F6 could contain virus particles at a level that yielded 5 ng ds RNA/g wet weight mycelium. At this concentration ds RNA was

not detectable by PAGE. Virus particles, however could be detected using immune electron microscopy. Therefore it is plausible that the conidial isolates of WWS2a and isolate 06S14 C may also be virally infected at a similarly low level. On the other hand conidiation may exclude virus infection in a proportion of conidia, presumably via a specific mechanism. A virus exclusion mechanism, (its mode of action is unidentified), has been shown to operate during ascospore formation in Ggt (McFadden, Buck and Rawlinson, 1983). Hence the conidial isolates 06S14C and those derived from WWS2a are quite possibly completely ds RNA-free. (It would be most interesting to analyse these isolates further using a more sensitive technique, e.g. nucleic acid hybridisation, to see if they are completely ds RNA-free).

The isolates 06S14 C, WWS2a A, WWS2a B, and WWS2a C, were significantly more pathogenic than 06S14 or WWS2a, respectively when they were assessed by the seedling disease index test. This strongly suggests that the ds RNA species found in 06S14 and WWS2a, or some of these ds RNA species, are suppressing the pathogenicity of isolates 06S14 and WWS2a. However it should be noted that isolate 06S14 B p also appeared to lack ds RNA, but did not appear to be highly pathogenic, (possibly due to its slow growth rate, or perhaps due to the influence of an undetected virus

infection); while the difference in pathogenic effect of WWS2a and its conidial progeny isolates was not so marked in the mature wheat plant assessment. On the other hand the difference in levels of pathogenicity of 06S14 and 06S14 C was maintained when assessed using fully-grown wheat plants. The ascospore isolate 3bla conDA3, (ds RNA-free), was also shown to be significantly more pathogenic than 3bla conD, from which the former was derived, in the seedling disease index assessment. However this difference was not maintained in the fully-grown wheat plant assessment. The other isolate of interest was 0881/14 B; this showed a significant decrease in pathogenicity in comparison with isolates 0881/14, 0881/14 A and 0881/14 C. This was presumably due to its slow growth, which is probably the result of self-inhibition. (From this observation it is evident that self-inhibition of Ggt isolates could have a considerable effect on pathogenicity test results from a group of such isolates. Therefore it is important that the pH of the medium in which the seedling roots and fungus exist, during any pathogenicity testing of Ggt isolates, is close to, and certainly not more acidic than, the natural pH of the soil from which the isolates were selected).

It is interesting to note that Deacon and Henry (1978a) reported that two single conidial isolates, (of a total of 35), from a Ggt isolate^a of unknown virus

content, were noticeably more pathogenic than the parental isolate, or the other conidial isolates. Furthermore the report of Lapierre et al. (1970) that Ggt isolates cured of ds RNA virus infection through ascospore isolation were considerably more pathogenic than the parental isolates, appears to be in agreement with the results of this investigation regarding virus-cured isolates. However Rawlinson et al. (1973) found no correlation between loss of virus and increased pathogenicity in ascospore isolates from two parental isolates of differing pathogenicity. The contrast between these reports is probably the result of only a few viruses or variant viruses being capable of reducing pathogenicity in Ggt, and hence their presence is not always detected.

Overall the results of the analysis of the single conidial and single ascospore isolates indicate that certain ds RNA viruses, e.g. those found in 06S14 and WWS2a are associated with reduced pathogenicity in Ggt. It should be pointed out that DNA species, such as those found by Honeyman and Currier (1983) may in fact be responsible for reduced pathogenicity, but their possible involvement in the pathogenicity of isolates 06S14 and WWS2a has not been investigated. The ds RNA species apparently responsible for reduced pathogenicity are worthy of further study as potential biological control agents of take-all of wheat.

3.3 ISOLATION, INFECTION AND REGENERATION OF GGT PROTOPLASTS.

Protoplasts were isolated from a number of related strains of Ggt and regenerated, often following inoculation with a virus suspension in the presence of polyethylene glycol (PEG). Single colonies of Ggt arising from regenerated protoplasts that had been inoculated were analysed to see if infection had been achieved. Newly infected isolates were tested, along with the uninfected parental isolates, using the seedling disease index assessment, to determine their pathogenicity ratings.

The isolates selected for study were 3bla, 3bla conD, 3bla conE, 3bla conDA3 and 3bla conEAl. Both 3bla conDA3 and 3bla conEAl are ds RNA-free, but are also presumably susceptible to infection by the four viruses of 3bla, since they were derived from 3bla, via the single conidial isolates, 3bla conD and 3bla conE, respectively (McFadden, Buck and Rawlinson, 1983). The development of a method of infecting Ggt protoplasts with ds RNA viruses yielded the means to examine the effect on pathogenicity of Ggt of introducing such viruses into ds RNA-free Ggt isolates.

Protoplasts were isolated and regenerated from each of these isolates listed above; while only 3bla conDA3 and 3bla conEAl were inoculated with purified virus preparations from 3bla prior to regeneration.

3.3.1 Isolation and Regeneration of Ggt Protoplasts.

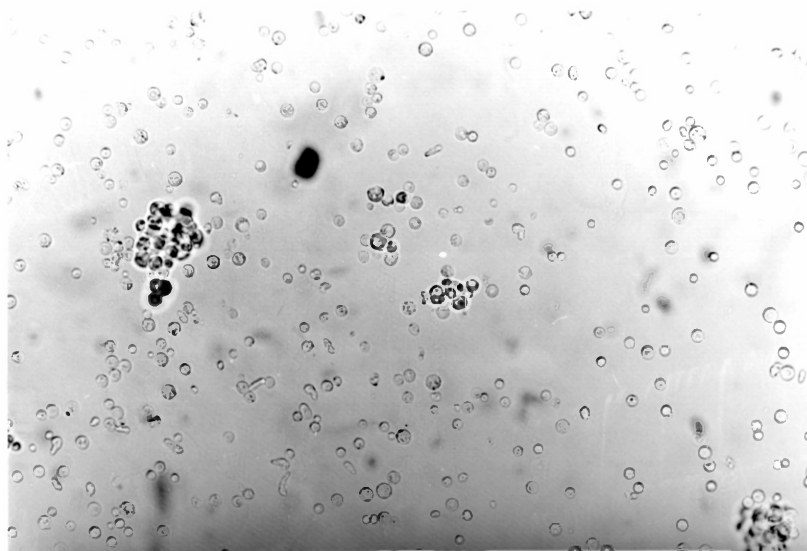
Protoplasts were isolated from the five isolates listed above using the method described in section 2.23. Figure 17 shows a selection of typical protoplasts of 3bla conDA3. Protoplasts from all of the isolates were similar in size, an average range in diameter being 5-11 μm .

The number of protoplasts isolated from each isolate was generally of the same order of magnitude; the exact figures are given in Table 18. In general the concentration in the final volume of buffer/stabiliser, was between 1.5 and 6.0×10^7 protoplasts per ml. Although all the isolates generally gave similar yields of protoplast numbers, it appeared that 3bla conDA3 and 3bla conEA1 could provide yields 4 to 5 times greater than the other isolates. This is demonstrated by the upper limit of the yield ranges of 3bla conDA3 and 3bla conEA1, (11.7×10^7 and 10.50×10^7 protoplasts/g mycelium respectively), compared with the upper limit of the yield ranges of 3bla, 3bla conD and 3bla conE, (2.25×10^7 , 2.20×10^7 and 2.25×10^7 protoplasts/g mycelium respectively).

Protoplast regeneration frequencies, using the technique where the protoplast suspension was mixed with warm molten regeneration agar, (Section 2.23), were in the range of 0.5 to 5%. This is not a particularly high rate of regeneration if it is compared with regeneration of Aspergillus nidulans

Figure 17. Protoplasts from 3bla conDA3.

a) Magnification x 100



b) Magnification x 800.

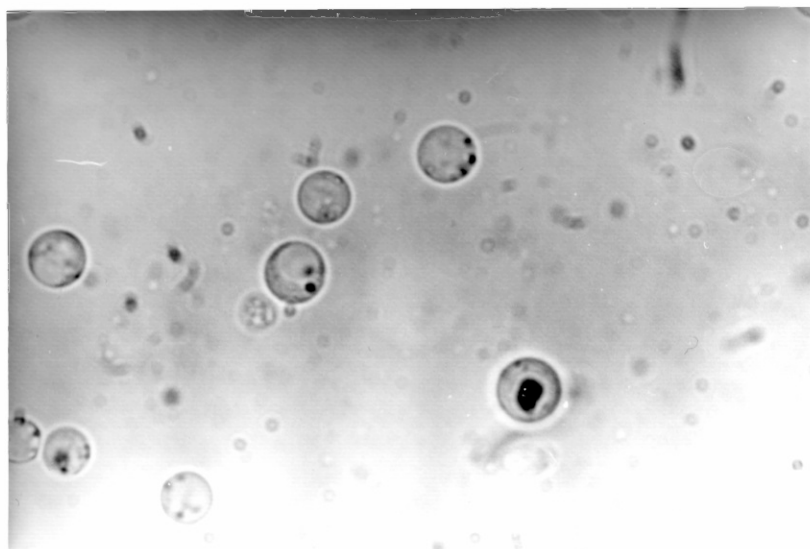


TABLE 18 Numbers of protoplasts isolated.

Isolate	Total Number of Protoplasts*	Protoplasts per g of Mycelium
3bla	$7.2-7.5 \times 10^7$	$2.20-2.25 \times 10^7$
3bla conD	7.2×10^7	2.20×10^7
3bla conE	7.5×10^7	2.25×10^7
3bla conDA3	$5.1-39.0 \times 10^7$	$1.50-11.7 \times 10^7$
3bla conEA1	$14.5-35.1 \times 10^7$	$4.40-10.50 \times 10^7$

* Normally collected from approximately 50 ml of filtrate after lytic incubation, i.e. two-thirds of incubation mixture.

(Peberdy, 1979). However, a regeneration frequency in this range generally yielded sufficient single colonies on the 10^{-2} , 10^{-3} dilution regeneration Petri dishes for analysis in an experiment where the protoplasts had been inoculated with virus. In one experiment the protoplast suspension, (from 3bla conDA3), was spread on pre-set regeneration agar. This led to a regeneration frequency of approximately 14.5%.

3.3.2 Inoculation of Ggt Protoplasts and Assessment for Infection of Regenerated Colonies.

The inoculation of protoplasts using PEG promoted protoplast fusion presumably resulted in incorporation of absorbed virus particles into the cytoplasm of the fusion product. PEG also caused a reduction in numbers of viable protoplasts. When 2 ml of 40% w/v PEG was used in the initial inoculation mixture, (giving a final PEG concentration of 32.4% w/v), between 60-65% of the protoplasts survived the inoculation process. (N.B. survival was assessed by viable counts of a known number of inoculated and uninoculated protoplasts). If 2 ml of 30% PEG was used, (final concentration of 24.3% w/v), then the survival rate increased to between 80 and 90%.

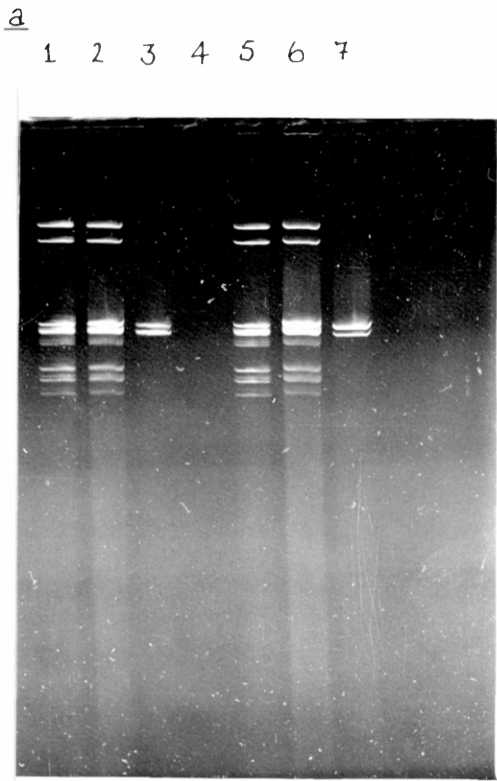
In a typical inoculation using 5×10^7 protoplasts and 65 to 90 μ g of virus inoculum, (from 3bla); the ratio of virus particles to protoplasts was approximately 400,000:1. It should be noted that 3bla

is infected with four viruses, A, B1, B2 and C (see Figure 18).

In two separate inoculation experiments, both using 3bla conDA3 as the source of protoplasts, regenerated colonies were found to be infected. In the first experiment, of 10 regenerated colonies examined using PAGE, one colony, (R1), was found to contain two ds RNA species that co-migrated with the two ds RNA species of virus B1 of 3bla (mol. wts $1.60, 1.54 \times 10^6$), (see Figure 18a). In the second experiment, 20 regenerated colonies were examined, and ds RNA species were detected in two colonies, (R2 and R3), using PAGE. The ds RNA species from R2, like those from R1, in PAGE co-migrated in PAGE with the two ds RNA species from virus B1, (Figure 18a), while the two ds RNA species from R3 co-migrated in PAGE with the two ds RNA species from virus B2, (mol. wts $1.45, 1.43 \times 10^6$), (see Figure 18b).

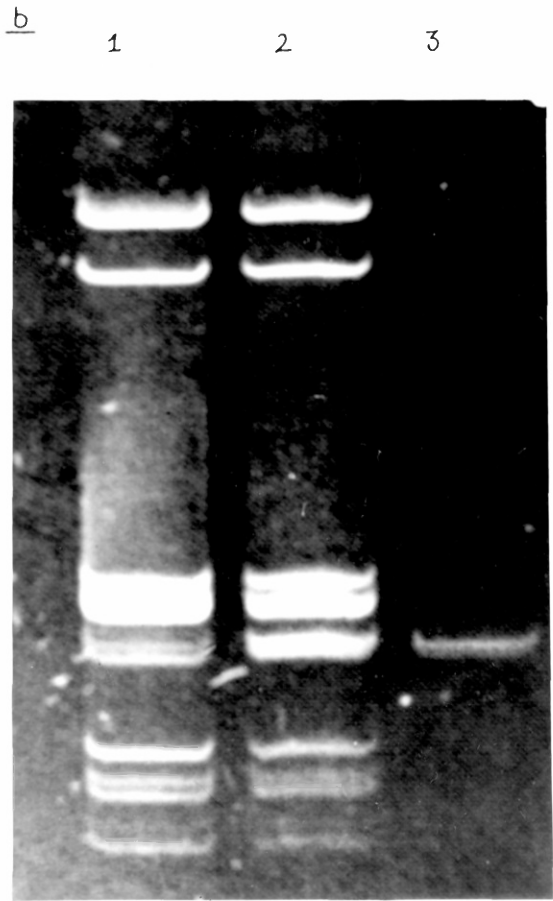
On examination by electron microscopy the three isolates R1, R2 and R3 were observed to contain virus particles of approximately 35 nm diameter, using virus T1-A as a standard, (known to have a particle diameter of 35 nm, whilst it should be noted that both viruses B1 and B2 have particle diameters of 35 nm Buck *et al.*, 1981). The ds RNA extracted from the virus particles of R1, R2 and R3 behaved identically in PAGE to the ds RNA directly extracted from the isolates, (shown in Figure 18). Examination of the capsid polypeptides

Figure 18. PAGE analysis of regenerated colonies R1, R2 and R3.



a

1: 3bla	ds RNA
2: 3bla and R1	ds RNA
3: R1	ds RNA
4:	
5: 3bla	ds RNA
6: 3bla and R2	ds RNA
7: R2	ds RNA



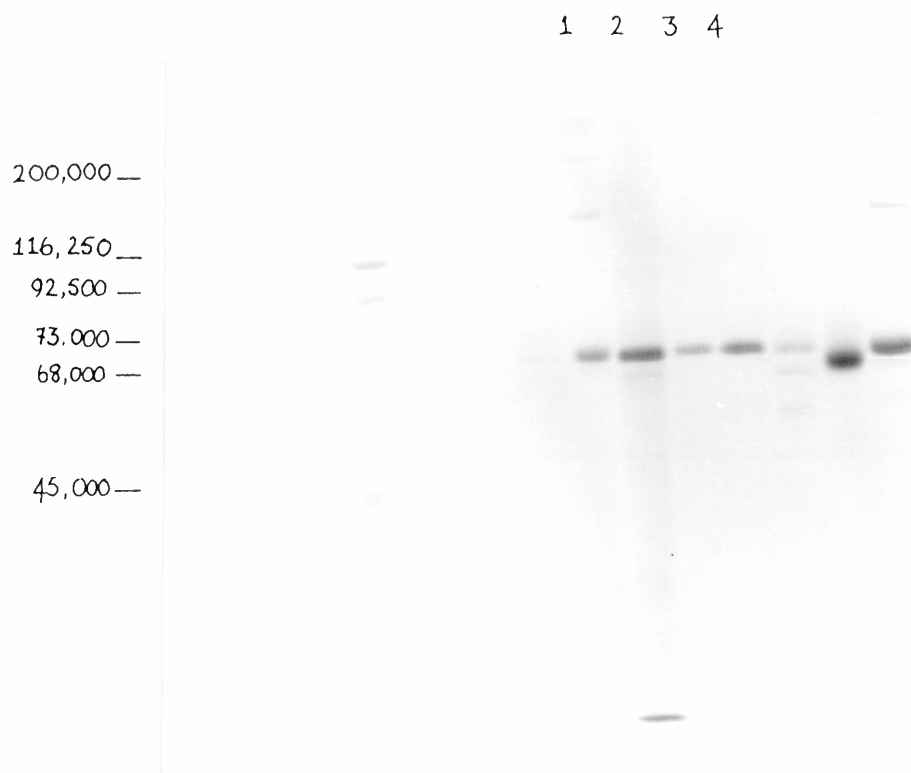
b

1: 3bla	ds RNA
2: 3bla and R3	ds RNA
3: R3	ds RNA

from the virus particles of the regenerated colonies by SDS-PAGE, (Section 2.21), revealed a single major polypeptide with a molecular weight of 73,000, (sized by using a range of protein standards, and the capsid polypeptide from virus T1-A; known to have a molecular weight of 73,000, Buck et al., 1981; see Figure 19). McFadden, Buck and Rawlinson (1983) demonstrated that viruses B1 and B2 both have a major capsid polypeptide of 73,000. Therefore from this evidence it was apparent that isolates R1 and R2 are infected with B1 virus of 3bla, while isolate R3 is infected with virus B2 of 3bla. Of the 30 regenerated isolates examined for infection, no cases of infection with virus A or C of 3bla was found.

Through three serial subcultures, each of the infected regenerated isolates yielded a constant quantity of ds RNA equal to, (if not slightly greater than), that found in the inoculum source isolate, 3bla from viruses B1 and B2. For instance between 8 to 10 μ g of B1 ds RNA was extracted from approximately 5 g wet weight mycelium of either R1 or R2, through the serial subculturing of both isolates. Similar results, with a lower but stable yield, were observed for isolate R3. Hence the infections of isolates R1, R2 and R3 are stable and replicating at levels similar, if not identical, to those observed in the original host. Inoculation of protoplasts isolated from 3bla conEAL with virus inoculum from 3bla using 2 ml of 30% PEG,

Figure 19. SDS-PAGE of capsid polypeptides of isolates R1, R2 and R3.



1: T1-AV

2: R1 V

3: R2 V

4: R3 V

(Protein standards and molecular weights: myosin 200,000; B-galactosidase, 116,250; phosphorylase B, 92,500; virus T1-A capsid polypeptide, 73,000; bovine serum albumin, 68,000; ovalbumin, 45,000).

(final concentration of PEG, 24.3%) appeared to be unsuccessful. Of the twenty regenerated colonies analysed, none contained any ds RNA. Similarly protoplasts of 3bla conDA3 inoculated with 3bla virus ds RNA, (20 µg/ml) and 40% PEG did not yield any infected, regenerated colonies, in a total of 20 that were analysed.

3.3.3 Assessment of Pathogenicity of the Infected Isolates.

The regenerated, infected isolates R1, R2 and R3 were pathogenicity-tested using the seedling disease index assessment (section 2.7b). The results from this test are shown in Table 19, along with a set of results obtained for other 3bla-derived isolates. Furthermore isolates R1 and R3 were pathogenicity-tested using fully-grown wheat plants, (see Table 20). In both assessments the regenerated infected isolates appear to be less pathogenic than 3bla conDA3, and similar in pathogenicity rating to 3bla conD and 3bla.

3.3.4 Discussion

Protoplasts were isolated from both virus-containing and virus-free isolates of Ggt and regenerated by the use of techniques developed for Aspergillus nidulans (Peberdy and Kevei, 1979b; Hamlyn et al., 1981). The yields achieved from 3bla and its derived isolates, (Table 18), compare well with those achieved by Hamlyn et al. (1981) for A.nidulans, which

TABLE 19. Seedling disease index values for regenerated infected isolates R1, R2 and R3 (section 2.7b).

Isolate	Average Seedling Disease Index Value ⁺
3bla*	2.25 (2, 3, 2, 2)
3bla conD*	1.25 (1, 1, 2, 1)
3bla conDA3*	3.00 (,3, 3, 3, 3)
R1	2.25 (2, 2, 2, 3)
R2	0.75 (1, 1, 1, 0)
R3	2.25 (2, 3, 2, 2)

* Also shown in Table 1⁴, test b.

⁺ Actual scores in brackets.

TABLE 20 Mature wheat plant disease assessment for regenerated infected isolates R1 and R3 (Section 2.7d).

Isolate	Ears of Grain		Observations	Disease Index
	Healthy+	Rudimentary+		
3b1a*	7	5	Roots largely healthy; 10% lesioned. NSH**.	6
3b1a conD*	6	4	Roots largely healthy; 10% lesioned. NSH**.	6
3b1a conDA3*	9	4	Roots slightly stunted; 50-70% lesioned. NSH.	8
R1	10	5	Roots largely healthy; 10% lesioned. NSH**.	4
R3	10	7	Roots largely healthy; 10% lesioned. NSH**.	6

* Also shown in Table 17.

+ Per three plants.

** NSH = Normal shoot height.

yielded $28 \pm 1.4 \times 10^7$ protoplasts/ml using the same lytic procedure as described in section 2.23. Although the lower yields from the virus-free isolates, (3bla conDA3 and EAl), are fairly close to the yields from the virus-infected isolates, it appears the former isolates are capable of producing considerably greater yields than the latter isolates.

Regeneration of both virus-free and virus-infected isolates did not occur at frequencies comparable with A.nidulans, unless the regeneration process used pre-set agar. In this case 3bla conDA3 protoplasts regenerated at a frequency of 14.5%, (compared with a rate of 0.5% to 5% using molten agar), which falls in the normally accepted range for A.nidulans of 10-30% (Peberdy, 1979). However a regeneration frequency of 0.5 to 5% was sufficient to yield numerous single colonies on the 10^{-2} , 10^{-3} dilution regeneration plates, and therefore did not present a problem. The success in isolating and regenerating protoplasts from both virus-containing and virus-free isolates of Ggt is in contrast with the report of Rawlinson and Muthyalu (1974). This is probably the result of different lytic enzymes and buffer/stabilizer.

The analysis of the regenerated isolates R1, R2 and R3 clearly demonstrates that R1 and R2 are stably infected with virus B1 of 3bla, while R3 is stably infected with virus B2 of 3bla. The biochemical and biophysical characteristics of the viruses B1 and B2

were the same as previously observed in 3bla (Buck et al., 1981), except that only one capsid polypeptide with a molecular weight of 73,000, was observed from the viruses of the regenerated infected isolates. In 3bla the B1 and B2 viruses appeared to have two capsid polypeptides, of molecular weights 73,000 and 68,000 (Buck et al., 1981). The 68,000 capsid polypeptide is perhaps therefore a degradation production; or possibly it is a prematurely terminated polypeptide, since it was the only capsid polypeptide found associated with the altered B1 virus Ascl/Asc2, which has lost a segment from both of its ds RNA species (McFadden, Buck and Rawlinson, 1983). This inoculation technique (Section 2.24) has overcome the problems encountered by various investigators, i.e. failure to infect protoplasts (Bozarth, 1975; Lai and Zachariah, 1975; Boissonet-Menes and Lecoq, 1976), or failure to produce stable infections (Lhoas 1971b; Pallett, 1976), or failure to produce infections at a reasonable frequency for detection (Ghabrial and Mernaugh, 1983). It is interesting to note that Ghabrial and Mernaugh (1983) have detected, at a very low frequency, transmission of virus particles of Helminthosporium victoriae, using immune electron microscopy, associated with newly-infected mycelium of H.victoriae. However they have not isolated the 190S and 145S virus particle types from newly-infected mycelium. Moreover the

concentration of virus particles in newly-infected isolates was very low (Ghabrial, 1985) possibly because the recipient strain was of a different genotype to that from which the virus particles were obtained and hence possibly more resistant to infection. Ghabrial (1984) has also reported that virus transmission into H.victoriae diseased isolates via virus inoculation of protoplasts was only at best equivocally demonstrated by a dot blot hybridization technique.

The infections achieved here have separated the viruses B1 and B2, and shown that they are functionally independent units, and may now be examined as such in the new isolates. Analysis of more isolates or perhaps changes in the inoculation procedure, (possibly different pHs for example), would have yielded isolates infected with viruses A or C of 3bla.

This technique should permit the examination of the effect of virus infection on pathogenicity in Ggt. For instance, the infection of isolate 06Sl4C with the viruses of 06Sl4, followed by pathogenicity testing of such re-infected isolates, in comparison with 06Sl4 and 06Sl4C, would provide detailed information on the relationship between the pathogenicity of 06Sl4 and its viruses. The isolates R1, R2 and R3 all appear to be less pathogenic than 3bla conDA3 in both the seedling disease index assessment and the mature plant assessment, (Tables 19 and 20). Hence it is plausible that infection of protoplasts derived from this virus

free isolate with viruses B1 and B2 has produced isolates that are of diminished pathogenicity. However due to the significance of such an assumption these pathogenicity tests would need to be repeated with greater numbers of plants, and also perhaps using a recipient virus-free isolate that has not been in culture as long as 3bla conDA3 and is more pathogenic. Moreover it would be important to test the pathogenicities of several virus-free and virus-infected single protoplast cultures derived from such a single ascospore isolate in order to assess possible somaclonal variation between individual protoplast colonies.

The development of this technique will also assist further study on the effects of mycoviruses on their hosts generally and may assist studies on host range of mycoviruses. An improved method of detection, e.g. a colony of nucleic acid hybridization or immunological assay would greatly facilitate such studies.

4. CONCLUSIONS AND FUTURE PROSPECTS.

In summary the major conclusions from this investigation into the effects of ds RNA viruses on the pathogenicity of Ggt can be defined as follows:

- i) Several methods of measuring the pathogenicity of Ggt isolates have been compared and an assessment of their reliability has been made. The two seedling disease index pathogenicity tests appeared to give results in close agreement with each other and also with the mature plant disease assessment test, implying that they present an accurate value for isolate pathogenicity. (It is noteworthy that the seedling disease index test of Cook and Naiki (1982) should be preferred, on balance, to the agar plate seedling disease index test, because of the more natural seedling growth conditions of the former). The seedling shoot dry weight pathogenicity test appears to be biased towards providing low pathogenicity ratings, although a reasonable representation of the relative pathogenicity ranking of the isolates tested seemed to be maintained.
- ii) Two methods for obtaining isogenic isolates of Ggt with and without ds RNA, have been developed. Firstly ds RNA-free protoplasts have been infected with two viruses (Stanway and Buck, 1984; see Appendix IV). Secondly single conidial isolates

of Ggt have been shown to be apparently ds RNA-free, presumably due to an exclusion mechanism acting during conidiogenesis, analogous to the exclusion mechanism believed to act during ascospore formation in Ggt (McFadden, Buck and Rawlinson, 1983).

- iii) Evidence has been obtained that specific ds RNA species may be responsible for significantly reducing pathogenicity of Ggt isolates, although other cytoplasmic factors, e.g. linear DNA plasmids (Honeyman and Currier, 1983) may also be involved.

From the results of this investigation various lines of further research would appear to be feasible and worthwhile.

The survey of ds RNA composition of the field isolates of Ggt has indicated that group A ds RNA species, (molecular weight range 7.0 to 4.6×10^6), are more frequent than previously observed (Buck et al., 1981; Rawlinson and Buck, 1981) and therefore it would be most interesting to study them further in order to elucidate their virological properties and also to permit their use as inoculum in further protoplast infection experiments, (see below). Other ds RNA species worthy of study include the 2.55×10^6 species found in isolate 0601/4, and also those species less than 1.00×10^6 , such as those seen in

isolate 06S14. It may well be that ds RNA species of the latter variety are satellite or defective viruses (Mossop and Francki, 1978; Romanos, Buck and Rawlinson, 1981; Huang and Baltimore, 1977) and may be involved in reducing pathogenicity in Ggt. For instance the satellite virus CARNA 5, of cucumber mosaic virus has been shown to alter the pathogenic effect of its helper virus (Waterworth et al., 1979).

Perhaps the most interesting area of potential research would involve development of the protoplast infection technique and/or further infection experiments using a variety of recipient isolates and virus inocula. (Evidently this technique will also be of use to the study of other fungal viruses as well). Detection of successful infection would be greatly facilitated by the development of colony hybridization technique, such as that routinely used for bacteria (Grunstein and Hogness, 1975). This could be quite possibly achieved by a static incubation of the regenerated isolates with lytic protoplasting enzymes in buffer stabilizer followed by nucleic acid transfer to nitrocellulose or a similar membrane.

With or without the development of colony hybridization technique, it would be feasible to

study other potential virus/isolate inoculations, since the frequency of infection observed in this study does not mean that culturing and ds RNA extraction of a relatively small number of regenerated colonies is likely to fail to detect infection when it has occurred. Hence it would be reasonable to attempt the re-infection of Ggt isolates that have been cured of infection, (either through formation of conidia or ascospores), with the ds RNA viruses found in their respective parental isolates, or to attempt infection of Ggt isolates that are naturally ds RNA-free with viruses of interest. (Both of these examples will be discussed later in terms of their potential for studying pathogenicity in Ggt). Protoplast infection could also be used to study the interaction of Ggt viruses of varying relatedness by attempting super-infection experiments, whilst the effect of the host isolate on virus replication could also be investigated. For instance isolate 02S9 β , where the host isolate appeared to suppress virus replication, could be cured of infection and then re-infected with its original viruses and/or other Ggt viruses and the resulting virus titres assessed.

Initially, further investigation into the relationship between reduced pathogenicity and particular ds RNA viruses should focus on whether

the phenomenon of reduced pathogenicity, (in the isolates O6S14 and WWS2a), is cytoplasmically transmissible. This would involve heterokaryon (or heteroplasmon) transfer experiments using genetically marked strains, such as was achieved with Ustilago maydis in the study of its killer factor (Wood and Bozarth, 1973; Koltin and Day, 1976). Prototrophic or fungicide-resistant mutants of Ggt isolates could either be induced by mutagenesis (Blanch, Asher and Burnett, 1981) or alternatively spontaneous fungicide-resistant mutants could be selected, as has been achieved with Ceratocystis ulmi ascospores at a frequency of ca. 1 in 10^6 (Brasier, C.M., personal communication to K.W. Buck). These genetically marked strains, (produced by either method), would have to be pathogenicity-tested prior to their use in heterokaryon transfer tests.

In order to provide more definitive evidence relating to the effect of ds RNA viruses on pathogenicity of Ggt, infected conidial isolates from field isolates O6S14, WWS2a and others, (i.e. those seen to be weakly pathogenic and ds RNA-containing), should be produced and then cured of infection by ascospore isolation. These resulting ascospore isolates should be shown to be completely ds RNA-free by a technique more

sensitive than PAGE, such as nucleic acid transfer hybridization, (as used by McFadden, Buck and Rawlinson, 1983, for ascospore isolates of 3bla). These ascospore isolates would yield direct evidence of the effect of ds RNA infection on pathogenicity in seedling disease index and mature plant assessment tests when compared with their respective infected parental isolates. Infection of these ds RNA-free ascospore isolates and naturally ds RNA-free highly pathogenic Ggt isolates with ds RNA viruses apparently associated with reduced host pathogenicity, using the protoplast infection technique, would demonstrate whether it is actually ds RNA viruses, or the influence of another cytoplasmic element, e.g. DNA plasmids (Honeyman and Currier, 1983) that can reduce pathogenicity of Ggt.

Identification of the precise nature of the cause of this phenomenon would yield a method of analysing control of pathogenicity in Ggt. For instance, isogenic isolates with and without the cytoplasmic element responsible could be examined for differences in their mRNA populations. This could possibly be achieved by electrophoresis and transfer of the mRNA fraction from an infected isolate followed by a pre-hybridization step using unlabelled cDNAs made to the mRNA fraction of the uninfected isolated. Hybridization with labelled

cDNA synthesised from the pathogenicity-reducing cytoplasmic element would identify any mRNAs involved in the phenomenon; these could be retrieved from gel electrophoresis and then cloned, (as cDNA), and/or translated in vitro. Alternatively the ds RNA species, (or DNA elements if this is shown to be the case), could be cloned as a first step permitting sequence analysis that would yield information relating to the protein(s) encoded therein, (if any). Identification of the nature of any such protein, (e.g. DNA binding potential), would obviously be most informative with regard to the mode of action of the pathogenicity reducing element. Hence an insight into the mechanism involved in reducing the pathogenicity of Ggt would be obtained and hence this understanding could possibly be used to identify the genes involved in pathogenicity of Ggt.

It is also obviously most important to develop the observation that ds RNA species are associated with reduced pathogenicity of Ggt as a biological control agent of take-all of wheat. This would principally involve a series of field trials to investigate various aspects that need clarification for a biological control method to become a realistic and economically worthwhile

proposition.

Firstly it would be necessary to develop a method for introducing the Ggt isolates carrying the pathogenicity reducing ds RNA species/DNA element into the rhizosphere. Inoculation of the field soil would probably prove to be labour intensive and slow to yield control. Hence research into this aspect should concentrate on seed treatment with mycelium or spores of the Ggt isolates of interest, this should yield immediate protection from highly pathogenic isolates of Ggt, since presumably the roots would be colonized by the former Ggt isolates as the roots emerge and grow. This would also accelerate contact and therefore increase opportunities for cytoplasmic transfer between the inoculated Ggt isolates and the Ggt isolates existing in the soil. Two potential candidates for the seed treatment should be the chlamydospores and microsclerotia of Ggt (Sivasithamparam and Parker, 1976) since these structures are presumably in essence survival structures.

Secondly the field trials would have to be monitored carefully to assess if the Ggt isolates that would be used to attempt biological control, (e.g. 06S14), are sufficiently debilitated to avoid causing damage to the grain yield^e of the crop. this could be achieved by the use of field

plots (previously not used for cereal cultivation to avoid any natural Ggt infestation), planted with wheat, one uninoculated, the other(s) inoculated with an isolate demonstrated to have a ds RNA-associated pathogenicity loss, followed by comparison of the final grain yields from these plots. Linked to this would be the need to assess the stability of such Ggt isolates in the field since there is a possibility that they may revert to high pathogenicity. This could be determined by re-isolation after a wheat crop followed by seedling disease index pathogenicity tests. Evidently in situations where the isolate of reduced pathogenicity might have come into contact with other Ggt isolates it may be a problem to identify the original isolate. This would most easily be solved by the selection of prototrophic or fungicide-resistant mutant isolates, (as previously discussed). It should be borne in mind that any mutant isolate would have to be pathogenicity-tested to ensure that its disease-causing potential was not altered. Of course another means of identification would be the ds RNA species pattern, however this would not be absolute since transfer of ds RNA species may occur between isolates of Ggt in the field (Jamil et al., 1984)

It would be of interest to study the likelihood and/or frequency of ds RNA species transfer in the field, especially from isolates carrying ds RNA ~~species~~^e associated with reduced pathogenicity. Co-inoculation of a series of uninfested field plots with two isolates, one infected with such ds RNA species, the other being ds RNA-free and highly pathogenic, followed by one season of wheat cultivation and then reisolation of Ggt from the infected wheat roots would yield valuable information in this respect, once the isolates were analysed for ds RNA content and also their v-c grouping. In the case of chestnut blight, Anagnostakis (1982; 1983) has found that due to limited ds RNA transfer between isolates of Endothia parasitica, of different v-c groups, biological control is most effectively achieved by inoculation of cankers of infected trees with mixtures of hypovirulent isolates from a number of v-c groups. Presumably this same approach using Ggt isolates from various v-c groups, each infected with ds RNA species-associated with pathogenicity loss, (using the protoplast infection technique if necessary), would provide improved biological control.

The observation of Jamil and Buck (1984) that identical ds RNA viruses can replicate in both Ggt and Phialophora sp. (lobed hyphopodia) suggests

that Phialophora sp. (lobed hyphopodia) isolates may well be able to act as a vector of ds RNA viruses under field conditions, and would therefore, due to Phialophora spp. being avirulent towards wheat, be a better biological control agent than debilitated Ggt isolates. It would be necessary, evidently, to study the rate of transfer of the ds RNA species of interest from Phialophora sp. (lobed hyphopodia), and presumably as suggested for Ggt, transfer would be improved by a multiplicity of infected isolates from different v-c groups.

The effect of transfer of the ds RNA species, associated with reduced Ggt pathogenicity, in terms of biological control, could be assessed by means of field trials of wheat, using replicate plots; one inoculated and one uninoculated. As a result an effective and economically worthwhile method of controlling take-all of wheat may be obtained. Lastly it is important to note that the potential for the natural spread of pathogenicity-reducing ds RNA species throughout the pathogen population, (as seen in the case of E.parasitica in Italy; Anagnostakis, 1978), would appear more attractive ecologically than other possible methods of biological control of take-all of wheat.

APPENDIX 1

Control-adjusted pathogenicity ratings of the field isolates of Ggt.

Test 1

<u>Isolate identity code</u>	<u>Pathogenicity rating</u>
02S2	0.35
02S3	0.20
02S5	0.18
02S6	0.19
02S7	0.06
02S8 α	0.00
02S8 β	0.30
02S8 γ	0.51
02S9 α	0.19
02S9 β	0.30
02S9 γ	0.27
02S11	0.38
02S14	0.27
02S15	0.39
02S16	0.14
02S18	0.14
02S19	0.13
03S2	0.29
03S3a	0.27
03S3b	0.16
03S5	0.31
03S6	0.16

TEST 1 cont.

<u>Isolate identity code</u>	<u>Pathogenicity rating</u>
03S2	0.29
03S3a	0.27
03S3b	0.16
03S5	0.31
03S6	0.16
03S8a	0.48
03S8b	0.19
03S10	0.58
03S13	0.48
03S14	0.43
03S15	0.20
03S17	0.52
03S18	0.44
03S20a	0.37
03S20b	0.33
06S2	0.35
06S3	0.35
06S4	0.39
06S7	0.43
06S8	0.27
06S9	0.29
06S10	0.13
06S11	0.25
06S12	0.32

TEST 1 cont.

<u>Isolate identity code</u>	<u>Pathogenicity rating</u>
06S14	0.19
06S15	0.32
06S16	0.12
06S17	0.05
06S18	0.29
07S1	0.32
07S2	0.44
07S4	0.36
07S5a	0.29
07S5b	0.38
07S6	0.27
07S7	0.25
07S8	0.25
07S9	0.25

TEST 2

<u>Isolate identity code</u>	<u>Pathogenicity rating</u>
07S10	0.00
07S11	0.01
07S12	0.00
07S14	0.13
07S15	0.24
07S16	0.00
07S17	0.00
07S18	0.12
07S20	0.13
14S3 α	0.12
14S3 β	0.00
14S4	0.00
14S6	0.00
14S7	0.05
14S8 α	0.05
14S8 β	0.18
14S10	0.00
14S11 α	0.07
14S11 β	0.30
14S12	0.69
14S13	0.41
14S15 α	0.53
14S15 β	0.61
14S15 γ	0.46
14S17a	0.36

TEST 2 cont.

<u>Isolate identity code</u>	<u>Pathogenicity rating</u>
14S17b	0.53
14S19	0.47
15S2	0.42
15S3	0.57
15S4 α	0.53
15S4 β	0.40
15S5 α	0.51
15S5 β	0.35
15S6 α	0.42
15S6 β	0.49
15S7	0.67
15S8	0.65
15S9	0.51
15S10	0.53
15S11	0.44
15S12a	0.22
15S12b	0.35
15S14	0.40
15S15	0.12
15S16	0.17
15S18	0.44
15S19	0.24
15S20	0.11
WWR2	0.23
WWS2a	0.07

TEST 2 cont.

<u>Isolate identity code</u>	<u>Pathogenicity rating</u>
WWS2b	0.20
WWS4	0.11

TEST 3

<u>Isolate identity code</u>	<u>Pathogenicity rating</u>
WWS6	0.00
WWS7a	0.09
WWS7b	0.05
WWS8	0.00
WWS10	0.00
WWS11	0.00
WWS12	0.00
0601/1	0.06
0601/2	0.05
0601/3	0.07
0601/4	0.00
0601/5	0.00
0601/6	0.00
0601/7	0.00
0601/8	0.00
0601/9	0.07
0601/10	0.00
0601/11	0.08
0601/12	0.16
0601/13	0.00

TEST 3 cont.

<u>Isolate identity code</u>	<u>Pathogenicity rating</u>
0601/14	0.13
0601/15	0.13
0601/16	0.00
0601/17	0.05
0601/18	0.03
0601/19	0.14
0601/20	0.13
0601/21	0.10
0601/22	0.26
0881/1	0.18
0881/2	0.29
0881/3	0.15
0881/4	0.21
0881/5	0.15
0881/6	0.23
0881/7	0.14
0881/8	0.29
0881/9	0.27
0881/10	0.15
0881/11	0.19
0881/12	0.08
0881/13	0.08
0881/14	0.08
0881/15	0.31

TEST 3 cont.

<u>Isolate identity code</u>	<u>Pathogenicity rating</u>
0881/16	0.26
0881/17	0.30
0881/18	0.45
0881/19	0.35
0881/20	0.27
0881/21	0.40

APPENDIX II

Compilation of the polyacrylamide gels used
to estimate the molecular weights of the ds RNA
found in the field isolates of Ggt.

All of the gels recorded here are 4% polyacrylamide gels composed and electrophoresed as described in section 2.16.

The molecular weights of the ds RNA standards used are listed below:

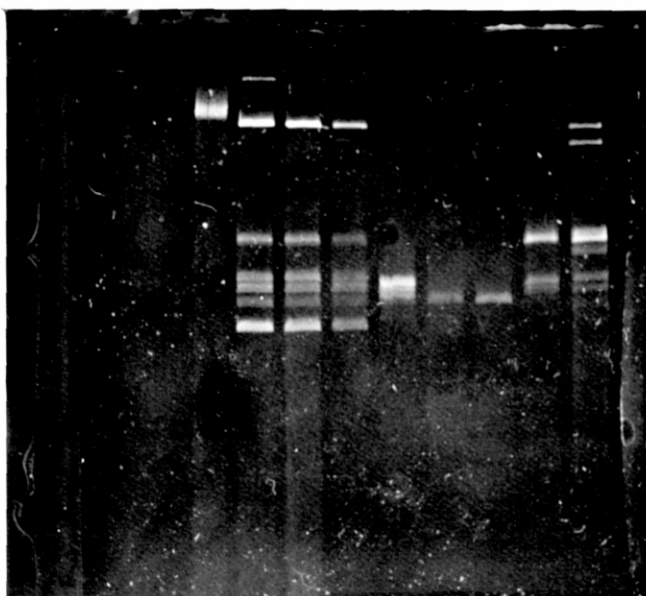
3bla : 4.1, 3.5, 1.6, 1.54, 1.45, 1.43, 1.27, 1.19,
1.11 (all $\times 10^6$).

AfV : 4.1, 2.7, 2.6, 1.87, 1.70, 1.44, 1.24
(all $\times 10^6$).

PsV : 1.11, 0.99, 0.94, 0.89, 0.46 (all $\times 10^6$).

Gel i)

1 2 3 4 5 6 7 8



— 4.1

— 1.11

1: O2S8 γ ds RNA

2: O2S8 β ds RNA

3: O2S8 α ds RNA

4: O2S7 ds RNA

5: O2S6 ds RNA

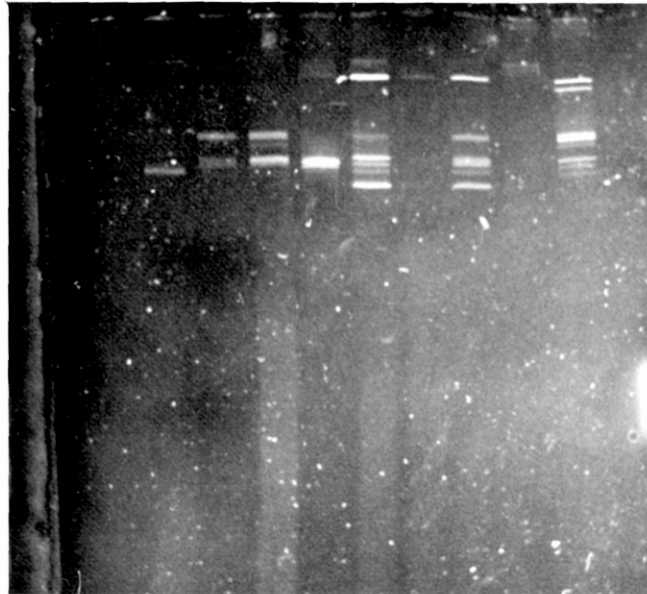
6: O2S5 ds RNA

7: O2S2 ds RNA

8: 3bla ds RNA

Gel ii)

1 2 3 4 5 6 7 8 9



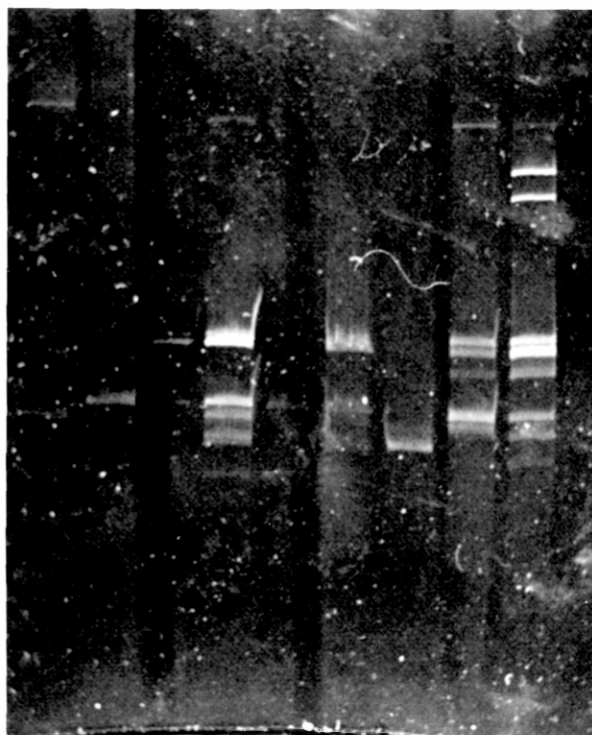
— 4.1

— 1.11

1:	O2S16	ds RNA
2:	O2S15	ds RNA
3:	O2S14	ds RNA
4:	O2S11	ds RNA
5:	O2S9 γ	ds RNA
6:	O2S9 β	ds RNA
7:	O2S9 α	ds RNA
8:	O2S3	ds RNA
9:	3bla	ds RNA

Gel iii)

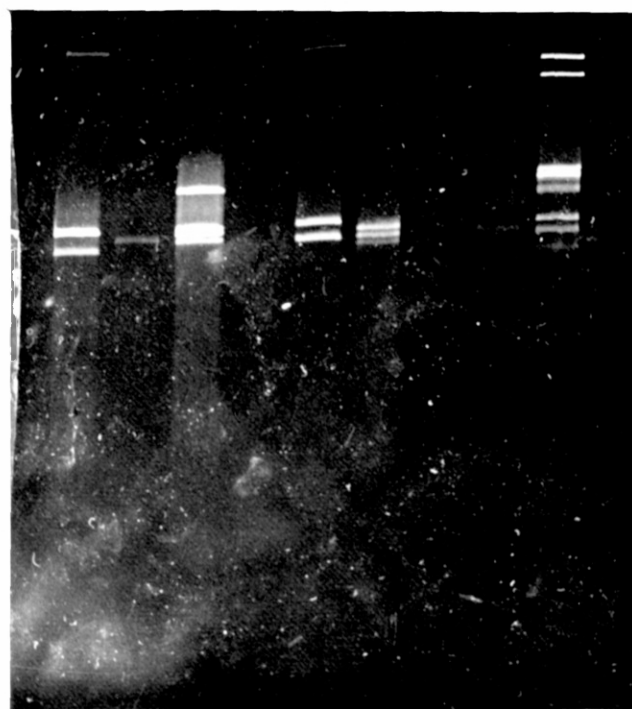
1 2 3 4 5 6 7 8



1:	O3S6	ds RNA
2:	O3S5	ds RNA
3:	O3S3b	ds RNA
4:	O3S3a	ds RNA
5:	O3S2	ds RNA
6:	O2S19	ds RNA
7:	O2S18	ds RNA
8:	3bla	ds RNA

Gel iv)

1 2 3 4 5 6 7 8 9



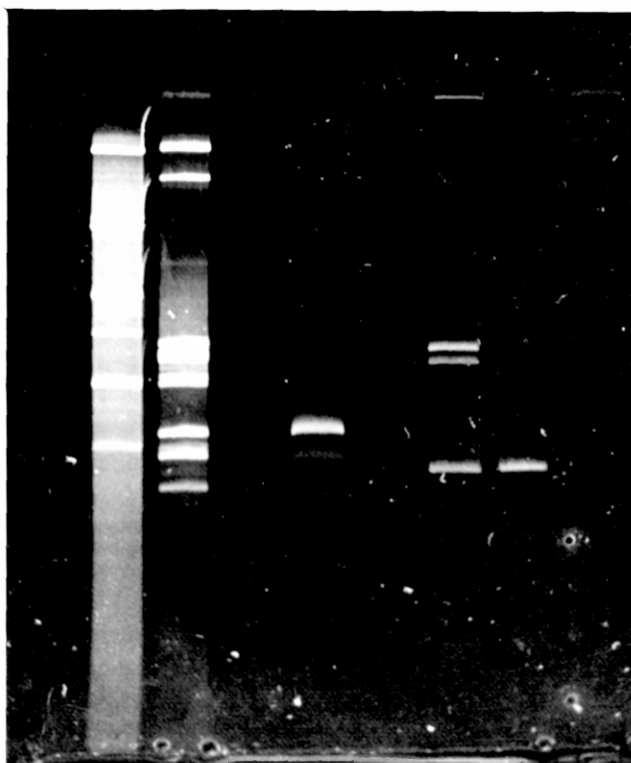
1:	O3S18	ds RNA
2:	O3S17	ds RNA
3:	O3S15	ds RNA
4:	O3S14	ds RNA
5:	O3S13	ds RNA
6:	O3S10	ds RNA
7:	O3S8b	ds RNA
8:	O3S8a	ds RNA
9:	3bla	ds RNA

Gel v)

1 2 3 4 5 6 7 8

4.1 —

1.11 —



1: AfV ds RNA

2: 3b1a ds RNA

3: O3S20a ds RNA

4: O3S20b ds RNA

5: O6S2 ds RNA

6: O6S3 ds RNA

7: O6S4 ds RNA

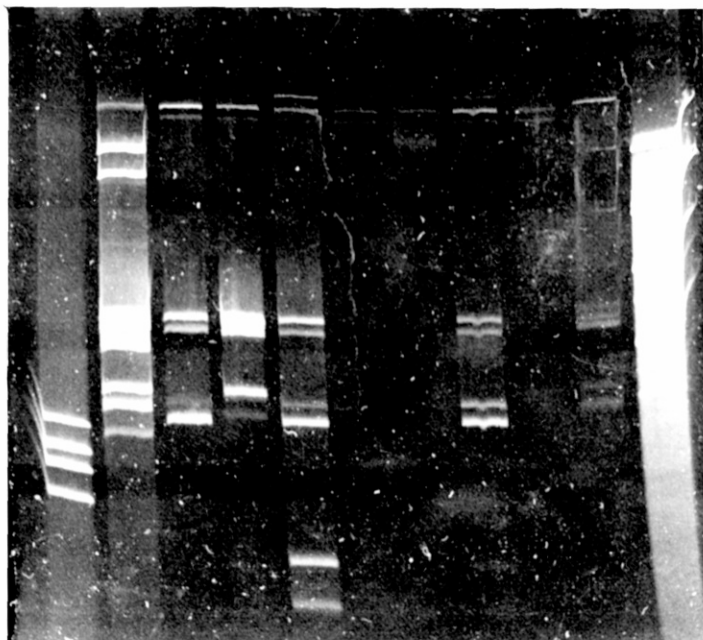
8: O6S7 ds RNA

Gel vi)

1 2 3 4 5 6 7 8 9 10 11

4.1—

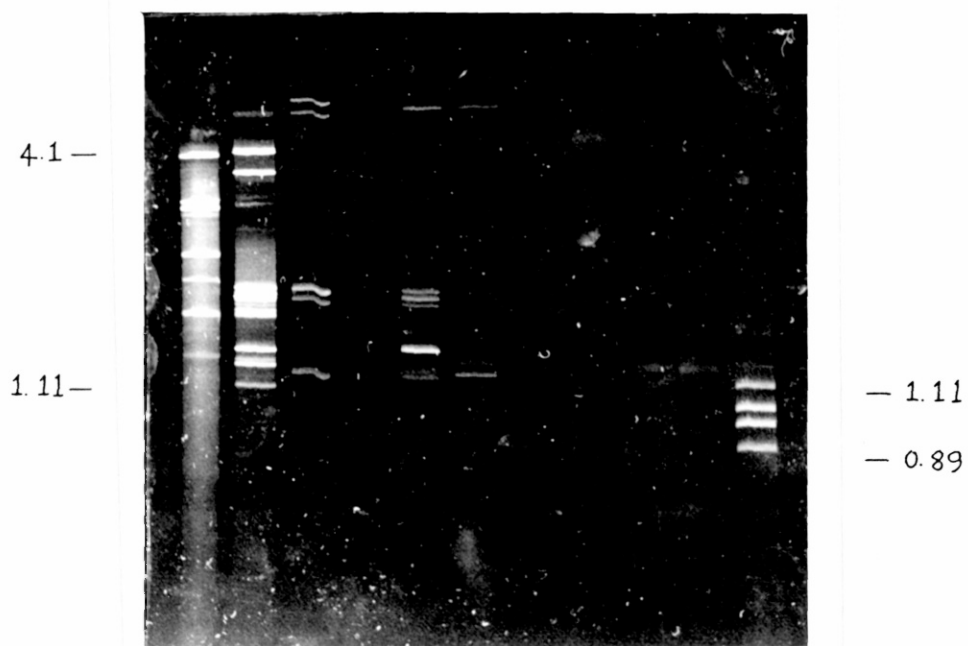
0.89—



1: PsV	ds RNA
2: 3bla	ds RNA
3: O6S16	ds RNA
4: O6S15	ds RNA
5: O6S14	ds RNA
6: O6S12	ds RNA
7: O6S17	ds RNA
8: O6S10	ds RNA
9: O6S9	ds RNA
10: O6S8	ds RNA
11: AfV	ds RNA

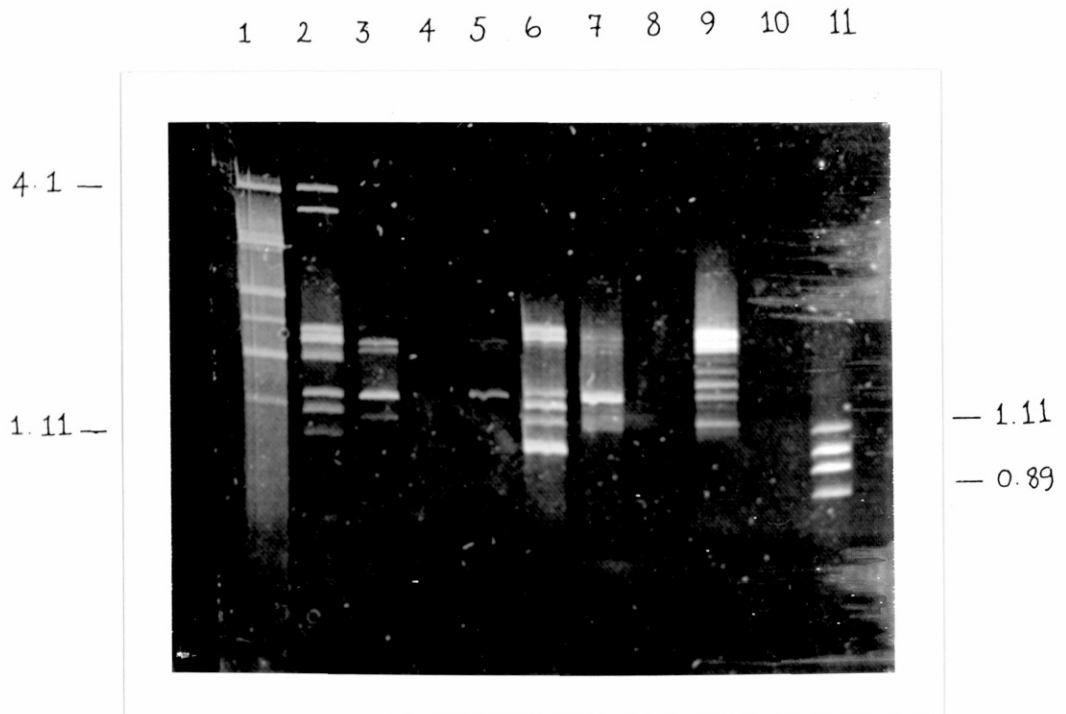
Gel vii)

1 2 3 4 5 6 7 8 9 10 11



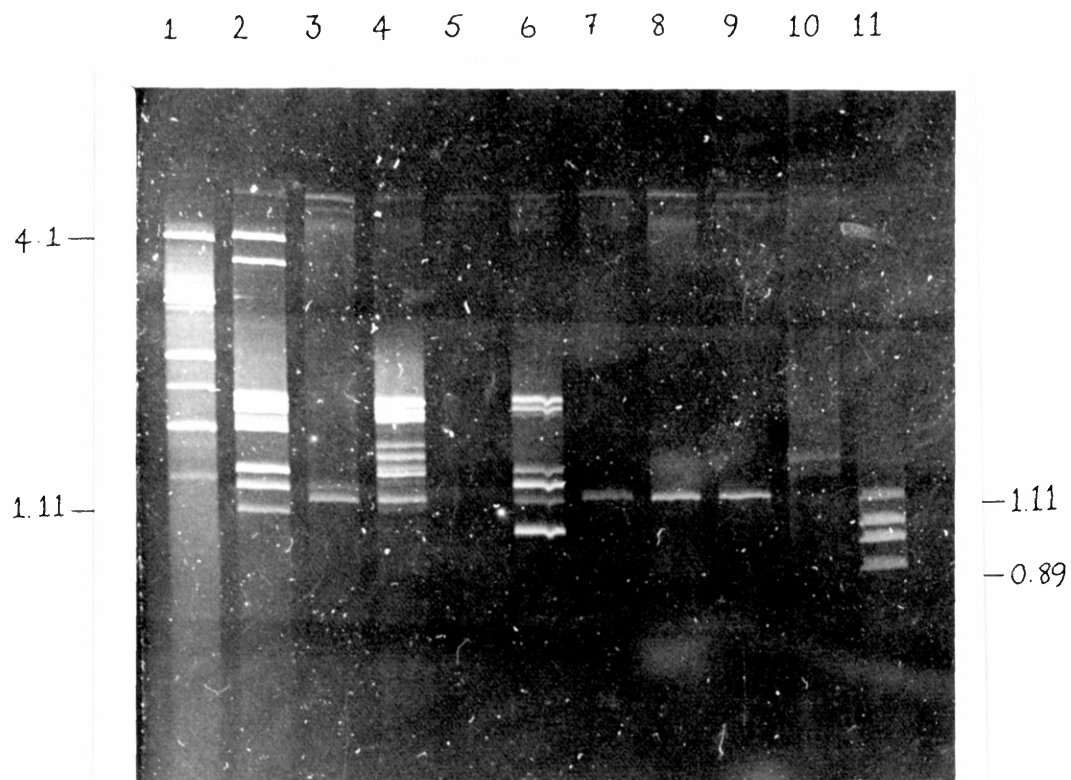
1: AfV	ds RNA
2: 3b1a	ds RNA
3: 06S17	ds RNA
4: 06S18	ds RNA
5: 07S1	ds RNA
6: 07S2	ds RNA
7: 07S4	ds RNA
8: 07S5a	ds RNA
9: 07S5b	ds RNA
10: 07S6	ds RNA
11: PsV	ds RNA

Gel viii)



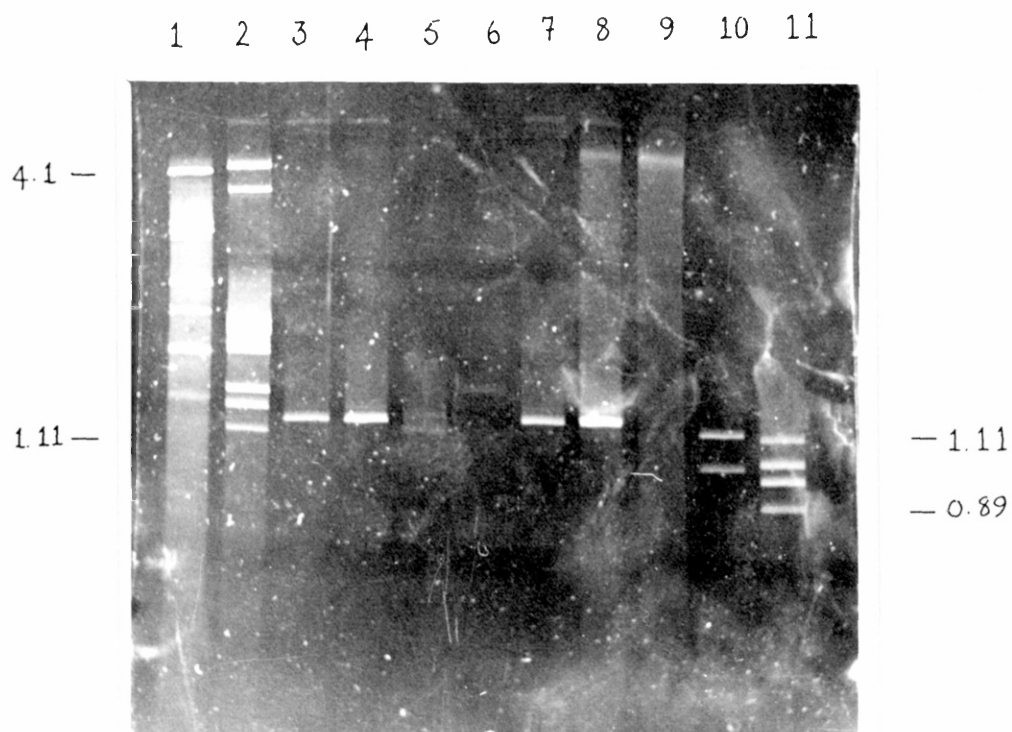
1:	AfV	ds RNA
2:	3bla	ds RNA
3:	O7S7	ds RNA
4:	O7S8	ds RNA
5:	O7S9	ds RNA
6:	O7S10	ds RNA
7:	O7S11	ds RNA
8:	O7S12	ds RNA
9:	O7S14	ds RNA
10:	O7S15	ds RNA
11:	PsV	ds RNA

Gel ix)



1: AfV	ds RNA
2: 3b1a	ds RNA
3: 07S16	ds RNA
4: 07S17	ds RNA
5: 07S18	ds RNA
6: 07S20	ds RNA
7: 14S3 β	ds RNA
8: 14S3 α	ds RNA
9: 14S4	ds RNA
10: 14S6	ds RNA
11: PsV	ds RNA

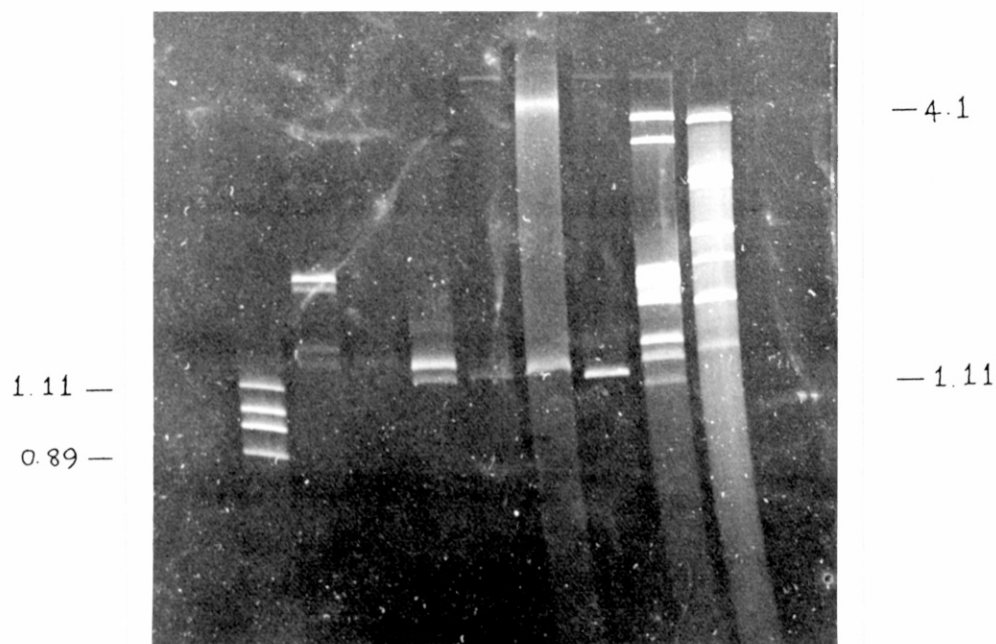
Gel x)



1: AfV	ds RNA
2: 3b1a	ds RNA
3: 14S7	ds RNA
4: 14S8 α	ds RNA
5: 14S8 β	ds RNA
6: 14S10	ds RNA
7: 14S11 α	ds RNA
8: 14S11 β	ds RNA
9: 14S12	ds RNA
10: 14S13	ds RNA
11: PsV	ds RNA

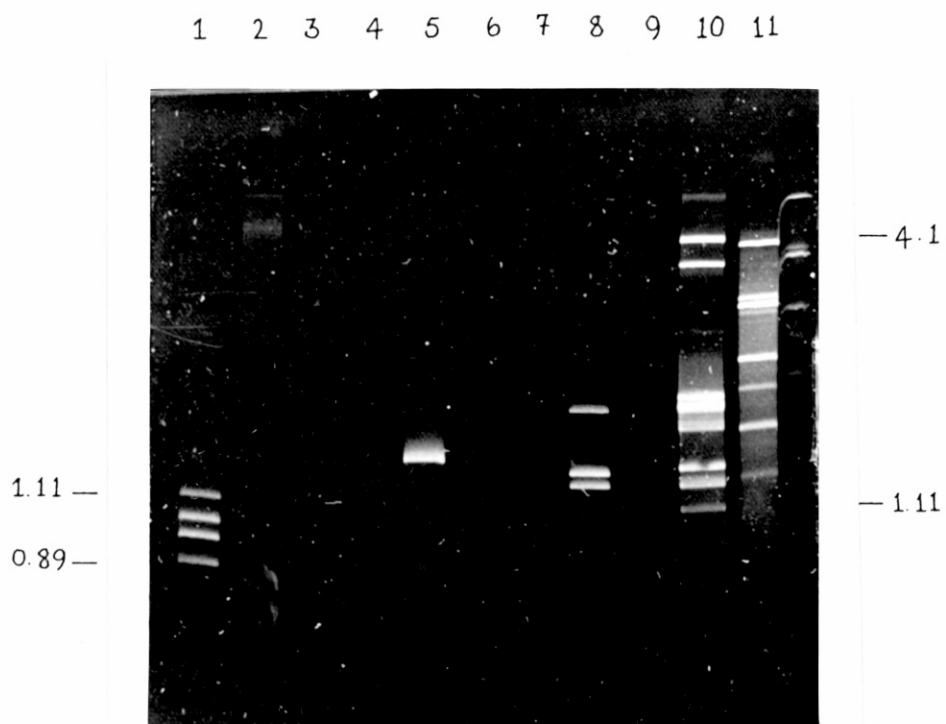
Gel xi)

1 2 3 4 5 6 7 8 9



1: PsV	ds RNA
2: 14S19	ds RNA
3: 14S17b	ds RNA
4: 14S17a	ds RNA
5: 14S15 γ	ds RNA
6: 14S15 β	ds RNA
7: 14S15 α	ds RNA
8: 3bla	ds RNA
9: AfV	ds RNA

Gel xii)



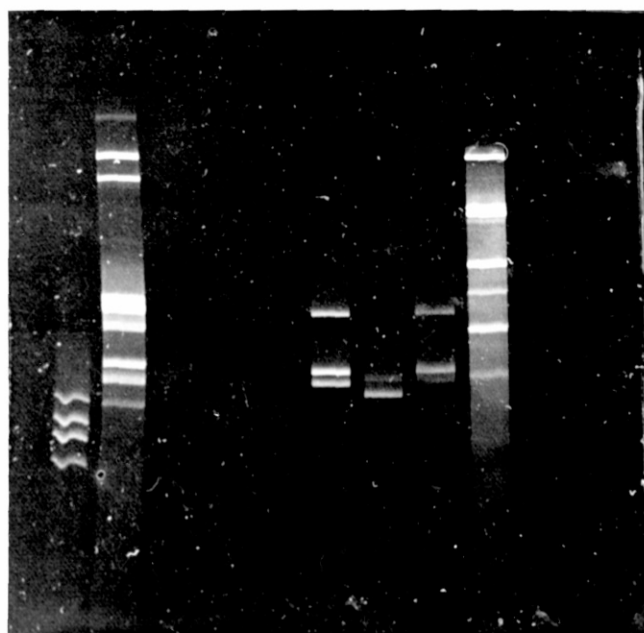
1:	PsV	ds RNA
2:	15S6 β	ds RNA
3:	15S6 α	ds RNA
4:	15S5 β	ds RNA
5:	15S5 α	ds RNA
6:	15S4 β	ds RNA
7:	15S4 α	ds RNA
8:	15S3	ds RNA
9:	15S2	ds RNA
10:	3b1a	ds RNA
11:	AfV	ds RNA

Gel xiii)

1 2 3 4 5 6 7 8 9

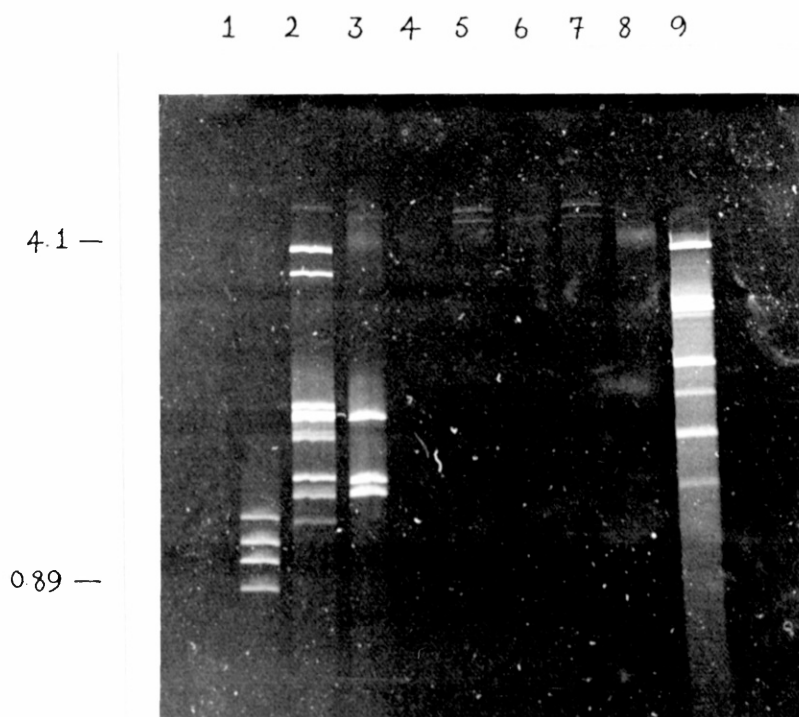
4.1 —

0.89 —



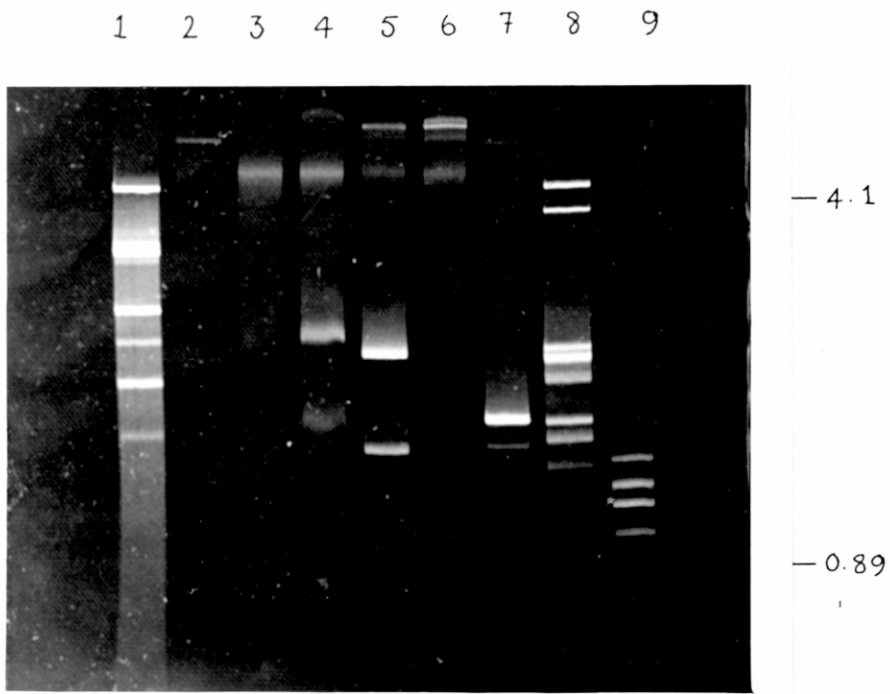
1: PsV	ds RNA
2: 3bla	ds RNA
3: 15S7	ds RNA
4: 15S8	ds RNA
5: 15S9	ds RNA
6: 15S10	ds RNA
7: 15S11	ds RNA
8: 15S12a	ds RNA
9: AfV	ds RNA

Gel xiv)



1: PsV	ds RNA
2: 3b1a	ds RNA
3: 15S12b	ds RNA
4: 15S14	ds RNA
5: 15S15	ds RNA
6: 15S16	ds RNA
7: 15S18	ds RNA
8: 15S19	ds RNA
9: AfV	ds RNA

Gel xv)



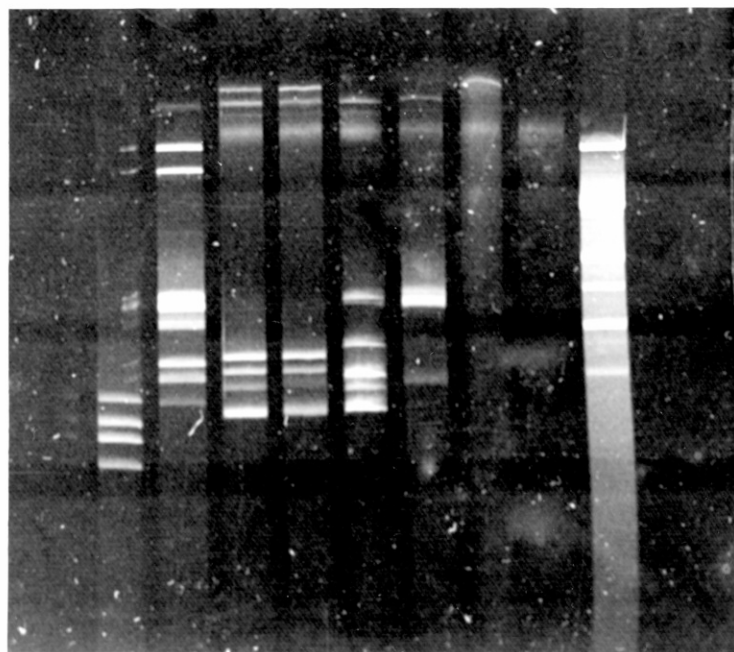
1: AfV	ds RNA
2: WWS6	ds RNA
3: WWS4	ds RNA
4: WWS2b	ds RNA
5: WWS2a	ds RNA
6: WWR2	ds RNA
7: 15S20	ds RNA
8: 3b1a	ds RNA
9: PsV	ds RNA

Gel xvi)

1 2 3 4 5 6 7 8 9

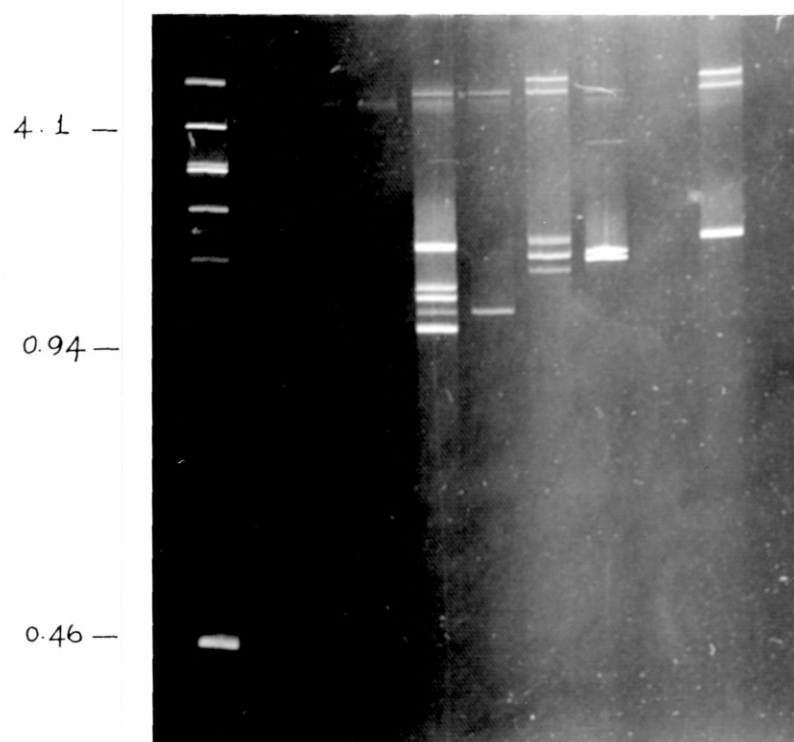
4.1 —

0.89 —



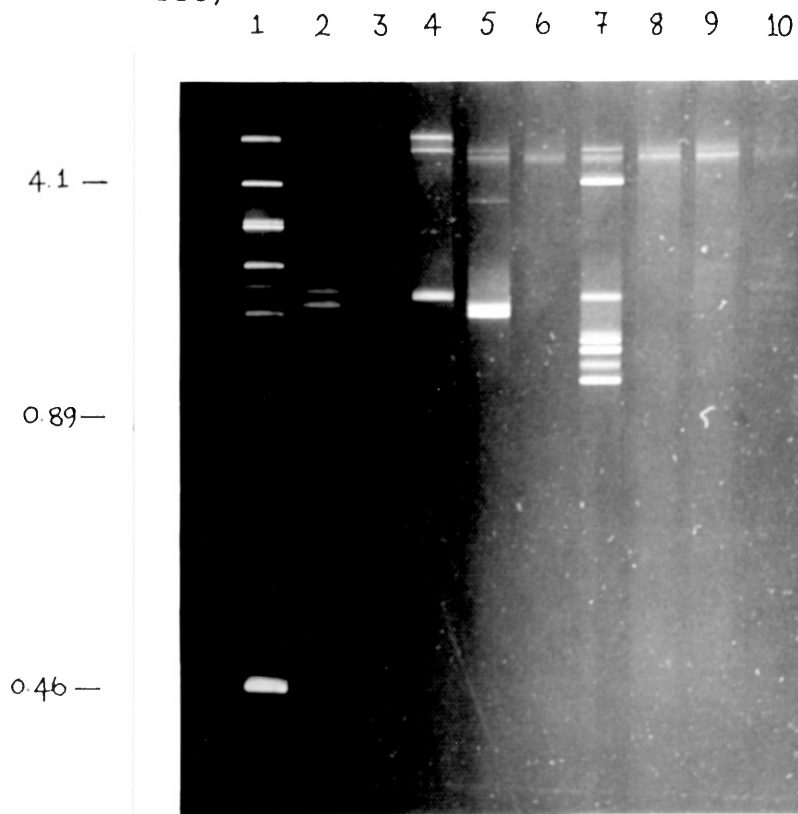
1: PsV	ds RNA
2: 3bla	ds RNA
3: WWS7a	ds RNA
4: WWS7b	ds RNA
5: WWS8	ds RNA
6: WWS10	ds RNA
7: WWS11	ds RNA
8: WWS12	ds RNA
9: AfV	ds RNA

Gel xvii) 1 2 3 4 5 6 7 8 9 10



1: HmV, AfV & PsV	ds RNA
2: 0601/1	ds RNA
3: 0601/2	ds RNA
4: 0601/3	ds RNA
5: 0601/4	ds RNA
6: 0601/5	ds RNA
7: 0601/6	ds RNA
8: 0601/7	ds RNA
9: 0601/8	ds RNA
10: 0601/9	ds RNA

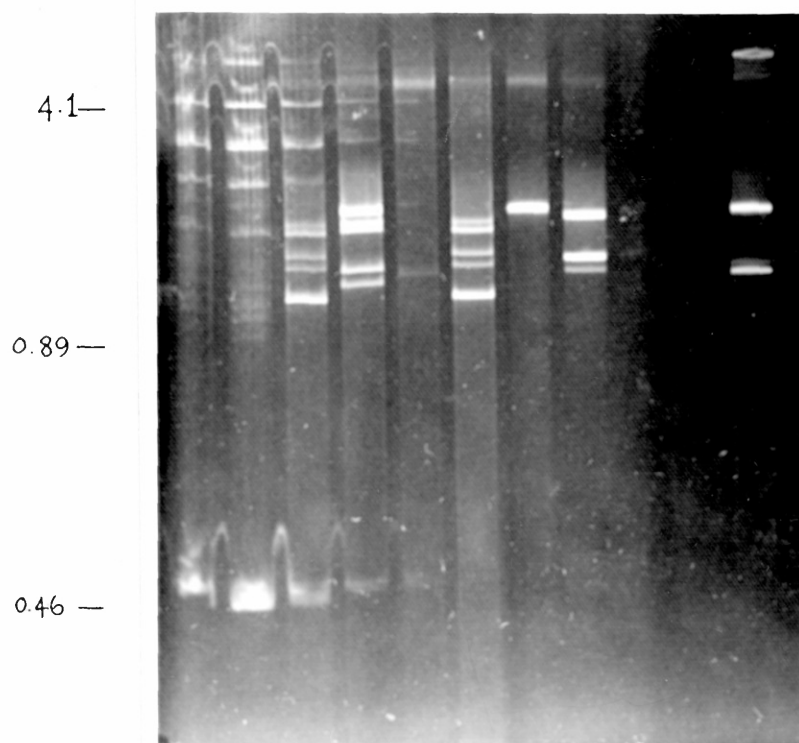
Gel xviii)



1:	HmV, AfV & PsV	ds RNA
2:	0601/10	ds RNA
3:	0601/11	ds RNA
4:	0601/12	ds RNA
5:	0601/13	ds RNA
6:	0601/14	ds RNA
7:	0601/15	ds RNA
8:	0601/16	ds RNA
9:	0601/17	ds RNA
10:	0601/18	ds RNA

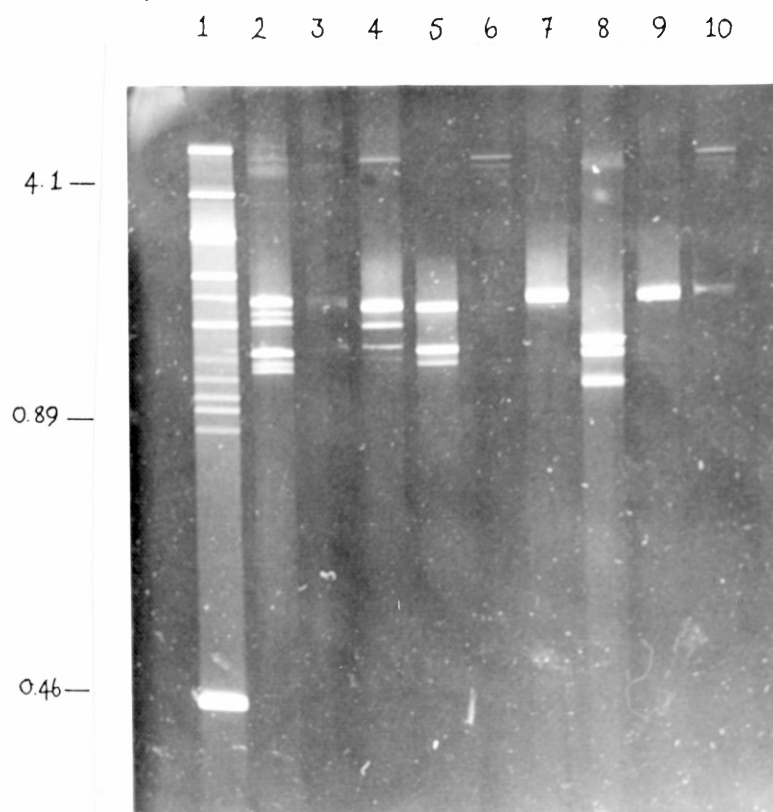
Gel xix)

1 2 3 4 5 6 7 8 9 10



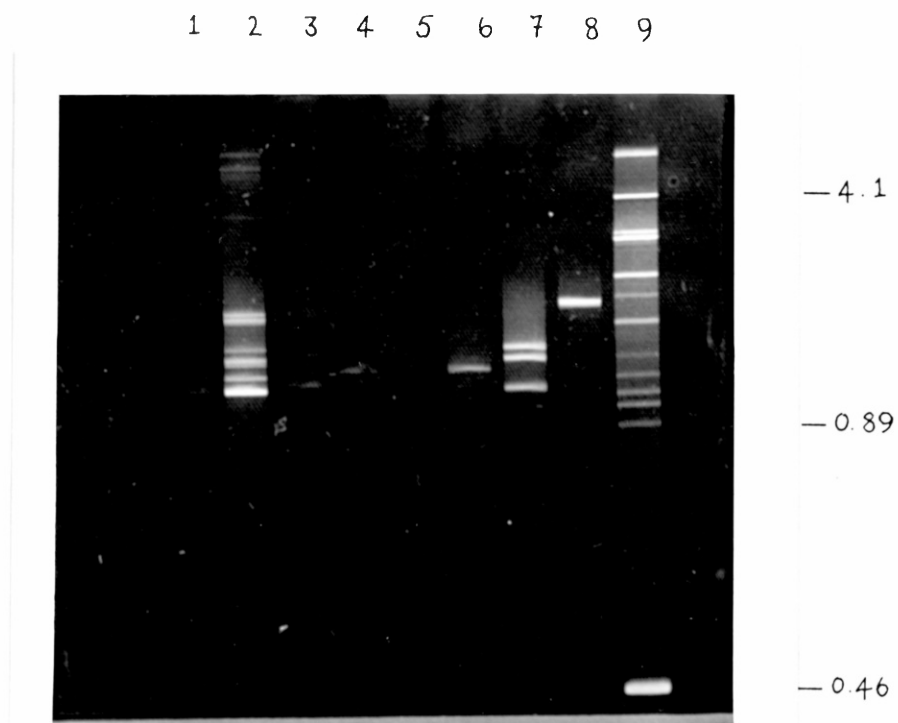
1: HmV, AfV & PsV	ds RNA
2: 0601/19	ds RNA
3: 0601/20	ds RNA
4: 0601/21	ds RNA
5: 0601/22	ds RNA
6: 0881/1	ds RNA
7: 0881/2	ds RNA
8: 0881/3	ds RNA
9: 0881/4	ds RNA
10: 0881/5	ds RNA

Gel xx)



1: HmV, AfV & PsV	ds RNA
2: 0881/6	ds RNA
3: 0881/7	ds RNA
4: 0881/8	ds RNA
5: 0881/9	ds RNA
6: 0881/10	ds RNA
7: 0881/11	ds RNA
8: 0881/12	ds RNA
9: 0881/13	ds RNA
10: 0881/14	ds RNA

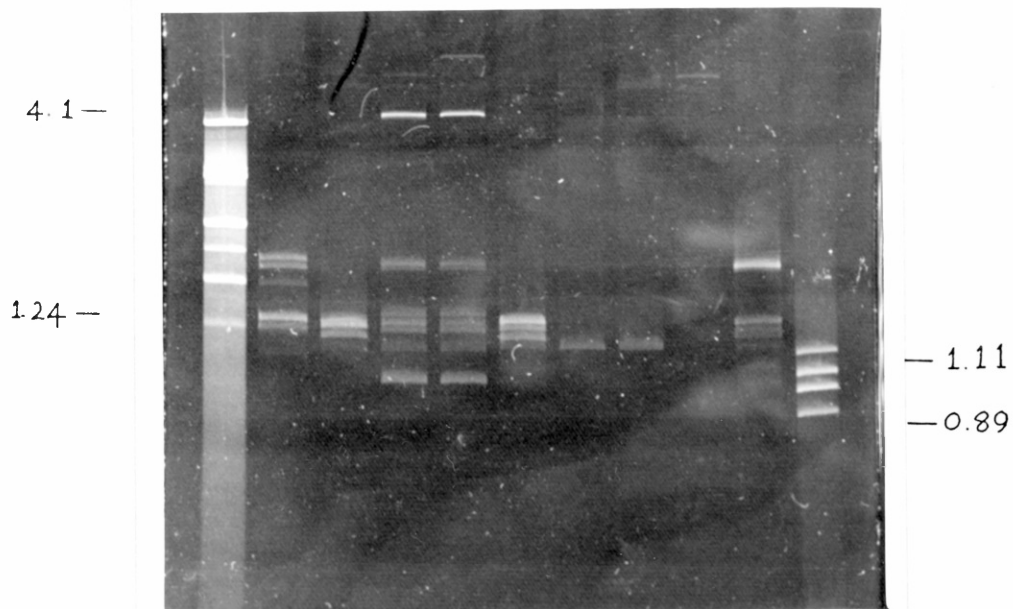
Gel xxi)



1: 0881/4	ds RNA
2: 0881/21	ds RNA
3: 0881/20	ds RNA
4: 0881/19	ds RNA
5: 0881/18	ds RNA
6: 0881/17	ds RNA
7: 0881/16	ds RNA
8: 0881/15	ds RNA
9: HmV, AfV & PsV	ds RNA

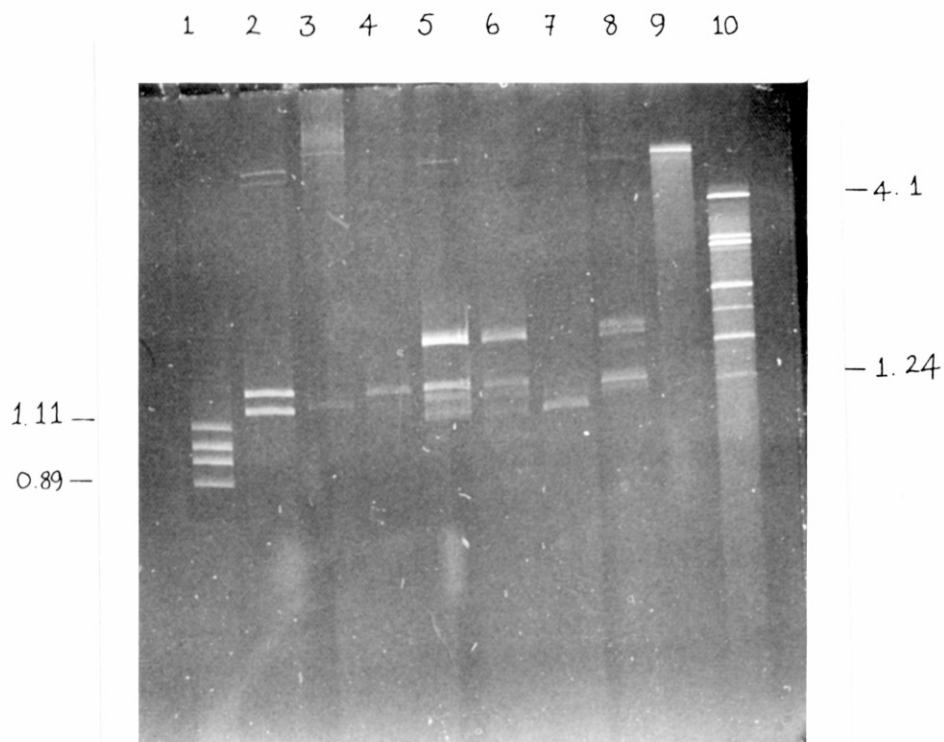
Gel xxii)

1 2 3 4 5 6 7 8 9 10 11



1:	AfV	ds RNA
2:	O2S14	ds RNA
3:	O2S11	ds RNA
4:	O2S9 α	ds RNA
5:	O2S8 α	ds RNA
6:	O2S7	ds RNA
7:	O2S6	ds RNA
8:	O2S5	ds RNA
9:	O2S3	ds RNA
10:	O2S2	ds RNA
11:	PsV	ds RNA

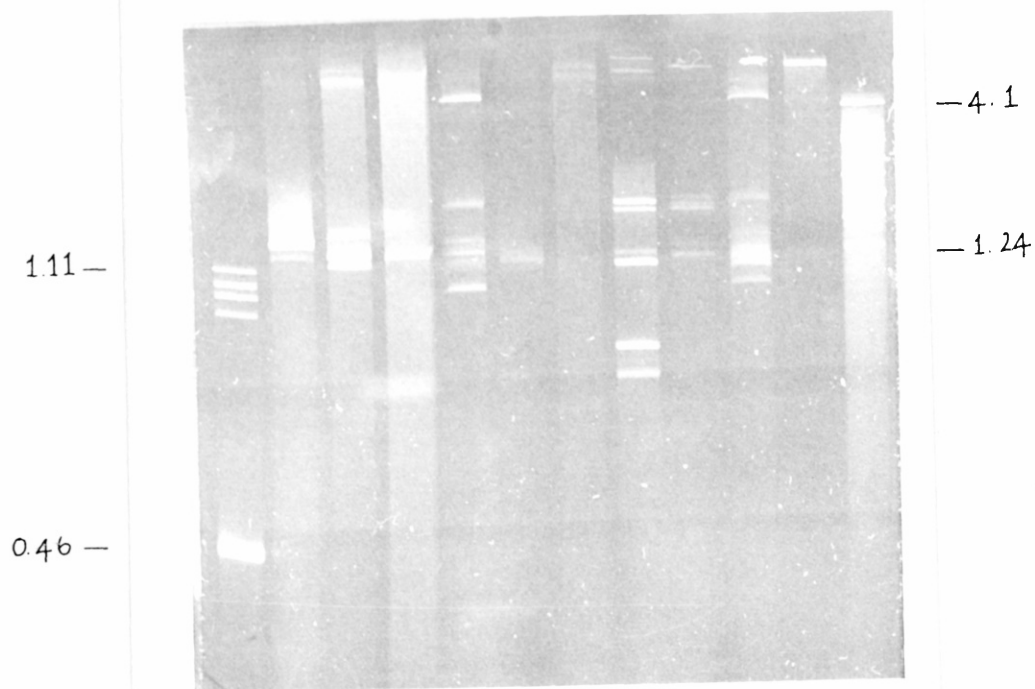
Gel xxiii)



1: PsV	dsRNA
2: O3S13	ds RNA
3: O3S6	ds RNA
4: O3S5	ds RNA
5: O3S3b	ds RNA
6: O3S2	ds RNA
7: O2S19	ds RNA
8: O2S18	ds RNA
9: HmV	ds RNA
10: AfV	ds RNA

Gel xxiv)

1 2 3 4 5 6 7 8 9 10 11 12



- 1: PsV ds RNA
- 2: 15S5 β ds RNA
- 3: 14S17b ds RNA
- 4: 14S15 β ds RNA
- 5: 14S8 β ds RNA
- 6: 07S6 ds RNA
- 7: 07S5a ds RNA
- 8: 06S14 ds RNA
- 9: 06S8 ds RNA
- 10: 02S9 β ds RNA
- 11: HmV ds RNA
- 12: AfV ds RNA

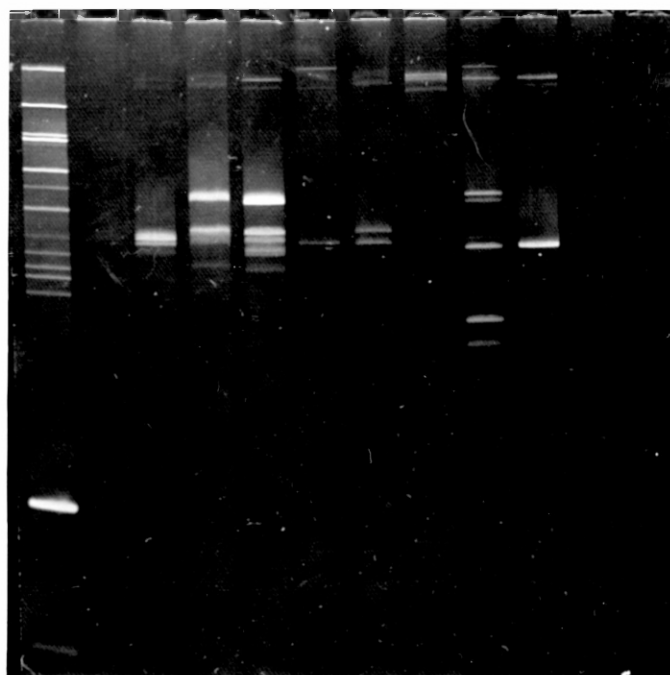
Gel xxv)

1 2 3 4 5 6 7 8 9 10 11

4.1—

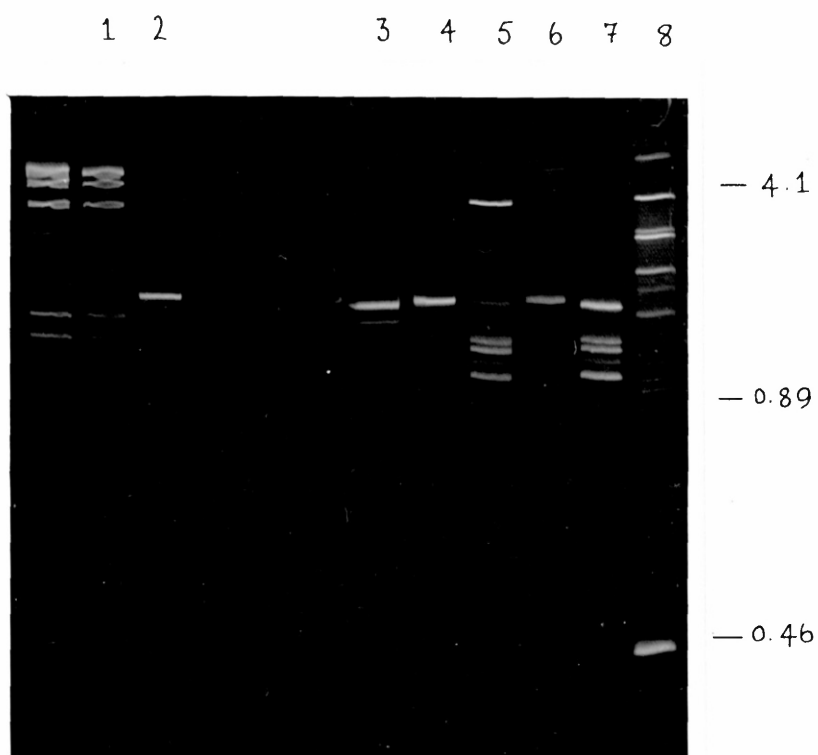
0.89—

0.46—



1:	HmV, AfV & PsV	ds RNA
2:	O2S5	ds RNA
3:	O2S11	ds RNA
4:	O3S2	ds RNA
5:	O3S3b	ds RNA
6:	O3S6	ds RNA
7:	O3S13	ds RNA
8:	O3S20a	ds RNA
9:	O6S14	ds RNA
10:	14S8 α	ds RNA
11:	15S6 α	ds RNA

Gel xxvi)



1: 0881/15	ds RNA
2: 0881/14	ds RNA
3: 0881/8	ds RNA
4: 0881/1	ds RNA
5: 0601/15	ds RNA
6: 0601/9	ds RNA
7: 0601/4	ds RNA
8: HmV, AfV & PsV	ds RNA

APPENDIX III

Ds RNA content of the Ggt field isolates

(* denotes an isolate used as a reference point;
see section 3.1.3c and end of this section.)

<u>Ds RNA</u>	<u>Isolates</u>
(μ g/g)	
0	03S3a, 03S8b, 03S14 06S18 07S4, 07S5a, 07S5b, 07S8, 07S12 14S12 15S2, 15S4 α , β , 15S6 α , β , 15S7, 15S8, 15S9, 15S14, 15S19 WWS4, WWS11, WWS12 0601/1, 0601/2, 0601/3, 0601/8, 0601/11, 0601/14 0881/3, 0881/18
0.01 - 1.00	02S3, 02S5*, 02S6, 02S16 06S2, 06S11 07S6, 07S15, 07S18 14S6, 14S10, 14S15 α , β , γ 15S11, 15S16, 15S20* WWS2b, WWS6 0601/18, 0601/21*, 0601/22 0881/4, 0881/7, 0881/15, 0881/17, 0881/19, 0881/20

Ds RNA

(µg/g)

Isolates

1.01 - 2.00

02S9β, 02S15, 02S19

03S5, 03S6, 03S8a, 03S17, 03S20b

06S4, 06S7, 06S9*, 06S12

07S9

14S3 α,β, 14S4*, 14S7, 14S8 α,β,

14S11 α,β*, 14S13, 14S17a,b, 14S19*

15S3*, 15S5 α,β, 15S10, 15S12a,b*,

15S15, 15S18*

WWS2a*, WWS10*

0601/5*, 0601/10, 0601/16, 0601/17*

0881/1, 0881/2, 0881/5, 0881/9,

0881/10, 0881/11, 0881/12*, 0881/13,

0881/14, 0881/16

2.01 -3.00

02S2, 02S7, 02S11*, 02S14*, 02S18*

03S2*, 03S3b*, 03S10, 03S20a

06S8

07S2, 07S11, 07S16

WWR2*

0601/4, 0601/6, 0601/7, 0601/9,

0601/12, 0601/13, 0601/19, 0601/20

0881/8

<u>Ds RNA</u> (µg/g)	<u>Isolates</u>
3.01 - 4.00	03S13*, 03S15, 03S18 06S3, 06S10, 06S14, 06S15*, 06S16, 06S17 07S1, 07S7, 07S20* 0601/15 0881/6*, 0881/21
4.01 - 5.00	02S8 α,β, γ, 02S9 α, γ 07S10*, 07S14, 07S17 WWS7a,b, WWS8

Ds RNA content of isolates used as reference points.

<u>Isolate</u>	<u>Ds RNA content</u> (µg/g)
02S5	0.4
15S20	0.6
0601/21	0.3
06S9	1.1
14S4	1.7
14S11β	1.5
14S19	1.1
15S3	1.4
15S12b	1.5
15S18	1.1
WWS2a	1.3
WWS10	1.7
0601/5	1.2
0601/17	1.9
0881/12	1.9
02S11	2.6
02S14	2.7
02S18	2.6
03S2	2.1
03S3b	2.4
WWR2	2.8

<u>Isolate</u>	<u>Ds RNA content (µg/g)</u>
03S13	3.6
06S15	3.7
07S20	3.1
0881/6	3.8
02S9 α	4.9
07S10	4.1
WWS7a	4.5

Key words: *dsRNA mycoviruses/Gaeumannomyces graminis/fungal protoplasts/PEG-mediated infection*

Infection of Protoplasts of the Wheat Take-all Fungus, *Gaeumannomyces graminis* var. *tritici*, with Double-stranded RNA Viruses

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(Accepted 19 July 1984)

SUMMARY

Protoplasts from a virus-free isolate, 3b1a conD/A3, of *Gaeumannomyces graminis* var. *tritici* were inoculated with a purified preparation of a mixture of four viruses, A, B1, B2 and C, from *G. graminis* isolate 3b1a in the presence of polyethylene glycol. Three out of 30 fungal colonies obtained by regeneration of inoculated protoplasts were found to be infected, two with virus B1 and one with virus B2. The concentrations of these viruses in the newly infected isolates were similar to their respective concentrations in isolate 3b1a and remained stable through three serial subcultures.

Infections of field isolates of the wheat take-all fungus *Gaeumannomyces graminis* var. *tritici* with double-stranded RNA (dsRNA) viruses are common (Rawlinson & Buck, 1981). Lapierre *et al.* (1970) and Lemaire *et al.* (1970) correlated virus infection with hypovirulence in *G. graminis*, but Rawlinson *et al.* (1973) found no consistent association of virus infection with weak pathogenicity of the fungus. These apparently conflicting reports could be explained if the variations in the serological properties (Frick & Lister, 1978) and in the numbers and mol. wt. of the dsRNA and capsid polypeptide components (Buck *et al.*, 1981; Buck, 1984) of viruses from different fungal isolates reflect variations in biotype.

To obtain unequivocal evidence for the effect of a particular virus on the pathogenicity of *G. graminis*, it is necessary to obtain virus-free and virus-infected isolates of the same fungal strain. Although protoplasts of a few fungi have been infected with partially purified virus preparations (Lhoas, 1971; Pallett, 1976; Ghabrial & Mernaugh, 1983) many investigators have not succeeded (Bozarth, 1975; Lai & Zachariah, 1975; Boissonnet-Menes & Lecoq, 1976). In attempts to transmit *G. graminis* viruses, Rawlinson & Muthyalu (1974) reported that uninfected isolates produced few protoplasts, from which they were unable to make any regenerate mycelium.

We now report the isolation and virus infection of *G. graminis* protoplasts and their regeneration to produce fungal colonies which remained stably infected through three serial subcultures.

For the preparation of virus inoculum, *G. graminis* isolate 3b1a (Buck *et al.*, 1981) was cultured for 10 days at 24 °C in two shaken flasks each containing 200 ml GP medium [2% (w/v) glucose, 0.5% (w/v) peptone, 0.25% (w/v) yeast extract]. After harvesting by vacuum filtration, the mycelium (20 g wet weight) was suspended in 60 ml ice-cold PK buffer (0.03 M-NaH₂PO₄, 0.15 M-KCl, adjusted to pH 7.6 with NaOH) and disrupted by shaking with 10 g of glass beads (0.45 to 0.5 mm diam.) in a Braun model MSK cell homogenizer at 4000 r.p.m. for 20 s. After addition of 140 ml PK buffer and stirring at 4 °C for 4 h the virus was isolated by two cycles of differential centrifugation (15000 g, 30 min; 96000 g, 3 h) and purified by centrifugation through linear density gradients of sucrose [10 to 45% (w/v) in PK buffer] at 149000 g for 1 h. Virus-containing fractions, identified by agarose gel electrophoresis (Buck *et al.*, 1981), were diluted with 2 vol. PK buffer and the virus was repelleted by centrifugation at 189000 g for 2 h and then resuspended in 1 ml PK buffer. Analysis of a sample by polyacrylamide gel electrophoresis (PAGE), after disruption of virus particles by heating in 1% SDS, revealed the

dsRNA components characteristic of the four viruses A, B1, B2 and C described previously (McFadden *et al.*, 1983).

G. graminis isolate 3b1a conD/A3, a single ascospore isolate derived from a single conidial isolate of 3b1a, was chosen as the host for virus infection because (i) it has been shown to be completely virus-free (McFadden *et al.*, 1983) and (ii) it should be susceptible to 3b1a viruses. For protoplast isolation, cultures were shaken in a liquid medium which contained (g/l): NaNO_3 , 6.0; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.52; KCl, 0.52; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.2; $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.2; glucose, 20; yeast extract, 5.0; casamino acids, 2.0, at 24 °C for 7 days. After harvesting by filtration, the mycelium (5 g wet weight) was resuspended in 25 ml of buffer/osmotic stabilizer (0.2 M-sodium phosphate pH 5.8, containing 0.6 M-KCl) (Hamlyn *et al.*, 1981) and then centrifuged at 730 g for 5 min. The pellet was resuspended in 75 ml of buffer/stabilizer containing the lytic enzymes Novozym 234 (Novo Enzyme Products, Windsor, U.K.) and Cellulase CP (kindly donated by J. & E. Sturge Ltd., Selby, U.K.), both at a concentration of 5 mg/ml and shaken orbitally at 24 °C for 4 h. The released protoplasts were separated from remaining mycelial fragments by filtration through glass wool, followed by filtration through a sintered glass filter (maximum pore size: 20 to 30 μm diam.). The protoplasts were then collected by centrifuging at 550 g for 5 min, washed twice in buffer/stabilizer and finally resuspended in 3 to 5 ml of buffer/stabilizer.

Protoplasts were inoculated using a modification of the methods of Cassells & Barlass (1978) and Maule *et al.* (1980) for virus infection of higher plant protoplasts. To 0.2 ml of protoplast suspension (approx. 5×10^7 protoplasts) in 0.2 M-sodium phosphate pH 5.8, containing 0.6 M-KCl, were added 2 ml of 40% (w/v) polyethylene glycol 6000 (PEG) containing 3 mM- CaCl_2 followed immediately by 0.5 ml of filter-sterilized virus suspension (130 to 180 $\mu\text{g}/\text{ml}$) at 20 to 22 °C. After 10 s, 20 ml of 0.2 M-sodium phosphate pH 5.8, containing 0.6 M-KCl, was added and the mixture was incubated for 1 h at 20 to 22 °C with occasional gentle shaking. Aliquots of 0.1 ml of 1:100 and 1:1000 dilutions of the inoculation mixture in 0.2 M-sodium phosphate pH 5.8, containing 0.6 M-KCl, were mixed with molten (48 °C) protoplast regeneration complete medium which contained (g/l): NaNO_3 , 6.0; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.52; KCl, 44.7; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.2; $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.2; glucose, 20; yeast extract, 1.0; Bactopeptone, 1.0; casamino acids, 1.0; biotin, 0.001; thiamine, 0.001; agar, 15, in Petri dishes and incubated at 20 to 22 °C. After 7 to 10 days, single fungal colonies about 3 mm in diameter and spaced about 1 cm apart, which were produced by regeneration of protoplasts, were picked off and shake-cultured in 200 ml GP medium at 24 °C. After 7 to 10 days when each single colony had produced about 5 g mycelium (wet weight after harvesting by filtration), about 10 mg was used for a subculture on a potato dextrose agar (PDA) slope and the remainder was subjected to extraction for dsRNA, essentially as described by Morris & Dodds (1979). No colonies were produced when filter-sterilized virus was plated undiluted on regeneration medium, showing that the inoculum was completely free from propagules of the infected parent isolate 3b1a. Hence all the colonies arose by regeneration of protoplasts.

In the first experiment 10 regenerated colonies were examined and dsRNA was detected by PAGE in one, designated R1. This dsRNA consisted of two components which co-migrated with the 2.33 and 2.24 kbp dsRNA of virus B1 (Fig. 1a, lanes 1, 2 and 3). In another experiment out of 20 regenerated colonies dsRNA was detected in two, designated R2 and R3. The dsRNA from R2 consisted of two components which co-migrated with B1 dsRNA (Fig. 1a, lanes 4, 5 and 6), whereas dsRNA from R3 consisted of two components which co-migrated with the 2.11 and 2.08 kbp dsRNA components of virus B2 (Fig. 1b, lanes 1, 2 and 3). Virus particles were extracted from further batches of mycelium of isolates R1, R2 and R3 grown in shaken liquid culture, using the PDA subcultures as sources of inoculum, and purified by sucrose density gradient centrifugation. Electron microscopy showed these particles to have diameters of 35 nm which is the same as those of virus B1 and B2 (Buck *et al.*, 1981). Double-stranded RNAs extracted from the virus particles had the same mobilities in PAGE as dsRNAs prepared from the isolates by direct nucleic acid extraction. Examination of the capsid polypeptides by SDS-PAGE showed in each case a major component with a mol. wt. of 73000 (Fig. 2), as found previously for both viruses B1 and B2 (McFadden *et al.*, 1983). It is clear therefore that regenerated isolates R1, R2 and R3 are infected with viruses B1, B1 and B2 respectively.

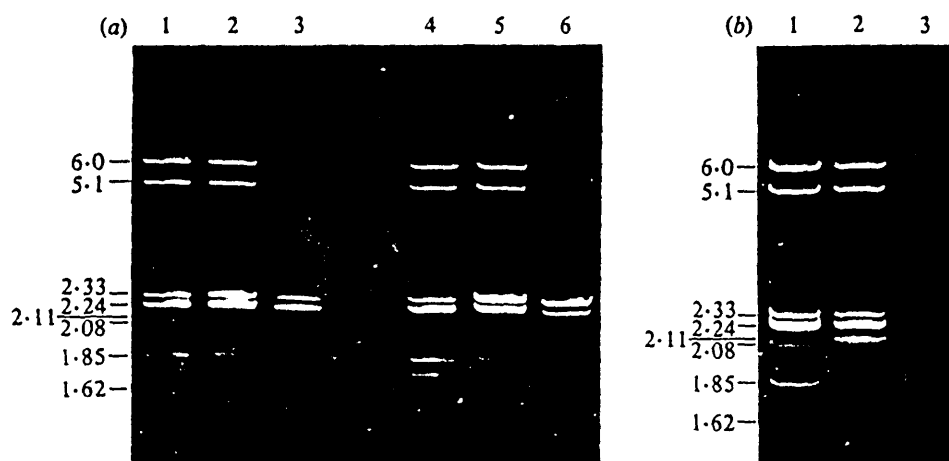


Fig. 1. PAGE of dsRNA from *G. graminis* isolates: (a) lane 1, 3b1a; lane 2, 3b1a + R1; lane 3, R1; lane 4, 3b1a; lane 5, 3b1a + R2; lane 6, R2; (b) lane 1, 3b1a; lane 2, 3b1a + R3; lane 3, R3. Electrophoresis was at 70 V for 16 h (a) and 60 V for 20 h (b). The gels were stained with ethidium bromide and photographed on a u.v. transilluminator. Sizes of dsRNA on the left of each gel are in kbp.

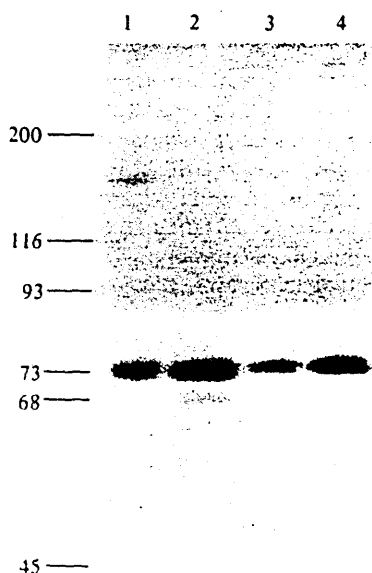


Fig. 2. SDS-PAGE of virus capsid polypeptides from *G. graminis* isolates: lane 1, T1 (mol. wt. 73000; Buck *et al.*, 1981); lane 2, R1; lane 3, R2; lane 4, R3. Electrophoresis was at 50 V for 16 h. The gel was stained with Coomassie Brilliant Blue. The numbers on the left are mol. wt. $\times 10^{-3}$ of standards which were included in the same gel (myosin, β -galactosidase, phosphorylase B, *G. graminis* virus T1-A, bovine serum albumin and ovalbumin).

The yields of B1 and B2 dsRNAs from isolates R1, R2 and R3 were similar to those of the respective dsRNA from the parent infected isolate 3b1a and remained stable after three serial subcultures extending over 3 months. About 10 μ g of B1 dsRNA was recovered from 5 g wet weight of mycelium of isolates R1 and R2, which is similar to the amount of B1 dsRNA (in virus particles) in the total inoculum. Since a maximum of 0.005% of the inoculated protoplasts were

plated on regeneration medium and a single colony represented about 0.2% of the surface area of a Petri dish, a single colony could not have carried more than 0.00001% residual inoculum. Virus B1 must therefore have replicated at least 10^7 -fold. At the first subculture 10 mg of the 5 g of mycelium was used to inoculate the PDA slope and about 5% of the subculture was used to inoculate shaken flasks for a second batch of mycelium from which about 9 µg of B1 dsRNA was again obtained. This represents a further 10^4 -fold replication of virus B1, giving an overall replication of 10^{11} . The stability of the concentrations of B1 dsRNA in isolates R1 and R2 after two further subcultures leaves absolutely no doubt that this dsRNA is replicating in the newly infected strains. Similar conclusions were reached with virus B2 dsRNA in isolate R3.

The virus concentration in the inoculum corresponds to >100000 particles per protoplast. Since only 10% of the regenerated colonies were infected and each with only one virus, it is likely that only a small percentage of the inoculum was taken up by protoplasts. However, the procedure has enabled individual viruses to be 'cloned' from a mixture of viruses and indeed virus B2 was separated in a soluble form for the first time (previously it was obtained only bound to bentonite; McFadden *et al.*, 1983). The failure to detect colonies infected with viruses A or C may be due to screening of an insufficient number of colonies and/or lower infectivity of these viruses. It is unlikely to be due to one virus excluding another, since infection was achieved with virus B2, which from PAGE of 3b1a dsRNA, appears to be in the lowest concentration (Fig. 1a, lane 1, 2.11 and 2.08 kb dsRNA). The requirement for a high PEG concentration for infection (probably to promote protoplast fusion) was shown by the absence of dsRNA in 20 colonies regenerated from protoplasts inoculated with 3b1a viruses using 30% (w/v) PEG in place of 40% (w/v) PEG. No dsRNA was detected in 20 colonies regenerated from protoplasts inoculated with 3b1a virus dsRNA (50 µg/ml) and 40% (w/v) PEG at 20 to 22 °C.

The procedure described will clearly be useful for infecting isolates of individual strains of *G. graminis* for testing the effect of virus infection on fungal pathogenicity. Since no selection method is available for infected colonies, further optimization of the conditions to increase the fraction of protoplasts which become infected and use of the procedure to infect large numbers of *G. graminis* isolates with a range of different viruses would be greatly facilitated by the development of a method for detection *in situ* of infected colonies on Petri dishes, such as a nucleic acid hybridization or immunological assay.

Little is known concerning the replication cycle of any dsRNA mycovirus because procedures for synchronous infection of cells have not been developed. It is possible that the procedure described could be adapted to culture protoplasts, after inoculation with individual viruses, in liquid medium. Virus replication could then be studied using methods similar to those employed with viruses in protoplasts of higher plants.

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