Characterization of double-stranded RNA (dsRNA) from *Rhizoctonia solani*

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Declaration

I declare that this thesis has been composed by myself
and the research presented is my own.

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Abbreviations

AG	anastomosis group
amp	ampicillin
ATA	aurintricarboxylic acid
ATP	adenosine triphosphate
bp	base pairs
BSA	bovine serum albumin
°C	degrees Celsius
cAMP	cyclic adenosine monophosphate
cDNA	complementary deoxyribonucleic acid
Ci	curies
Da	daltons
dATP	deoxyadenosine triphosphate
dCTP	deoxycytosine triphosphate
dGTP	deoxyguanosine triphosphate
dTTP	deoxythymidine triphosphate
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dsRNA	double-stranded ribonucleic acid
EDTA	ethylenediaminetetraacetate
g	grams
dH_20	distilled water
IPTG	isopropyl-β-D-thiogalactoside
kbp	kilobase pairs
LB	Luria broth
M	molar
µ	micro
min	minutes
mm	millimetres
mM	millimolar
mg	milligrams
ml	millilitres
MW	molecular weight
µl	microlitres
µm	micrometres
nm	nanometres
ORF	open reading frame
PDA	potato dextrose agar
PDCA	potato dextrose charcoal agar
PIPES	piperazine-N,N'-bis [2-ethanesulfonic acid]
psi	pounds per square inch
RDRP	RNA-dependent RNA polymerase
RNA	ribonucleic acid
RNase	ribonuclease
rpm	revolutions per minute
SDS	sodium dodecyl sulphate

SDW	sterile distilled water
sp	species
ssRNA	single-stranded RNA
TEMED	N,N,N,N'-tetramethylethylenediamine
Tris	tris (hydroxymethyl) aminomethane
UV	ultraviolet light
VCG	vegetative compatibility group
VLP	virus-like particles
v/ v	volume to volume ratio
w/ v	weight to volume ratio
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactoside

Abstract

The existence, nature and possible functions of double-stranded RNA (dsRNA) were studied in strains of *Rhizoctonia solani* anastomosis group (AG) 3, which infects potatoes, and in some other AGs of *R. solani*. The aim was to determine whether dsRNA might be exploited as a basis for reducing the virulence of *R. solani* strains, as occurs in *Cryphonectria parasitica*, a pathogen of chestnut trees.

Isolates of *R. solani* AG 3 were obtained from potato tubers from a single field site, and from geographically distant sites. DsRNA was found to be ubiquitous, with multiple elements present in each strain, as determined by CF11 cellulose chromatography. Similar gel banding patterns were observed between strains isolated from separate tubers within a single field site; however, banding patterns differed between isolates from diverse sources. All the AG 3 isolates were assessed as being weakly virulent in seedling assays on six host crops (carrot, cress, lettuce, onion, radish, tomato). Attempts to "cure" strains of dsRNA by repeated hyphal tip subculturing or by growing strains in the presence of cycloheximide were generally unsuccessful; although some individual dsRNA bands were lost, they sometimes reappeared, potentially indicating the presence of a chromosomally, integrated copy of the dsRNA. Partially cured strains were unaltered in virulence compared with their respective parental strains. To determine whether dsRNA elements might be transmitted throughout field populations by hyphal anastomosis, strains were paired in various combinations on agar and examined microscopically. Strains from single tubers were compatible with one another; but isolates from different tubers showed a high degree of incompatibility with one another, and isolates from separate fields were incompatible with each other.

Pathogenicity-related enzymatic activities were compared between different strains. Activity of phenol oxidases and pectic enzymes was similar between dsRNA-containing isolates. Cellulolytic activity varied up to 3 fold between isolates. Further examination of cellulolytic activity between isolates from all but two (AG 10 and BI) AGs of *R. solani* indicated different levels both within and between AGs.

Plasmid pXH9 containing complementary DNA of hypovirus CHV1-713 of *C. parasitica* was used as a probe in hybridization studies with dsRNA of *R. solani*, to determine potential sequence similarity. At low stringency (42°C) no hybridization was observed, indicating no homology between CHV1-713 and dsRNA elements of *R. solani*.

A protoplast production protocol, using Novozyme 234 with 1.0 M $MgSO_4$ as an osmotic stabilizer, was successfully developed for *R. solani*. The protoplasts were transformed with plasmids pES200 and pAXHY2, both of which encode hygromycin B resistance. Following PEG-mediated transformation, the regenerated strains were hygromycin resistant. However, transformation was non-integrative, with the hygromycin resistance phenotype being lost upon subculture.

Overall, the study indicated the ubiquity of dsRNA in *R. solani* strains, but no firm correlation could be found between the presence of dsRNA and the degree of virulence of strains. Moreover, vegetative incompatibility seen in this study would indicate the difficulty of achieving transmission of any hypovirulent dsRNA in field populations of *R. solani* AG 3. This would form a major barrier to the potential use of dsRNA for biological control of this important plant pathogen.

CHAPTER 1

INTRODUCTION

1. Introduction

Rhizoctonia solani Kühn (teleomorph *Thanatephorus cucumeris* (Frank) Donk) is an economically important plant pathogen, infecting a diverse array of crops e.g. field, ornamental, vegetable, nursery and greenhouse crops (Baker, 1970), causing seed decay, damping-off of seedlings, stem cankers, root rots, fruit decays and foliage diseases (Ogoshi, 1985). Currently control of *Rhizoctonia*-incited diseases is achieved using chemical treatments such as tolclofos-methyl and Vitavax. A more desirable alternative, especially for consumer products, would be a reliable and economical biocontrol strategy. A novel way of potentially achieving this in *R. solani* involves utilizing double-stranded RNA (dsRNA) elements which reside naturally within some isolates. This has been achieved successfully with chestnut blight, caused by *Cryphonectria parasitica* (Murr.) Barr, and several other phytopathogens e.g. *Ophiostoma ulmi* (Buisman) Nannf. and *Helminthosporium victoriae* Link. might be controlled in a similar manner.

1.1. *General Introduction to* Rhizoctonia solani

The Basidiomycete genus *Rhizoctonia* (from the Greek "death of roots") was first described by De Candolle in 1815 (Carling and Sumner, 1992), with the subsequent description of *R. solani* by Julius Kühn in 1858 (Carling and Sumner, 1992). The amalgam of isolates characterized as *R. solani* form a species complex which share the following characteristics (Parmeter and Whitney, 1970). (i) Multinucleate cells with dolipore septa. (ii) Constriction at the point of right-angle hyphal branching. (iii) Formation of a septum near the origin of branch points. (iv) Absence of clamp connections. (v) Lack of conidia. (vi) Sclerotial tissue not differentiated into rind and medulla. (vii) Vegetative cells 4-12 μm diameter and 50-250 μm length (Palo, 1926). (viii) Aerial and surface hyphae ranging in colour from hyaline through yellow to brown (Butler and Bracker, 1970). (ix) No rhizomorphs.

1.2. *Subdivision of the species complex* Rhizoctonia solani

Isolates of *R. solani* are diverse with respect to pathogenicity, sclerotial morphology, physiological characteristics, cultural appearance etc. Many efforts have been made to subdivide the species complex of *R. solani* into logical groups, for example by pathogenicity, ecology or morphology (Ogoshi, 1987). A lack of authentic cultures has led to repeated misidentifications, resulting in the use of anastomosis groups for subdivision of the *R. solani* species complex.

1.2.1. What is an anastomosis group?

An anastomosis group (AG) can be defined as a group of isolates capable of recognizing each other and subsequently undergoing hyphal anastomosis (Vilgalys and Cubeta, 1994). Experimentally this can be observed by placing isolates from the same anastomosis group 2 to 3 cm apart on agar plates. Hyphae as far apart as 100 μm (Ogoshi, 1987) will show mutual attraction towards one another, with subsequent tip-to-tip hyphal fusion (Figure 1. 1a). If hyphae are from differing AGs, neither attraction nor fusion will be observed (Figure 1. 1b). Currently 12 AGs are recognized (Cubeta and Vilgalys, 1997); AG 1 to 11, plus the bridging isolate AG-B1, which fuses frequently with AG 2-2 and occasionally with AG 2-1, AG 3 and AG 6 (Kuninaga *et al.*, 1979). AG 2 is subdivided into AG 2-1 and AG 2-2 based on the frequency of anastomosis between isolates (Ogoshi, 1987), whereas AG 1, 3, 4, 6, 8 and 9 have subgroups which differ in one or more biochemical, genetic or pathogenic characteristics (Laroche *et al.*, 1992; Lui *et al.*, 1993; Lui and Sinclair, 1993; MacNish *et al.*, 1993; Schneider *et al.*, 1997). It is expected that with further research, the remaining AGs (AG 5, 7, 10, 11 and B1) will have subgroups identified.

Hyphal anastomosis reactions are categorized according to vegetative compatibility groups (VCGs). A vegetatively incompatible reaction results in vacuolation and death of several cells on each side of the fusion point, known as a "killing reaction" (Flentje *et al.*, 1967). In contrast a vegetatively compatible reaction involves no cell death at the fusion point. Here cytoplasmic continuity exists which may potentially allow transfer of cytoplasmic elements such as double-stranded RNA (dsRNA) between isolates. Various terminologies have been proposed to characterize these reactions (Matsumoto *et al.*, 1932; Flentje and Stretton, 1964; Parmeter *et al.*,

1969), with the current classification being that of Carling *et al.* (1988). Four classes of reaction are recognized: C0 (different AGs), C1 (hyphal contact occurs with no evidence of wall or membrane dissolution; this reaction may be accompanied by cell death), C2 (same AG, but different VCG i.e. "killing reaction") and C3 (perfect fusion, same VCG).

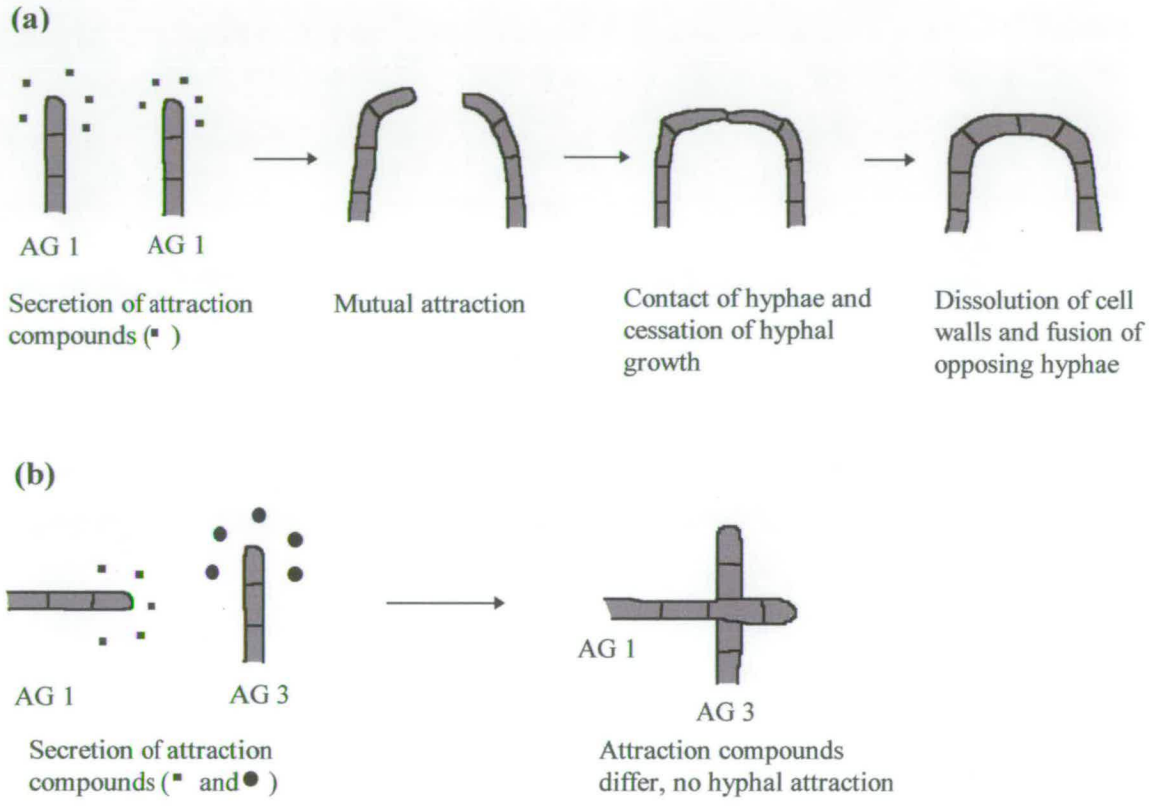


Figure 1. 1 Anastomosis reactions. (a) Isolates of the same anastomosis group (AG) release the same attractant compounds, leading to mutual attraction and fusion of hyphae (b) Isolates of differing AG perhaps release different attraction compounds, resulting in no attraction nor fusion of hyphae.

1.2.2. Genetics of vegetative compatibility reactions

Karyotyping of the genome of *R. solani* indicates that 11 to 16 chromosomes are present, with an estimated genome size of 39 to 46 Mb (Keijer *et al.*, 1996), although the underlying genetic mechanism for the vegetative compatibility reactions (C2 and C3) in *R. solani* is undetermined. Vegetative compatibility is generally governed by several genetic loci (*vic* loci: vegetative incompatibility loci); for example in *Cryphonectria*

parasitica, 7-8 *vic* genes are involved which are scattered throughout the genome (Anagnostakis, 1995). This makes C3 compatible reactions less likely than C2 "killing reactions", because if, for example, 10 *vic* loci with two alleles per locus are segregating in a population, then over 1 000 different VCGs will be found in the offspring. In *Podospora anserina* Niessl, nine genetic loci are involved in vegetative compatibility (Esser, 1974; Labarere *et al.*, 1974). Six of the loci have two alleles each and three loci have five, four and three alleles each, such that the expected offspring produced will be of 7680 genotypes. For a C3 reaction to occur, it is believed that two isolates would need to be identical (or near identical) at all the *vic* loci responsible for mediating compatibility reactions. The greater the differences within *vic* loci, the greater is the possibility of an incompatible response, observed as a "killing reaction". It is interesting to note that in *C. parasitica* isolates that differ at a single *vic* locus, the killing reaction is slow enough that dsRNA associated with hypovirulence can be transmitted from one strain to another (Anagnostakis, 1987). The mechanism resulting in the killing reaction is undetermined. In *P. anserina* it is brought about by secreted proteases (Delettre and Bernet, 1976; Labarere and Bernet, 1977) and in *Neurospora crassa* it is mediated by pre-formed proteins (Wilson *et al.*, 1961; Williams and Wilson, 1966).

1.2.3. Are AGs "separate" biological species?

A species can be defined as a group of morphologically similar organisms capable of undergoing genetic exchange with one another. Classification schemes over the years have resulted in 33 synonyms of *R. solani*, e.g. *R. alli*, *R. dimorpha*, *R. napi* (cited in Carling and Sumner, 1992). The isolates currently recognized as *R. solani* are classified as a single species divided into AGs, rather than several different species, due to their sexual states being indistinguishable (Ogoshi, 1987). However, since isolates from different AGs are not able to undergo hyphal fusion with one another, it has been proposed that AGs should be recognized as separate biological species due to the lack of genetic exchange between them (Anderson, 1982; Ogoshi, 1987; Vilgalys and Cubeta, 1994; Adams, 1996). This view has been supported by DNA-DNA hybridization studies (Vilgalys, 1988). Between isolates of different AGs, DNA hybridization values were usually low (< 25 %), whereas intragroup DNA hybridization was typically higher (> 70 %), again indicating that different AGs are genetically

isolated. However, if we consider the bridging isolates, some AG 8 and AG B1 isolates are able to "bridge" with isolates of five other AGs, whereas some isolates of AGs 2, 3, 6 and 11 are able to "bridge" with certain isolates from two other AGs (Figure 1. 2) (Carling, 1996). Thus, the only AGs which are genetically isolated from any other are AG 1, 4, 5, 7, 9 and 10. Potentially these could be recognized as individual species, with the amalgam of AGs connected by bridging isolates forming a separate species. However, it is not inconceivable that, given time, bridging isolates will be found which are capable of fusing between AG 1, 4, 5, 7, 9 and 10. Further opposition to defining AGs as separate biological species involves non-self-anastomosing isolates (NSAI). These isolates will anastomose with other isolates from their respective AGs (Hyakumachi and Ui, 1987), but not with themselves. It is then unreasonable to define their progeny as separate species due to lack of fusion, hence genetic exchange, with their parental colony. Tentatively, AGs should not yet be classified as separate biological species.

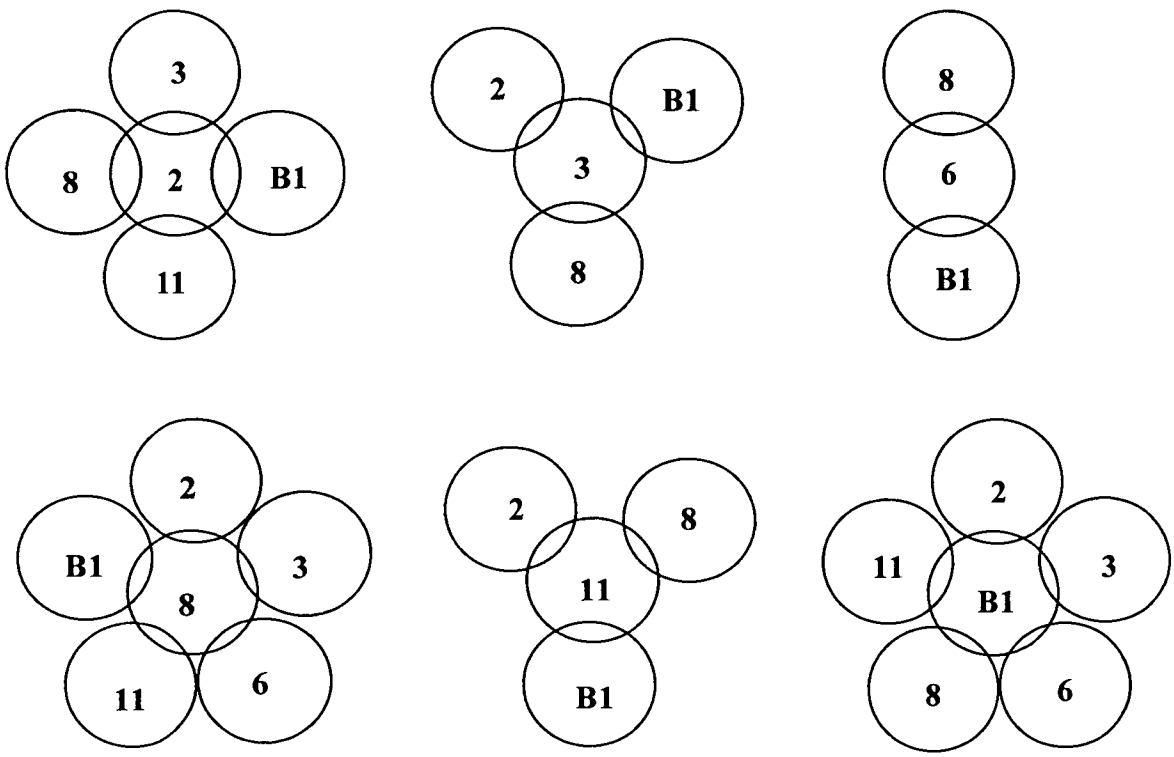


Figure 1. 2 Bridging relationships between anastomosis groups (AG) which contain certain isolates capable of fusing with isolates from more than one AG (Carling, 1996).

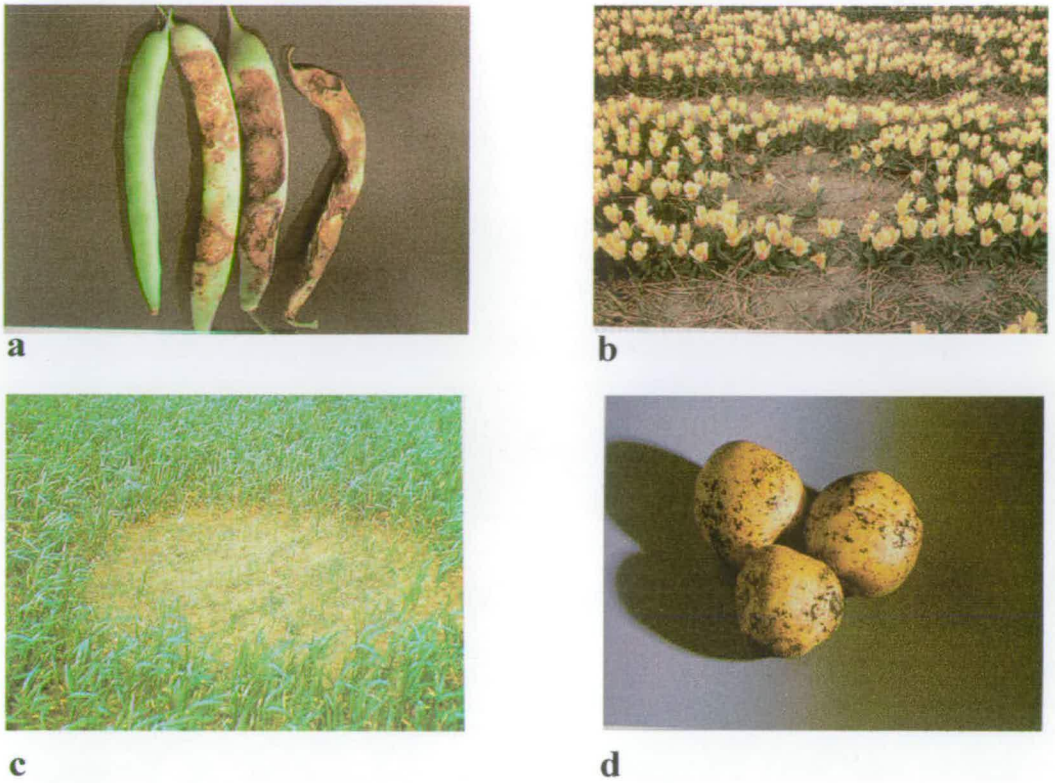


Figure 1. 3 Disease symptoms caused by *R. solani* over a range of crops. (a) Lesions on bean (*Phaseolus vulgaris*) pods by an AG 1-1B isolate (b) Bare patch in tulip (*Tulipa gesneriana*) beds by an AG 2t isolate (c) Bare patch in wheat (*Triticum aestivum* L.) incited by an AG 8 isolate (d) Black scurf of potato (*Solanum tuberosum*) incited by an AG 3 isolate (Verma *et al.*, 1996).

1.3. Phytopathogenicity of R. solani

The species complex *R. solani* is distributed worldwide causing more diseases over a wide range of crops than any other plant pathogenic species (Baker, 1970). Disease symptoms vary (Figure 1. 3) from seed decays through damping-off of seedlings, root rot, stem cankers and crown rot to aerial blight (Ogoshi, 1985). For example, in oilseed rape the disease manifests itself as a "brown girdling root rot" which circumscribes the tap root and lateral roots, subsequently resulting in poor pod development. In Alberta (1983-1984) this has reduced annual crop yields by 30 % (Verma, 1996). In rice, sheath blight occurs as lesions on the sheaths of the lower leaves. *R. solani* currently infects 32 to 50 % of the total world rice fields (Hashiba and Kobayashi, 1996). Damping-off has been observed on a range of crops including conifer and broad-leaved trees e.g. *Pinus*, *Ulmus* and *Eucalyptus* sp. (Hietala and Sen, 1996).

The host range of individual isolates is varied, with certain AGs preferentially infecting different hosts. For example, AG 1 isolates characteristically infect Leguminosae and Gramineae; AG 2-1 infects Cruciferae, whereas AG 2-2 infects Chenopodiaceae and Gramineae; AG 3 infects Solanaceae, including potatoes (*Solanum tuberosum*), causing the economically important black scurf disease of potatoes; AG 8 infects Gramineae causing stunting diseases of cereals (Ogoshi, 1987).

1.3.1. Mechanism of infection

Infection can be initiated from basidiospores or sclerotia, both of which germinate into a mycelial phase, which subsequently causes infection. The overall infection mechanism typically involves: growth towards the plant, attachment to the plant, directed growth and formation of T-shaped branches leading to production of an infection structure, followed by penetration and ultimately tissue colonization. Between AGs there are slight variations in certain stages of the infection process, which are not observed between isolates from the same AG.

Hyphae are initially attracted to the host plant by a combination of plant exudates such as amino acids, carbohydrates and phenolics. Once contact is achieved, attachment rapidly ensues. This can be visualized after 10 to 12 hours, when the round hyphae become flattened to the host surface (Armentrout and Downer, 1987). In some AGs (e.g. AG 1), attachment is aided by the production of a mucilaginous material (Matsuura, 1986). Following attachment, directed growth occurs along the anticlinal walls of epidermal cells (Dodman and Flentje, 1970; Armentrout and Downer, 1987). Side branches are then formed at right angles, and they branch again at right-angles to form "feet" structures (Armentrout and Downer, 1987). These form the basis of infection structures, which are of two types (Weinhold and Sinclair, 1996): (i) lobate appressoria characterized by further lateral branching, hyphal swelling and coiling and additional "feet" formation; (ii) dome-shaped infection cushions similar to lobate appressoria, except they are several cell layers thick. The infection structures tend to vary between AGs as opposed to within AGs. For example AGs 2 and 4 form dome-shaped infection structures, whereas AG 1 isolates tend to form lobate appressoria (Matsuura, 1986).

The next stage involves penetration of the plant tissues. Again this varies between AGs and can be mechanical, enzymatic (See Section 1.3.2) or a combination of both. In general a mechanical response involves formation of thin infection pegs from tightly adhered, swollen hyphal tips. In AG 1 and AG 4 these pegs are 1-2 μm in diameter (Fukutomi and Takada, 1979; Matsuura, 1986). The infection pegs exert hydrostatic pressure, which in other fungi has been measured at > 8 mega Pascals (Howard *et al.*, 1991). Penetration of the plant tissues is achieved as the strong adherence response generates counter-pressure. In AG 3, penetration is accompanied by enzymatic processes, which are observed as lighter coloured "halos" on scanning electron micrographs (Hofman and Jongebloed, 1988). Following penetration, hyphae return to "normal" size, branch within the cell lumen and rapidly colonize neighbouring cells. The extent of this is variable depending on the plant host and fungal isolate. For example in potatoes infected with AG 3, penetration initially involves two cell layers, which gradually increases to ultimately reach the vascular bundle, girdling the sprout and causing death (Hofman and Jongebloed, 1988). The above describes the normal mode of penetration through healthy tissue. However, penetration can also occur through wounds, lenticels and stomata (Akino and Ogoshi, 1995; Keijer, 1996).

1.3.2. Enzymes involved in pathogenicity

Tissue invasion by *R. solani* is partly mechanical and partly enzymatic. The enzymes are released prior to mechanical penetration and include polygalacturonase (Bateman, 1963b), trans-eliminases (pectin lyase and pectate lyase) (Ayers *et al.*, 1966), cutinase (Baker and Bateman, 1978; Trail and Koller, 1990), cellulases (Bateman, 1964a) and hemicellulases such as arabinases, galactanases, galactomannases and xylanases (Bateman *et al.*, 1969). The first to be released is polygalacturonase, for which a high correlation exists between the ability of isolates to produce this enzyme and their degree of pathogenicity (Barker and Walker, 1962; Bateman, 1963a). Polygalacturonase is released after the infection cushions have formed (t = 18 h), but prior to cuticle penetration (Brookhouser and Weinhold, 1979). Once released, polygalacturonase is responsible for "tissue maceration" (Bateman, 1963b; Bateman, 1964b), by degrading the pectin of the middle lamellae of the plant cell walls. Although

"tissue maceration" is eliminated in the absence of polygalacturonase (Bateman, 1963a), it is thought that trans-eliminases aid the process considerably (Ayers *et al.*, 1966). These degrade pectates and pectinates by breaking the glycosidic linkage at carbon 4 and simultaneously eliminating the hydrogen from carbon 5, resulting in oligouronides that contain an unsaturated galacturonyl unit. It is noteworthy that Marcus *et al.* (1986) have reported that pectin lyase was present only in virulent but not hypovirulent isolates. Cellulase release follows that of polygalacturonase. This delay is observed in several other pathogenic species, for example, *Colletotrichum* sp. (English *et al.*, 1971), *Pyrenochaeta* sp. (Horton and Keen, 1966) and *Fusarium* sp. (Jones *et al.*, 1972). The delay in cellulase release is thought to result from cellulases adhering more strongly to the cell walls than polygalacturonases, as higher concentrations of buffers and more intensive washings are required to release cellulases *in vitro* (Lisker *et al.*, 1975a; Lisker *et al.*, 1975b). The release of polygalacturonases and cellulases is triggered by pectic substances in host exudates (Bateman, 1963; Brookhouser and Weinhold, 1979), although Gupta (1962) describes an isolate which produced polygalacturonase in the absence of pectic substances.

Cellulose is the major structural component of plant cell walls, forming the microfibrillar structure of the primary and secondary wall. This is evidenced by microscopic examination of *R. solani*-infected hypocotyls, using polarized light, which showed that cell walls of infected hypocotyls lost their birefringent properties, indicating degradation of crystalline cellulose. Uninfected hypocotyls, however, were still birefringent due to the presence of intact cellulose (Bateman, 1964a). Subsequent studies indicate that cellulases are involved in intracellular penetration and the ultimate collapse of the cells following tissue maceration by polygalacturonase (Bateman, 1970).

The role of enzymes in pathogenesis is limited due to the plant's defence mechanisms. Polygalacturonase action is prevented in two ways. Firstly, at the zone of infection, plant respiration increases which leads to an accumulation of calcium and other cations. These in turn release and activate host pectin methylesterases which demethylate the host pectin. The demethylated pectic substances subsequently form insoluble salts with calcium, which are then resistant to degradation by fungal polygalacturonases, thus forming a barrier to infection (Bateman, 1964b). A secondary mode of infection limitation is inhibition of polygalacturonase by oxidized phenolics secreted by the host (Cole, 1958). For example, catechin content in host plants

increases during infection, and it is subsequently oxidized. The oxidized catechin then inhibits polygalacturonase activity (Akino and Ogoshi, 1995). Older hypocotyls show further resistance to polygalacturonase action. Stockwell and Hanchey (1983) propose this to be due to a thicker cuticle, which reduces the amount of host exudates released, such that they no longer form a trigger for infection cushion formation and subsequent release of polygalacturonase.

1.4. Control of Rhizoctonia-incited diseases

Marketable yield losses from soil-borne plant pathogens result in an annual loss of $ 4 to 5 billion in the USA alone (Lewis *et al.*, 1998). More specifically, in crops such as potatoes marketable yield losses of 30 % frequently occur due to *Rhizoctonia* infection (Carling *et al.*, 1989; Read *et al.*, 1989), which is of great agroeconomical significance, considering that potatoes form the staple diet of many western civilizations. Control of *Rhizoctonia*-incited diseases is achieved in a variety of ways using cultural, chemical, biological and integrated techniques. The oldest, most commonly used cultural technique is that of crop rotation. Ideally rotations of 3 years, or longer, are recommended for crops such as potatoes (Banville *et al.*, 1996), with intervals of 5 years effectively eliminating damage from soil-borne sources of *R. solani* (Jager *et al.*, 1991). A further cultural approach which is useful for small areas of high value crops, such as greenhouse and nursery crops, is soil sterilization via heat treatment. However, problems with re-infestation subsequently occur (Herr, 1995). An alternative method of achieving control is to use disease-free seed. This is usually established using chemical treatments, the exact nature of which is dependent on the country and the crop. Some fungicides commonly employed against *R. solani* include quintozene, tolclofos-methyl, carboxin, formaldehyde, benodamil, benomyl, triazoles (e.g. propiconazole, hexaconazole), triadimefon, fenpropimorph, pencycuron, validimycin A and PCNB (pentachloronitrobenzene) (Kataria and Gisi, 1996). Of these, quintozene was most frequently utilized between 1935 and 1965 (Kataria and Gisi, 1996), although tolclofos-methyl (Rizolex; Schering-Aagrunol) and pencycuron (Monceren, Bayer AG) currently monopolize the market. Tolclofos-methyl acts by reducing the capacity of *R. solani* to penetrate sprouts, stems and stolons (Jager *et al.*, 1991) and by inhibiting sclerotia

germination (Yamada, 1986). This fungicide is popular as it is active against virtually all types of *Rhizoctonia* disease over a large number of crop species growing in diverse environments (Kataria and Gisi, 1996). This is in stark contrast to fungicides such as triadimefon which is highly inhibitory to *R. solani* at 22°C, but not at 27°C (Kataria and Gisi, 1989), and pencycuron which is active against *R. solani* AGs 1, 2-1, 2-2, 3, 6 and 9, but inactive against AGs 5, 7 and 8 (Sumner, 1987). The main drawback with tolclofos-methyl is the appearance of fungicide-insensitive *R. solani* isolates (van Bruggen and Arneson, 1984). Validimycin A is highly effective in control of rice sheath blight, but to date is used solely in Japan (Kataria and Gisi, 1996). In Australia, formaldehyde is widely used to control *Rhizoctonia* diseases of potato. This has been shown to be highly effective in killing sclerotia. However, 100 % death required treatment for 20 minutes in a 2 % formaldehyde solution (Wicks *et al.*, 1995). The toxicity of formaldehyde subsequently leads to problems with handling and disposal.

Although adequate control is currently established using chemical treatments, many problems arise from their usage. From an environmental viewpoint fungicides, either directly or indirectly, pose a certain risk to human health, livestock, local ecosystems and water quality. For example, tolclofos-methyl, as with other organophosphorus compounds, has toxicity towards mammals. Ingestion of tolclofos-methyl results in inhibition of acetyl-cholinesterase which leads to accumulation of acetylcholine at nerve end-plates (Tomimaru *et al.*, 1996). This condition can be treated with a combination of atropine and the acetylcholinesterase reactivator, pralidoxime (Taylor, 1990). In addition, many fungicides prevent the use of organic manures (Bandyopadhyay *et al.*, 1982; Kataria and Sunder, 1985). Manures contain large amounts of humic and fulvic acids which sequester substantial amounts of fungicides, rendering them inaccessible for disease control. A further problem with many fungicides is that extensive, prolonged usage may result in resistance. This has already been observed in experimental conditions with tolclofos-methyl (van Bruggen and Arneson, 1984). Therefore, if chemical control is to remain a viable option, two or preferably three fungicides should be available which elicit control in different ways to delay the onset of resistance. However, a more environmentally sound approach would be to utilize a successful biological control system.

1.4.1. Potential for biological control of *Rhizoctonia*-incited diseases

Although biological control is more environmentally friendly than chemical control, surprisingly few products are available commercially due to high developmental costs, in relation to market value. In the USA microbial biological control agents must be registered as "microbial pesticides", and undergo extensive toxicological tests to determine potential health hazards. Registration routinely costs $US 100 to 200 thousand (Cook, 1993), which is too great to justify development of many disease or environment specific agents. In certain circumstances registration can be avoided by marketing the product as a "plant growth improver" rather than a "microbial pesticide". In 1993 there were seven microbial biocontrol agents registered in the US for use against phytopathogens. These included (i) *Agrobacterium radiobacter* K-84 for use against crown gall (Kerr, 1980), (ii) *Pseudomonas fluorescens* marketed as Dagger G for use against *Rhizoctonia* and *Pythium* damping-off of cotton (Howell and Stipanovic, 1979), (iii) *Gliocladium virens* marketed as GlioGard for use against seedling diseases of ornamental and bedding plants (Cook, 1993), (iv) *Trichoderma harzianum* marketed as F-Stop and used as a seed treatment to improve stands of corn, beans and other vegetable crops (Harman *et al.*, 1991), (v) *Trichoderma harzianum/ polysporum* sold under the tradename of BINAB T effective against wood decay (Ricard, 1981), (vi) *Bacillus subtilis* marketed as Kodiak for use as a seed treatment, being active against *Rhizoctonia* on beans, cotton, peanuts and soybeans (Cook, 1993), (vii) *Burkholderia cepacia* marketed as Blue Circle and used as a seed treatment which is active against *Rhizoctonia* on corn, melon, cotton and beans (Lewis and Kulik, 1996). Of these, *Ps. fluorescens*, *B. subtilis*, *G. virens* and *B. cepacia* show a certain degree of activity against *Rhizoctonia* isolates. *G. virens* is a suppressant of *R. solani*-incited damping-off diseases (Lumsden and Locke, 1989), although the suppression mechanism is undetermined. Howell *et al.* (1993) showed that viridin and gliovirin produced by *G. virens* had no effect, so subsequent studies by Howell and Stipanovic (1995) focused on gliotoxin produced by some strains of *G. virens*. The production of UV-induced gliotoxin mutants indicated that gliotoxin activity is not the primary mechanism of suppression. Later studies by Wilhite and Straney (1996) indicate that gliotoxin is produced only for a short 16 hour period during replicative growth, thus limiting its

biocontrol capacity. *Ps. fluorescens* is thought to exert control partly through the antibiotics pyrrolnitrin and pyoluteorin (Homma *et al.*, 1989). *B. subtilis* controls *R. solani* on cotton in sub-tropical climates (McKnight, 1993), where the temperature favours bacterial growth and metabolism; however, Fiddaman and Rossall (1995) demonstrated that within temperate regions, where the soil temperature is lower, suppression was not achieved. *B. cepacia* is used in horticultural situations where it is employed as a suspension in polyfoam rooting cubes in the planting of *Poinsettia* (*Euphorbia pulcherrima* Willd.) (Cartwright and Benson, 1995). Unfortunately these developed biocontrol agents are limited to specific crops in addition to specific environments. Various other strategies are currently under investigation for their potential to suppress *Rhizoctonia*-incited diseases. For example, *Laetisaria arvalis* can prevent *Rhizoctonia*-incited damping-off of sugar beet (Odvody *et al.*, 1980), peppers (Conway, 1986), seedling disease of cotton (Lartey *et al.*, 1991) and fruit rot of cucumbers (Lewis and Papavizas, 1980). Two further highly promising avenues exist to developing further biocontrol strategies effective against *Rhizoctonia*. The first involves using the mycoparasite *Verticillium biguttatum* Gams. (Section 1.4.1.1) and the second by harnessing double-stranded RNA (dsRNA) genetic elements (Section 1.4.1.2).

1.4.1.1. *Verticillium biguttatum* as a biocontrol agent

The biotrophic mycoparasite (van den Boogert and Deacon, 1994) of *R. solani*, *Verticillium biguttatum*, is a common soil organism with worldwide distribution, being able to survive in different soil types ranging from purely mineral to peat soils (van den Boogert and Saat, 1991). Infection of *R. solani* involves trophic hyphae of *Verticillium* penetrating host hyphae (Figure 1. 4). The internal hypha becomes orientated along the axis of the host hyphae (van den Boogert and Deacon, 1994), where it interacts biotrophically with *R. solani* for a short period, following which it rapidly degrades the host cytoplasm. The septa of the invaded cell becomes plugged (van den Boogert *et al.*, 1989), such that further cells tend to be invaded from external clasping side branches of *Verticillium* formed at the penetration point. The main macroscopic effect of this interaction is a large reduction in sclerotial production by *R. solani* (van den Boogert *et al.*, 1994). The economically important black scurf disease of potatoes results from *R.*

solani sclerotia forming scabs on the tuber surface, so the reduction in sclerotial production by *V. biguttatum*, plus the ability of *V. biguttatum* to kill sclerotia (Jager and Velvis, 1984), indicates that *V. biguttatum* may be a potential biocontrol agent.

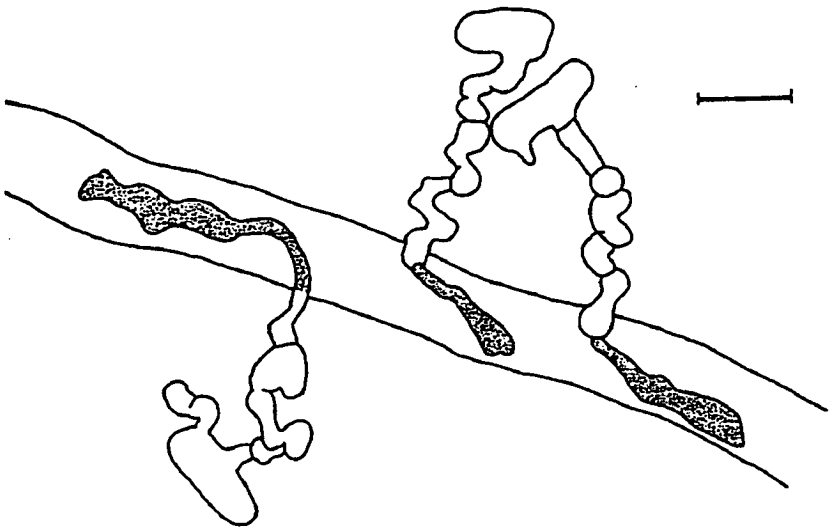


Figure 1. 4 Infection of *Rhizoctonia solani* hyphae from three germinating spores of *Verticillium biguttatum*. Bar = 10 μm (van den Boogert and Deacon, 1994).

This concept seems promising but, as with all proposed biocontrol mechanisms, problems arise. The main difficulty as indicated by Velvis and Jager (1983) is that the suppressive effect of *V. biguttatum* is dramatically decreased at temperatures below 15°C and absent at 10°C (Table 1. 1). The lack of growth at low temperatures is problematic, as *Rhizoctonia* isolates can grow at temperatures as low as 4°C, so *Rhizoctonia* can initiate growth in the spring, when the temperature is too low to permit the suppressive activity and growth of *V. biguttatum*.

van den Boogert *et al.* (1994) have tried to overcome this setback using green crop harvesting. Daughter tubers develop *Rhizoctonia* scurfy lesions relatively late in the season. This formation can only occur once the tuber begins to form a melanized skin. Green crop harvesting involves lifting the potato crop while the tubers are still white, coating them with *V. biguttatum* and subsequently reburying them under fresh ridges, to enable development of the skins, which is independent of attachment to the

plant. This technique prevents black scurf developing, as the temperature of the soil is greater than 15°C enabling *Verticillium* to display its mycoparasitic effect.

Temperature	Inoculum	Sclerotia (%) showing the indicated numbers of emerging hyphae				
		0	1-5	6-10	11-25	>25
10°C	None	11	6	8	29	46
	V.b.	14	6	13	24	43
15°C	None	15	5	13	18	49
	V.b.	94	4	0	0	0
20°C	None	19	20	6	12	43
	V.b.	95	4	0	1	0

Table 1. 1 Viability of sclerotia after treatment with *Verticillium biguttatum* M73 (V.b.) following 7 weeks incubation at differing temperatures (Velvis and Jager, 1983).

Green-crop harvesting appears to establish control so this could be used in conjunction with dsRNA-mediated control (Section 1.4.1.2). Morris *et al.* (1995) proposed that antifungal hydroxymethyl phenols produced by *V. biguttatum* may be responsible for sclerotial suppression. However, studies by van den Boogert *et al.*, (1994) showed that suppression could occur when hyphae of *R. solani* grew across a trench in agar plates and the mycoparasite *V. biguttatum* was inoculated on only one side of the trench. This indicates that the suppression is not due to volatile or water-diffusible inhibitors. Indeed suppression is thought to be mediated via *R. solani* hyphae, as *V. biguttatum* could cause suppression in two adjoining vegetatively compatible isolates, but not in the adjoining colony if it was vegetatively incompatible. This led van den Boogert and Deacon (1994) to propose that infection by *V. biguttatum* generates a continuing nutrient sink within the mycelium of *R. solani*, such that nutrients are diverted from sclerotial production to *V. biguttatum*. Thus, if in field situations we have isolates of *R. solani* infected with dsRNA, it may be possible to use the nutrient mobilization effect of *V. biguttatum* to "pull" dsRNA through adjoining colonies of the *R. solani* mycelial network, and to establish dsRNA induced hypovirulence in a larger proportion of the field population.

1.4.1.2. A potential biocontrol system using dsRNA

The biocontrol methods described so far require repeated applications, or complex cropping procedures. Ideally a biocontrol strategy should require one-time inoculation. For example *Phyllachora huberi*, which causes black crust on rubber foliage, is maintained in a suppressive state by the mycoparasites *Cylindosporum concentricum* and *Dicyma pulvinata* (Sutton and Peng, 1993). This was achieved by a single inoculation. A further example is that of control of *Sclerotinia minor* by the mycoparasite *Sporidesmium sclerotivorum* in lettuce monoculture in New Jersey (Adams, 1990). Again this was self-sustaining following inoculative release. A further intriguing example of self-sustaining biocontrol involves control of *Cryphonectria parasitica*, the causative agent of chestnut blight in Europe (Section 1.6). This strategy involved harnessing dsRNA naturally resident in certain *C. parasitica* isolates. The presence of dsRNA alters the phenotype of this fungal pathogen to a weakly virulent (hypovirulent) state. This strategy has the advantage that hypovirulent isolates are able to occupy the same ecological niche as virulent strains, thus it should not be an environment specific control system. The hypovirulent isolates were artificially released throughout infected chestnut coppices in Southern Europe, whereby dsRNA spread throughout natural field populations via hyphal anastomosis such that biological control of chestnut blight was achieved. This will be discussed in more detail in Section 1.6 as it forms the basis for a potential biological control that might be developed against *R. solani*.

1.5. Double-stranded RNA (dsRNA) in fungi

dsRNAs, of potential mycovirus origin, have a widespread incidence in fungi, being reported in all the major taxonomic groups (Buck, 1986). They occur either as encapsidated viral genomes or more rarely as naked unencapsidated molecules (Zhang *et al.*, 1994). They are presumed to be of viral nature (mycoviruses), since they have RNA-dependent RNA polymerase (RDRP) activity associated with the encapsidated virions, or their sequence contains an RDRP-related open reading frame (ORF) (Rubio *et al.*, 1996). Ghabrial (1998) proposed that mycoviruses should be classified within four groups: (i) *Totiviridae*, characterized by a single dsRNA (4.6-7 kbp) encapsidated in isometric particles of approx. 30 to 40 nm diameter, an example of which is the

Saccharomyces cerevisiae virus (ScV-L). (ii) *Partitiviridae*, characterized by several unrelated encapsidated dsRNA segments (1-3 kbp), the smaller of which are considered satellite dsRNAs. An example is the virus of *Gaeumannomyces graminis* (Sacc.) von Arx & Olivier var. *tritici* Walker. (iii) *Hypoviridae* which contain several dsRNA segments which have no virus capsid, but are enclosed in polymorphic vesicles 50 to 80 nm in size. They are similar in genetic organization and expression strategy to plant viruses, the main example being hypovirus CHV1-713 of *C. parasitica*. (iv) Unclassified viruses such as the La France isometric virus (LIV) associated with the La France disease of mushrooms.

dsRNA elements are generally non-symptomatic, as occurs in many rust fungi (Zhang *et al.*, 1994), but some have been reported to alter the fungal phenotype. For example, a study of 22 isolates of *Phytophthora infestans* de Bary from Mexico concluded that dsRNA-containing strains exhibited significantly higher levels of virulence than dsRNA-free isolates (Nuss and Koltin, 1990). However, from a biocontrol perspective, dsRNA-containing strains showing reduced virulence (hypovirulence) are of greater interest. Examples of this are found in *Ophiostoma ulmi* (Brasier, 1983), *Gaeumannomyces graminis* var. *tritici* (Nuss and Koltin, 1990), *Cochliobolus victoriae* (Ghabrial, 1994), *Chalara elegans* Nag Raj and Kendrick (Bottacin *et al.*, 1994), *Leucostoma persoonii* (Hammar *et al.*, 1989), *C. parasitica* (Nuss, 1992) and *Sclerotinia sclerotiorum* (Boland, 1992). Some of these will be discussed in further detail below.

1.5.1. dsRNA and hypovirulence in phytopathogenic fungi

1.5.1.1. Gaeumannomyces graminis

Gaeumannomyces graminis var. *tritici* is the causative agent of take-all diseases in wheat (*Triticum aestivum*) and barley (*Hordeum vulgare*). Symptoms range from chlorosis of leaves in late spring to severe stunting of plants and heavy reduction of grain yield. Successive crops show an increase in disease to a maximum (often in the third crop), followed by a reduction in disease level in subsequent crops, which has been termed "take-all decline" (Slope and Cox, 1964). Initial reports by Lapierre *et al.* (1970) found that weakly virulent (hypovirulent) strains isolated during the third crop were associated with virus-like particles (VLP). This was confirmed by subsequent

studies by Lemaire *et al.* (1970) and Spire (1970) which indicated two types of *G. graminis* var. *tritici* isolates: (i) isolates containing VLP of 29 ± 2 nm which were hypovirulent and (ii) isolates without VLP which were strongly virulent. Rawlinson *et al.* (1973) contradicted this by reporting isolates containing virus particles of sizes 27, 35 and 40 nm in diameter, which displayed no consistent correlation between presence of virus and virulence. The degree of virulence remained unchanged following curing of isolates of VLP with elevated temperatures (Rawlinson *et al.*, 1973). Later studies on 20 different isolates revealed that isolates contained multiple (1 to 12) dsRNA segments with molecular weights ranging from 1 to 3 x 10^6 daltons, with no two isolates containing identical dsRNA patterns (Almond *et al.*, 1977). Thus, if hypovirulence is indeed mediated by dsRNA, it is probably the effect of specific segments, or an interaction between several segments.

The potential for using hypovirulent *G. graminis* var. *tritici* isolates for biological control looks optimistic. In field conditions hypovirulent isolates occur naturally at low frequency (Naiki and Cook, 1983), and this increases gradually during the crop growing season concomitant with an increase in abundance of dsRNA (Rawlinson *et al.*, 1973). To determine how effective these may be for biocontrol, virus-free virulent strains were mixed with hypovirulent, virus-infected isolates and applied to both greenhouse and field plots of French wheat (cited by Day and Dodds, 1979). Disease severity was greatly reduced compared with that in control plots exposed to virus-free strains. Further studies by Duffy and Weller (1996), in Washington, improved yield of spring wheat by 20.8 % using hypovirulent *G. graminis* var. *tritici* strain L108hv. For hypovirulent isolates to be effective biological control agents dsRNA must be transmissible. Day and Dodds (1979) propose that dsRNA transfer occurs between isolates by vegetatively compatible anastomosis reactions. However, spread throughout field populations is likely to be limited as *G. graminis* var. *tritici* does not spread far through soil (Wehrle and Ogilvie, 1956) and pathogenically dissimilar isolates rarely anastomose (Chambers and Flentje, 1967). Thus, application of several different *G. graminis* var. *tritici* isolates over large areas would be required for biological control to succeed. In addition, the interaction of dsRNA with the host fungus requires to be elucidated. How does dsRNA suppress fungal virulence? Is it mediated by a single dsRNA segment, or a complex interplay between products of

multiple dsRNA segments? A secondary level of control is achieved through competition, as hypovirulent isolates will compete for the same ecological niche as virulent isolates. In addition, hypovirulent isolates may trigger the plant defence mechanism resulting in greater lignification and suberization of the endodermis and xylem vessels, thus decreasing the degree of invasion of the vascular system and the pathogenic effect of fully virulent isolates (Duffy and Weller, 1995).

1.5.1.2. *Helminthosporium victoriae*

Helminthosporium victoriae (teleomorph *Cochliobolus victoriae*) is the causal agent of Victoria blight of oats (*Puccinia coronata*). A "diseased" form of the pathogen is observed which is characterized primarily by lysis of aerial mycelium and reduced levels of the toxin "victorin" (Lindberg, 1960). In addition, reduced growth rate, hypovirulence and excessive sectoring are observed (Ghabrial, 1986). Studies by Sanderlin and Ghabrial (1978) reported the diseased forms to contain virus particles designated 190S and 145S, both of which are isometric particles of 40 nm diameter (Ghabrial, 1994). The 190S particle contains a single dsRNA segment of 5178 bp (Huang and Ghabrial, 1996), whereas the 145S particle contains four dsRNA segments of 2.6, 2.8, 3.0 and 3.4 kbp (Ghabrial, 1994). It is thought that the 145S particle is responsible for the "diseased" state of *H. victoriae*. Two lines of evidence support this: (i) diseased isolates always contained 145S, whereas normal, healthy isolates were either virus-free or contained only the 190S particle and (ii) healthy colonies prepared from colonies using protoplast techniques, always lacked the 145S particle (Sanderlin and Ghabrial, 1978). The virus-particles were shown to be cytoplasmically-transmissible concomitantly with the diseased state (Ghabrial, 1986). Isolates which acquire both the 190S and 145S particles secrete a broad-spectrum antifungal polypeptide (6-8 KDa), designated "victoriocin" (Ghabrial, 1994), being responsible for the death of aerial mycelium in *H. victoriae*. This is thought to be dsRNA-encoded, but the exact mechanism is yet to be determined. The 190S particle, which is a member of the family Totiviridae (Ghabrial *et al.*, 1995), has been sequenced and found to encode two open reading frames (ORFs); ORF1 encodes coat proteins and ORF2 encodes an RNA-dependent RNA polymerase (RDRP) (Huang and Ghabrial, 1996). Other Totiviruses

express these two gene products via a translational frame shift, but the 190S particle differs, as it lacks the heptamer slippery site and pseudoknot structures required (Huang and Ghabrial, 1996). It is thought the RDRP is expressed using a fungal host factor (Huang *et al.*, 1997). No evidence of coding capacity for the "diseased" state was found on 190S, thus re-affirming that it may be encoded on the 145S dsRNA segments. Work is currently underway to sequence these elements (Ghabrial, 1994). Isolates containing 145S particles in the absence of 190S particles have not been reported. It may be that these are "satellite" dsRNA segments dependent on 190S particles for function (Ghabrial, 1994). If "vitoriocin" is dsRNA encoded, it may be analogous to killer systems reported in yeasts (Wickner, 1986), with diseased *H. victoriae* isolates being analogous to "suicide" strains of yeast, which are sensitive to the toxin they produce (Buck, 1986). If this is the case, not only will it form the first example of a dsRNA-mediated "killer" system in filamentous fungi, but also a highly effective means of biological control against Victoria blight of oats may be established.

1.5.1.3. *Ophiostoma ulmi*

Ophiostoma ulmi is the causative agent of Dutch elm (*Ulmus procera*) disease. The disease is initiated by bark beetles (Coleoptera: Scolytidae) which feed in the crotches of twigs during spring and summer. This results in deposition of *O. ulmi* ascospores, which germinate and invade the xylem. The xylem rapidly becomes blocked and death of the tree ensues. During this century two pandemics have occurred: the first, during 1920 to 1940, caused by *O. ulmi* (Buisman) Nannf., and the second from 1940 to the present day, caused by *O. novo-ulmi* (Brasier, 1991). *O. novo-ulmi* is a highly aggressive pathogen and is split into two biotypes; (i) the North American (NAN) race and (ii) the Eurasian (EAN) race. These two races are partially reproductively isolated and differ in a range of morphological, physiological and molecular characteristics, as well as differing in geographical distribution (Jeng *et al.*, 1988). Within both of these races, diseased isolates are reported which display slow growth rate, abnormal "amoeboid" morphology, reduced production of the hydrophobin cerato-ulmin, reduced viability of conidia, impairment of sexual reproduction, production of a red-brown pigment and reduced infection potential (Brasier, 1983; Sutherland and

Brasier, 1997). In addition, large reductions in the levels of mitochondrial cytochrome oxidase occur (Rogers *et al.*, 1987), and the *de novo* generation of DNA plasmids from mitochondrial DNA (Charter *et al.*, 1993) is observed in diseased isolates. The factors that cause the diseased states are referred to as d-factors, of which many have been identified, and are numbered sequentially from d^1 to d^n (Brasier, 1986). The diseased state was found to be cytoplasmically transmissible via hyphal anastomosis (Rogers *et al.*, 1986). Both healthy and diseased isolates were found to contain dsRNA genetic elements, but transmission of a set of ten of these elements (3.49, 3.03, 2.69, 2.43, 2.33, 2.21, 0.95, 0.92, 0.48 and 0.33 kbp respectively) resulted in a healthy, uninfected recipient displaying the "d^2" diseased phenotype (Rogers *et al.*, 1986; Rogers *et al.*, 1988). These elements were not transmitted during sexual reproduction and progeny displayed a healthy phenotype (Rogers *et al.*, 1988). Observations of isolates where not all dsRNA segments were transmitted indicated that the diseased phenotype is invoked by segments 4 (2.43 kbp), 7 (0.95 kbp) and 10 (0.33 kbp), acting either individually or in combination (Rogers *et al.*, 1988). Of these, segments 7 and 10 have recently been sequenced (Hong *et al.*, 1998).

Since diseased isolates display poor pathogenicity, they may form good biocontrol agents. This is exemplified by *in vitro* studies which indicate that 500 to 1000 spores of healthy *O. novo-ulmi* are required to cause xylem infection in the English elm (*Ulmus procera*) (Webber, 1987; Webber, 1993; Sutherland and Brasier, 1997). In contrast d^6 and d^{13} diseased isolates required 5 000 and 50 000 spores, respectively, to elicit infection (Webber, 1987; Webber, 1993). Further studies by Sutherland and Brasier (1997) grouped d-factors into four categories according to their ability to reduce xylem infection of *U. procera*; (i) minimal effect as in d^1 isolates, (ii) mild effect as in d^4 isolates (NAN), (iii) moderate effect as in d^5 to d^8, d^{10}, d^{11} (EAN) and d^{12}, d^{13} (NAN), and (iv) severe effect as displayed by d^2 (NAN), d^3 and d^9 (EAN). Spore numbers required for infection by isolates exerting a moderate and severe effect, are greater than the number of spores carried by the majority of vector beetles (Webber, 1987). For example, in Europe only about 25 % of *Scolytus scolytus* beetles commonly carry more than 5 000 spores, with only 15 % carrying more than 10 000 spores (Webber, 1990; Webber, 1993). In the USA, these figures are greatly reduced due to the smaller size of the *S. multistriatis* beetle (Sutherland and Brasier, 1997). The success of control in this manner is increased on *U. x hollandica* cv. 'Commelin', which is a hybrid showing

moderate resistance to Dutch elm disease. For d^2 and d^9 infected *O. novo-ulmi* to elicit infection, 50 000 to 500 000 spores would be required (Sutherland and Brasier, 1997). Thus it is hoped that release of artificially bred beetles carrying diseased *O. novo-ulmi* isolates will establish control. Following release they should subsequently convert natural, healthy isolates to the diseased phenotype, thus limiting further infection.

It is believed that diseased isolates have reduced the effect of the current pandemic of Dutch elm disease (Brasier, 1988; Brasier, 1991). However, limitations to using diseased isolates as artificially introduced biocontrol agents are expected. The main barrier will be a limitation in the transmission of the diseased phenotype due to vegetative compatibility groupings (VCGs). In regional collections of *O. novo-ulmi*, most isolates belong to differing VCGs (Buck, 1988). Since spread of d-factors occurs in only 4 % of incompatible reactions (Brasier, 1984; Brasier, 1986) this is likely to severely limit dissemination. However, in other cases v-c supergroups exist where the majority of isolates are all vegetatively compatible. For example, in Portugal and Poland more than 90 % and 47 % respectively, belong to the same VCG (Buck, 1988). Where incompatibility is a problem, it may potentially be overcome by releasing isolates of several different VCG and including bridging isolates in the release, thus making diseased isolates a highly promising potential means of biocontrol.

1.5.2. Biological control using dsRNA

dsRNA genetic elements have been shown to reduce the pathogenicity of various fungal phytopathogens (Buck, 1988; Nuss and Koltin, 1990; Nuss, 1992). Many of these have the potential to be good biological control agents, as previously discussed. The main problem to be overcome in many potential systems lies in the dissemination of dsRNA which occurs via hyphal anastomosis. Transmission occurs readily when isolates contain vegetative compatibility (*vic*) genes which are the same at every locus. With increasing numbers of different *vic* genes, the frequency of dsRNA transmission decreases. For a fully incompatible reaction, transmission frequency has been reported at only 4 % in *Ophiostoma novo-ulmi* (Brasier, 1984; Brasier, 1986). Some fungal pathogens have simple population structures, such as *Helicobasidium mompa* and *Rosellinia necatrix* (Matsumoto, 1998). However, since many natural fungal populations contain diverse VCGs, vegetative compatibility is likely to pose a problem.

To a certain extent, these barriers can be overcome, for example, by applying several hypovirulent isolates from differing VCGs, in addition to "bridging" isolates. More elaborate means could involve suppressing vegetative incompatibility reactions using anti-sense messenger RNA (mRNA) (Benedetti *et al.*, 1987), which can block the expression of *vic* genes. In *C. parasitica*, these drawbacks have been circumvented, such that a highly successful dsRNA-mediated biocontrol strategy might be developed against chestnut blight, which is discussed in detail below.

1.6. Chestnut blight: a model biocontrol system

1.6.1. Chestnut blight

Chestnut blight is caused by the wound pathogen *Cryphonectria parasitica* (initially called *Diaporthe parasitica*, then renamed as *Endothia parasitica*) which infects branches and stems, then grows in and under the bark, resulting in a canker (Figure 1. 5a), which gradually spreads around the branch/ stem. This girdling results in death of everything distal to the canker. The roots, however, are not infected and the stumps retain the ability to resprout.

1.6.2. Control of chestnut blight in Europe

Chestnut blight was first reported on the European chestnut (*Castanea sativa* Mill.) in 1938 near Genoa and Avellino in Italy, and by 1950 it was widely distributed throughout the northern and southern chestnut plantations of Italy (Anagnostakis, 1992). In 1951, Biraghi discovered a chestnut coppice that looked "surprisingly healthy" despite 85 % of the shoots being infected (Biraghi, 1953). In this coppice, superficial cankers (Figure 1. 5b) formed where the fungal mycelium was restricted to the outer layers of the bark. Prior to 1964, it was assumed this was due to a resistant variety of chestnut; however, Grente (1965) isolated altered forms of *C. parasitica* from the healing cankers. These new forms displayed lower pigmentation (Figure 1. 6), sporulation and virulence. They were termed hypovirulent by Grente, because when they were inoculated onto chestnut trees, the resulting canker spread so slowly that the trees' natural defences had time to contain the infection. When hypovirulent strains

were coinoculated with heavily virulent isolates on agar, the virulent strain was converted to hypovirulent form. Thus, hypovirulence is cytoplasmically controlled, with dsRNA subsequently being shown by Day *et al.* (1977) to be the causative agent. In Italy, natural spread of hypovirulence has led to a substantial reduction in the incidence of chestnut blight, such that it is no longer a problem (Mittempergher, 1978). It is proposed that the hypovirulent isolates were disseminated naturally by wind, birds, insects, timber movement and tree climbing slugs (gastropods: *Lehmannia marginata* Müller) (Turchetti and Chelazzi, 1984; Heiniger and Rigling, 1994). Following natural control in Italy, Grente in the 1960s initiated a biological control program of chestnut blight in France. The program involved successive treatments of 10 cankers per hectare for a 3 year period, followed by treatment of 5 cankers per hectare for 2-3 years. This cured plantations of virulent cankers within a 10 year period (Grente and Berthelay-Sauret, 1978), thus proving to be an effective biocontrol system. To date, chestnut blight has spread throughout most of central Europe, including Portugal, Switzerland, Germany and Greece (Heiniger and Rigling, 1994). However, in many of these plantations, the frequency of hypovirulent isolates is high. For example, in chestnut coppices in Switzerland 59 % of isolates recovered were hypovirulent (Bissegger *et al.*, 1997), therefore chestnut blight is no longer a major problem in Europe.

1.6.3. Control of chestnut blight in the USA

In America, chestnut blight was first reported in 1904 on American chestnut (*Castanea dentata* (Marsh.) Borkh.) trees in Bronx zoo, New York City (Merkel, 1905). Here, as in Europe, the pathogen was thought to have been imported from Asia (Newhouse, 1990) such that American trees had no natural resistance, enabling the pathogen to spread rapidly through American chestnut stands, destroying several billion mature trees within a 50 year period (Anagnostakis, 1982). Initial attempts to prevent chestnut blight becoming established involved pruning and spraying using Bordeaux mixture (Murrill, 1906). This failed to control the disease, and it spread rapidly throughout American chestnut plantations. Following the success in controlling

a b

Figure 1. 5 Canker development caused by *Cryphonectria parasitica* on chestnut (*Castanea sativa*). (a) Canker resulting from infection with a fully virulent isolate of *C. parasitica*. (b) Healed canker, following infection with a hypovirulent *C. parasitica* strain (Heiniger and Rigling, 1994).

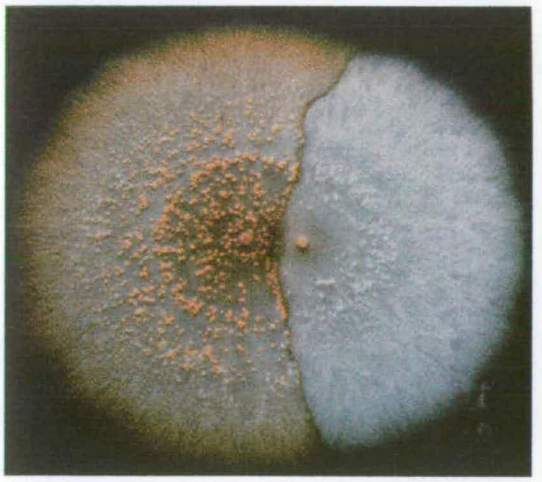


Figure 1. 6 Hypovirulent (white) and virulent (orange/ brown) forms of *Cryphonectria parasitica* (Heiniger and Rigling, 1994).

chestnut blight in Europe, it was hoped that the same could be achieved in the USA. Anagnostakis and Jaynes (1973) found that European hypovirulent strains could diminish the growth of cankers in greenhouse seedlings. This led to field trials involving 300 trees being inoculated with an American hypovirulent strain derived from the original French strain (Jaynes and Elliston, 1980). This proved successful in that 86 % of the inoculated cankers were controlled after one year. However, new cankers forming at other points on the trees were not cured and were lethal. Virulent infection levels, therefore, remained high with long-term survival of trees and natural dissemination not being achieved.

The movement of dsRNA between hypovirulent and virulent isolates depends on the vegetative compatibility of the isolates (MacDonald and Fulbright, 1991). Vegetative compatibility is controlled by *vic* (vegetative incompatibility) genes, which are estimated to be at 7-8 loci (Anagnostakis, 1995). If two strains have the same alleles at all of the *vic* genes then anastomosis will occur. The ability to undergo viable anastomosis decreases with the increasing number of alleles which differ. This has been shown by Lui and Milgroom (1996) who found that the frequency of transmission between groups differing by one *vic* gene was 0.49, and that transmission frequency decreased to 0.13 when *vic* genes differ at two loci. When more than two *vic* genes differ, then the transmission frequency decreased to 0.03. It has been shown that American isolates possess a great deal of vegetative incompatibility with more than 100 vegetative compatibility groups present, compared to only a handful of such groups in Italy (Newhouse, 1990). This complex population structure is thought to have limited the dissemination of dsRNA in field trials under natural conditions in the Eastern United States, thus preventing dsRNA being used as a successful biological control agent, as occurred in Europe.

1.6.4. Molecular basis for hypovirulence

When dsRNA elements associated with *C. parasitica* were examined, it was found that the number of segments, size and sequence homology differed greatly between isolates (Dodds, 1980; L'Hostis *et al.*, 1985). For example, dsRNA elements from different *C. parasitica* populations throughout Europe appear to represent a single species, which does not hybridize to North American dsRNA elements (Enebak *et al.*, 1994; Peever *et al.*, 1997). Within the North American continent, some similarity is

observed; for example, dsRNA from Michigan isolates has been observed to cross-hybridize with dsRNA from Ontario isolates (Melzer *et al.*, 1997). This variability has led to the European-derived hypovirulent strain EP713 being utilized as a type strain. EP713 harbours several dsRNAs, of which the largest is termed L-dsRNA (12 712 bp), and also several smaller segments termed M-dsRNA (8-10 kbp) and S-dsRNA (0.6-1.7 kbp) (Hiremath *et al.*, 1986; Shapira *et al.*, 1991a). The M- and S- dsRNA elements are reported by Shapira *et al.* (1991a) to be internally deleted forms of L-dsRNA, thus indicating that all the genetic information resides within L-dsRNA. L-dsRNA (CHV1-713) was recently shown to contain two contiguous open reading frames (ORFs); ORFA (622 codons) and ORFB (3165 codons) (Shapira *et al.*, 1991b), both of which encode polyproteins that undergo autoproteolytic processing during translation (Choi *et al.*, 1991).

To determine whether hypovirulence was due to a general debilitation of fungal metabolism, or due to dsRNA specifically altering the expression of certain fungal genes, Choi and Nuss (1992b) transformed virulent *C. parasitica* EP155 with the vector pAXHY2 (Figure 1. 7a). This vector contains the ORFA coding domain of L-dsRNA. The control vector pAXHY5 (Figure 1. 7b) contained ORFA in the reverse orientation. Transformation with pAXHY5 resulted in no phenotypic alterations. However, transformation with pAXHY2 resulted in decreased pigmentation, reduced laccase accumulation and suppressed conidiation, similar to that of natural dsRNA- containing hypovirulent strains. They were not, though, reduced in virulence, indicating an apparent uncoupling of hypovirulence-associated traits from hypovirulence. ORFA only represents 16 % of the coding capacity of CHV1-713, thus hypovirulence may be conferred by a different segment. This was demonstrated by Choi and Nuss (1992b) who transformed virulent *C. parasitica* strains with a cDNA copy of CHV1-713 in the vector pXH9 (Figure 1. 7c), which resulted in the expression of both hypovirulence and hypovirulence-associated traits e.g. reduced pigmentation and suppressed conidiation. The uncoupling of hypovirulence from associated traits is highly significant, as it raises the possibility that specific properties of hypovirulent isolates can be altered. For example, it may be possible to engineer hypovirulent strains which sporulate abundantly as opposed to the decreased sporulation levels of naturally hypovirulent isolates, in addition to displaying increased hypovirulence.

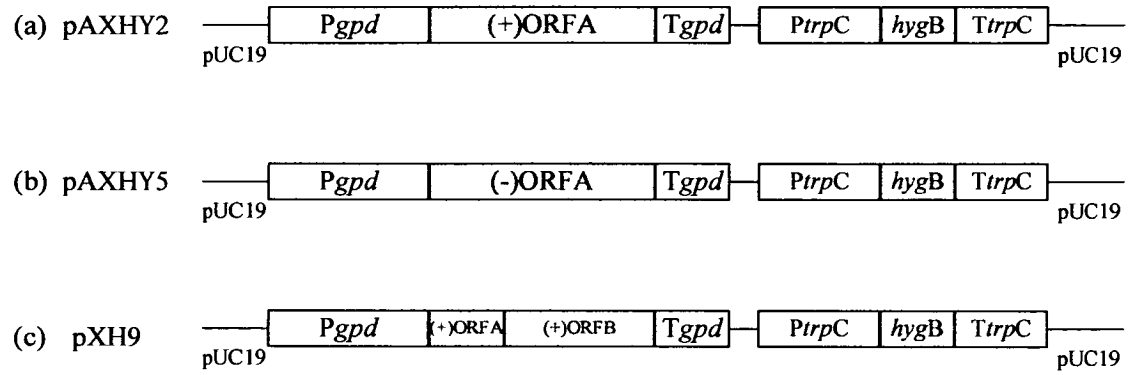


Figure 1. 7 Transformation vectors utilized for transforming virulent *Cryphonectria parasitica* isolates. (a) Plasmid pAXHY2 contains ORFA, flanked by the *C. parasitica* glyceraldehyde-3-phosphate dehydrogenase gene promoter (P*gpd*) and terminator (T*gpd*). The plasmid also contains the *E. coli* hygromycin B phosphotransferase gene (*hygB*) preceded by the *A. nidulans trpC* promoter (P*trpC*). (b) Plasmid pAXHY5 is the same as pAXHY2 except that ORFA is in the antisense direction. (c) Plasmid pXH9 is the same as pAXHY2 except that ORFA is substituted by the entire L-dsRNA sequence, which includes ORFA and ORFB (Choi and Nuss, 1992b).

1.6.5. A novel means of biological control

Choi and Nuss (1992b) have successfully transformed virulent *C. parasitica* strains with cDNA copies of CHV1-713, producing hypovirulent strains. These novel strains contain a chromosomally integrated copy of the viral RNA as well as cDNA-derived cytoplasmic dsRNA forms, which are fully transmissible via anastomosis. The presence of the chromosomally integrated copy of the viral RNA enables the potential additional transmission routes via conidia and via ascospores through nuclear inheritance. Chen *et al.* (1993) have demonstrated that the integrated viral cDNA is stably maintained through repeated rounds of conidiation and can be faithfully transmitted to ascospore progeny. This breakthrough overcomes the previous barrier to dissemination posed by vegetative compatibility, as CHV1-713 cDNA can be engineered into field isolates representing the range of vegetative compatibility groups present in a given ecosystem. The subsequent engineered strains due to the novel means of transmission should have improved dissemination and persistence.

As mentioned earlier, it should be possible to manipulate the viral cDNA to generate novel hypovirulent strains. Initial studies by Craven *et al.* (1993) have shown this to be possible by constructing strains possessing increased conidiation levels, and remaining hypovirulent.

The L-dsRNA copy may find broader usage as a biological control agent. Recent studies by Chen *et al.* (1994; 1996) indicate that CHV1-713 cDNA can be transformed into other species. The fungi tested were *C. cubensis* (Bruner) Hodges, *C. havanensis* (Bruner) Barr, *C. radicalis* (Schw. Ex Fries) Barr and *Endothia gyrosa* (Schw. Ex Fries) Fries, none of which contain natural dsRNA elements. Upon transformation, isolates displayed altered morphology, reduction in phenol oxidase production (except *E. gyrosa*) and attenuation of fungal virulence. Subsequent studies indicated *C. cubensis* and *E. gyrosa* were unable to elicit infections, indicating that biocontrol could be successfully achieved in several other species using cDNA derived from CHV1-713. Again the cDNA transformants have additional transmission routes via ascospore progeny and asexual spores.

dsRNA seems to be able to elicit hypovirulence in closely related species, so an interesting and important factor to be elucidated is: how does the presence of dsRNA lead to the phenotypic traits of hypovirulence, reduced laccase accumulation, reduced conidiation, lower pigmentation etc.?

1.6.6. How does dsRNA alter fungal phenotype?

Infection of *C. parasitica* with dsRNA leads to certain altered phenotypic traits; for example, decreased pigmentation, suppressed conidiation, hypovirulence and reduced laccase accumulation. It has recently been reported that several genes (e.g. *lac1* encoding phenol oxidase laccase, *vir2* encoding a putative pheromone, *cbh1* encoding cellobiohydrolase I and *cpg1* encoding a G-protein α-subunit), are down-regulated in hypovirulent isolates compared with virulent isolates (Rigling and van Alfen, 1991; Larson *et al.*, 1992; Zhang *et al.*, 1993; Choi *et al.*, 1995; Wang and Nuss, 1995). Of these *cpg1* encodes a heterotrimeric guanidine nucleotide binding protein (G protein) CPG1. G-proteins are a family of regulatory proteins which play an essential role in the response of eukaryotic cells to a variety of environmental stimuli, so it was proposed that CPG1 is involved in virulence attenuation. This was examined by

transforming sense and antisense copies of *cpg1* into virus-free *C. parasitica* isolates. Transformants were assessed for their degree of virulence using standard assays on chestnut stems (Choi *et al.*, 1995). Transformants containing sense copies of *cpg1* produced lesions of similar size to hypovirulent isolates (0.61 cm^2), whereas transformants containing antisense copies of *cpg1* produced lesions of similar size to virulent isolates (7.13 cm^2). These results indicated that a G protein mediated regulatory pathway is affected by dsRNA elements resulting in reduced fungal virulence. Subsequent studies by Wang and Nuss (1995) indicate that down-regulation of the virulence enzyme cellobiohydrolase (*cbh1*) upon dsRNA infection is dependent upon *cpg1* being down-regulated. Thus CPG1 may down-regulate several virulence factors in a regulatory cascade as proposed by Kazmierczak *et al.* (1996). To determine whether this is the case Chen *et al.* (1996) proposed that CPG1 may be a member of the G_i family of G protein α subunits, which inhibit adenylyl cyclase, and consequently reduce intracellular cAMP levels. Measurements of intracellular cAMP levels in virulent and hypovirulent isolates confirmed this by indicating a four fold greater level of cAMP in virulent strains (Chen *et al.*, 1996). This, combined with evidence obtained by Larson *et al.* (1992) and Larson and Nuss (1994) which indicated that laccase production was suppressed following disruption of an inositol triphosphate/ Ca^{2+}/ calmodulin signalling pathway, led Gao and Nuss (1996) to propose a model for hypovirus-mediated suppression of virulence. In a virulent virus-free *C. parasitica* isolate (Figure 1. 8a), an unknown ligand binds a transmembrane receptor, activating $G_{i\alpha}$ (*cpg1* encoded), which in turn negatively regulates adenylate cyclase, such that intracellular cAMP is decreased. Protein kinases are then inactive, so no inhibition is placed on phospholipase C, and phospholipase C can then convert phosphatidylinositol (PI) 4-5 phosphate (PIP_2) to inositol 1, 4, 5 trisphosphate (IP_3). IP_3 then activates virulence genes such as laccase. In hypovirulent *C. parasitica* isolates (Figure 1. 8b), $G_{i\alpha}$ is not produced, due to down-regulation of *cpg1*, such that adenylate cyclase is not activated and intracellular cAMP levels are therefore high. This leads to protein kinases being activated, which in turn

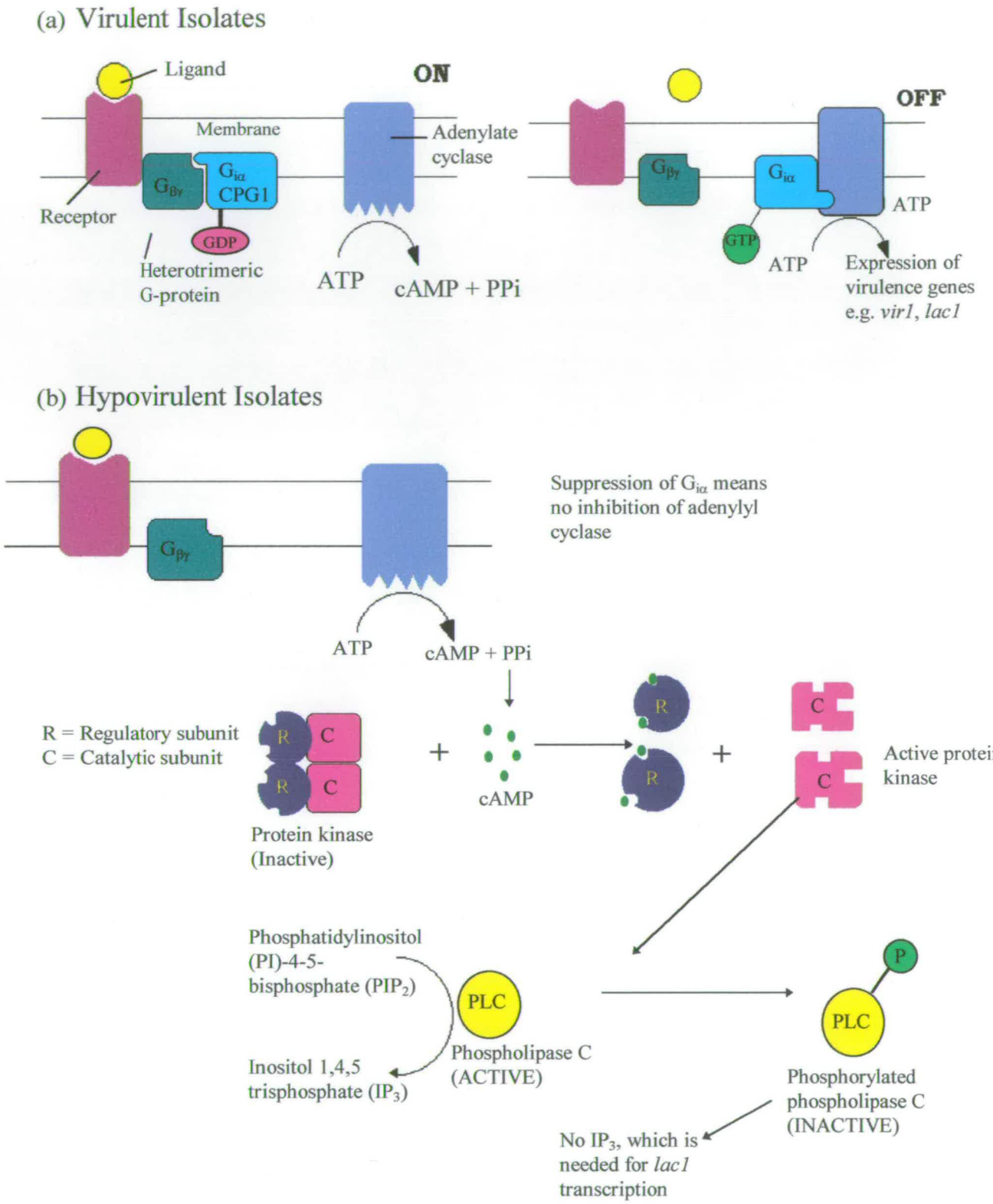


Figure 1. 8 Proposed mechanisms for virulence and hypovirulence in *C. parasitica* (a) In virulent isolates, a ligand binds a receptor, activating an inhibitory heterotrimeric G-protein. $G_{i\alpha}$ (encoded by *cpg1*) negatively regulates adenylyl cyclase, such that expression of virulence genes e.g. *lac1* can occur (b) In hypovirulent isolates $G_{i\alpha}$ (*cpg1*) is not produced, such that adenylyl cyclase is permeantly active, generating cAMP. This in turn activates a protein kinase, which inhibits phospholipase C, resulting in no IP_3 generated, which would otherwise enable *lac1* transcription.

de-activate phospholipase C. This results in a decrease in IP_3, preventing virulence genes from being transcribed, so the isolate is hypovirulent. It is likely that similar modifications of signaling cascades are resposible for the phenotypic changes observed when *C. cubensis*, *C. havanensis*, *C. radicalis* and *E. gyrosa* were successfully transformed with cDNA generated from L-dsRNA from *C. parasitica*.

This forms a highly plausible model. However, direct linkage between CPG1 and the IP_3/ Ca^{2+} transcription pathway of virulence genes is yet to be established.

1.7. dsRNA and R. solani

dsRNA was first reported to be associated with *R. solani* by Castanho and Butler (1978a). Their studies first described a degenerative disease of *R. solani* termed *Rhizoctonia* decline, characterized by slow growth, fewer sclerotia, loss of pigmentation, presence of dsRNA and reduced virulence compared with normal isolates. Of 16 isolates tested by Castanho and Butler (1978b) 13 lacked dsRNA and were virulent, whereas the remaining 3 contained dsRNA of variable sizes, and were avirulent. They also found that the disease causing factor of the hypovirulent isolate 189a could be transferred by hyphal anastomosis to the healthy isolate 189HT5. This prompted studies in which sugar beet (*Beta vulgaris*) seeds were disinfected in 1 % sodium hypochlorite for 10 minutes and subsequently coated with both isolates 189a and 189 HT5. This resulted in suppression of damping-off of seedlings and indicated a potential for biocontrol. Further studies by Zanzinger *et al.* (1984) indicated that dsRNA was highly abundant in *R. solani* isolates. Of 50 strains tested, 49 contained dsRNA ranging from 0.6 kb to over 8.4 kb. They found no consistent correlation between degree of virulence and presence of dsRNA. Studies by Bharathan and Tavantzis (1990) tested isolates from five different AG, and again found no correlation between degree of virulence and dsRNA. These results, contradicting those of Castanho and Butler (1978b), are further supported by the results of Hyakumachi *et al.* (1985), Washington and Martin (1991) and Kousik *et al.* (1994), all of which found dsRNA to be ubiquitous in natural populations of *R. solani* and there was no apparent correlation between the presence of dsRNA and the degree of pathogenicity. In further contradiction to the studies

mentioned is that of Finkler *et al.* (1985) which suggests that dsRNA is associated with virulence. This study examined 139 Israeli isolates. Of these, 23 % were hypovirulent, and dsRNA was only detected in the virulent group. In further support, transmission studies indicated that hypovirulent isolates could be converted to the virulent phenotype upon transmission of dsRNA (Finkler *et al.*, 1988). Recent studies by Jian *et al.* (1997) and Jian *et al.* (1998) are in support of this, and indicate that the presence, or acquisition, of a 6.4 kb dsRNA segment leads to enhanced virulence. Thus, it seems that any relationship between dsRNA and the degree of virulence is part of a complex genetic phenomenon, which warrants further investigation. It is likely that the relationship is similar to that in *Ophiostoma ulmi* (Nuss and Koltin, 1990) where many isolates contain dsRNA but only 3 of 10 segments are required to maintain the hypovirulent state, whereas strains lacking the critical 3 segments are virulent.

In *R. solani*, it may be that certain segments lead to virulence; for example, the 6.4 kb segment reported by Jian *et al.* (1998); whereas other segments may result in the hypovirulent phenotype. Several other dsRNA segments may have no effect on virulence. To determine the amount of genetic variability between dsRNAs and thus try to ascertain whether there were common segments associated with hypovirulence, Bharathan and Tavantzis (1990) and Bharathan and Tavantzis (1991) determined the degree of cross hybridization of dsRNA from 51 isolates of AGs 1-5. They found that in all but one case, cross hybridization did not occur between isolates of different AGs (but within an AG, cross hybridization occurred), which is consistent with the theory that the AGs of *R. solani* are genetically isolated (Anderson, 1982). The exceptions were 3 dsRNA segments (1.8, 2.3 and 6.4 kb) which cross-hybridized from isolates Rhs 47 (AG 2), Rhs 1A1 (AG 3) and Rhs 1 (AG 5) respectively. All three isolates were hypovirulent suggesting that their common sequence may be responsible for their hypovirulence.

Thus it is hoped that *R. solani* dsRNA segments can be used to exert biological control, in the same way as was achieved for *C. parasitica*, following the example of control in Europe or in the USA. Prior to control being achieved, factors such as the vegetative compatibility of field populations requires to be studied to determine whether it will pose a barrier to dissemination of dsRNA. In addition, the effects of individual, and combinations of, dsRNA elements requires to be elucidated. This can be examined via curing of isolates, although an alternative approach would be to introduce cloned

dsRNA into *R. solani*. However, a suitable transformation system requires to be developed before these studies can be completed.

1.8. Aims of the Thesis

Biological control has been achieved against *C. parasitica* in Europe using dsRNA elements naturally resident within the fungus (reviewed by Nuss, 1992). dsRNA has been reported in *R. solani*, although there is much controversy regarding its effect on the host phenotype (Castanho and Butler, 1978b; Zanzinger *et al.*, 1984; Finkler *et al.*, 1985; Bharathan and Tavantzis, 1990; Kousik *et al.*, 1994). The work of this study aims to examine the presence and abundance of dsRNA within natural field isolates of *R. solani*. Subsequent work aims to generate isolates either partially or fully cured of dsRNA, such that a potential correlation between dsRNA and host virulence can be drawn. Host virulence involves a battery of enzymes which will be examined in several dsRNA-containing AG 3 isolates, with a view to comparing levels with cured isolates to determine whether any alterations of the enzyme levels are attributable to dsRNA. A secondary approach to elucidating the function of dsRNA in *R. solani* involves comparison to dsRNA elements which have been previously characterized. This work examines the relationship between hypovirus CHV1-713 of *C. parasitica* and dsRNA from *R. solani*.

For dsRNA elements to form a successful biological control agent against *R. solani*, dsRNA must be transmissible throughout field populations. dsRNA transmission is reported to occur following compatible anastomosis (Finkler *et al.*, 1988), with transmission frequency reported to decrease to only 4 % of incompatible anastomosis reactions in *Ophiostoma novo-ulmi* (Brasier, 1984; 1986). This work, therefore, aims to examine the vegetative compatibility structure of natural field isolates of *R. solani*, to determine whether dsRNA transmission would be impeded.

To establish a biological control strategy against *R. solani* using dsRNA, it may be possible to transform cDNA of hypovirus CHV1-713 into *R. solani*, and have the hypovirulent phenotype expressed. However, prior to this, a suitable transformation procedure needs to be developed for *R. solani*, which this work aims to do. Additionally this would enable cDNA of dsRNA from *R. solani* to be re-introduced into

R. solani isolates devoid of dsRNA and thus provide direct evidence for the role of dsRNA in *R. solani*.

CHAPTER 2

MATERIALS AND METHODS

2.1. Materials

All chemicals, unless otherwise stated, were analytical grade and were purchased from one of the following suppliers: Sigma (Poole, Dorset, UK), British Drug Houses (BDH) (Poole, Dorset, UK), Gibco-BRL (Paisley, UK), Fisons (Loughborough, UK), Rathburn (Walkerburn, UK) or Pharmacia (Milton Keynes, UK). Enzymes for nucleic acid manipulations were purchased from Boehringer Mannheim (Lewes, East Sussex, UK). α-^{32}P dCTP (3000 Ci mM^{-1}, 10 μCi ml^{-1}.) was purchased from Amersham (Little Chalfont, Buckinghamshire, UK), as were nucleic acid hybridization membranes (Hybond N).

2.2. Media

All media was sterilized by autoclaving at 121°C for 20 minutes.

2.2.1. Fungal growth media

***Rhizoctonia* Selective Agar**

Oxoid agar no. 3 (20 g), K_2HPO_4 (1 g), $MgSO_4.7H_2O$ (0.5 g), KCl (0.5 g), $FeSO_4.7H_2O$ (10 mg), $NaNO_2$ (0.2 g) and gallic acid (0.4 g) were dissolved in distilled water (dH_2O) to give a final volume of 1 litre. After autoclaving, filter-sterilized stock solutions of streptomycin and chloramphenicol were added to give final concentrations of 50 mg l^{-1} each.

Potato Dextrose Agar

Oxoid potato dextrose agar (39 g) was added to dH_2O to give a final volume of 1 litre.

Cellulose Minimal Agar

Cellulose powder (5 g) was added to $NaNO_3$ (2 g), KH_2PO_4 (1.23 g), KCl (0.5 g), $MgSO_4.7H_2O$ (0.5 g), $FeCl_3.6H_2O$ (0.001 g), $ZnSO_4.7H_2O$ (0.0009 g), $MnSO_4.4H_2O$ (0.0004 g) and Oxoid no. 3 agar (20 g). These were dissolved in dH_2O to give a final volume of 1 litre.

Starch Minimal Agar

Soluble starch (3.88 g), $NaNO_3$ (2 g), KH_2PO_4 (1.23 g), KCl (0.5 g), $MgSO_4.7H_2O$ (0.5 g), $FeCl_3.6H_2O$ (0.001 g), $ZnSO_4.7H_2O$ (0.0009 g), $MnSO_4.4H_2O$ (0.0004 g) and Oxoid no. 3 (20 g) agar were dissolved in dH_2O to give a final volume of 1 litre.

Agar-Coated Microscope Slides

Oxoid no. 3 agar (20 g) was added to dH_2O to give a final volume of 1 litre. After autoclaving, sterile microscope slides were thinly coated with agar by briefly immersing them in the hot agar. Excess agar was allowed to drip off then the agar coated slides were placed on water agar plates.

Water Agar

Oxoid no. 3 agar (20 g) was added to dH_2O to give a final volume of 1 litre.

Cellulose Film Overlaid Agar

7 cm diameter discs of Rayophane Pu525 were immersed in water and autoclaved. Following autoclaving, single discs were placed upon water agar plates.

Mineral Nutrients

$NaNO_3$ (5 g), K_2HPO_4 (1 g), $MgSO_4.7H_2O$ (0.5 g) and Oxoid yeast extract (0.1 g) were added to dH_2O to give a final volume of 1 litre.

Potato Dextrose Charcoal Agar

Oxoid potato dextrose agar (39 g) and activated charcoal (5 g) was added to dH_2O to give a final volume of 1 litre.

***Rhizoctonia* Broth**

Oxoid malt extract (5 g), Oxoid yeast extract (5 g) and D-glucose (5 g) was added to dH_2O to give a final volume of 1 litre.

Pectin Enzyme Broth

$(NH_4)_2SO_4$ (2.64 g), K_2HPO_4 (0.34 g) and $MgSO_4.7H_2O$ (0.14 g) were dissolved in 500 ml of dH_2O. Citrus pectin (10 g) was added to the solution with vigorous magnetic stirring. Once dissolved, dH_2O was added to give a final volume of 1 litre.

Cycloheximide Agar

Oxoid potato dextrose agar (39 g) was added to dH_2O to give a final volume of 1 litre. After autoclaving, a filter sterilized cycloheximide stock solution was added to give a final concentration of 100 $\mu g\ ml^{-1}$.

Gallic Acid Agar

Oxoid malt extract agar (50 g) was dissolved in 1 litre of dH_2O containing 0.5 % gallic acid solution adjusted to pH 4.5.

Cellulose Agar

Cellulose powder (20 g) was suspended in 1 litre of water agar.

Pectin Agar

Citrus pectin (20 g) was dissolved in 1 litre of water agar.

2.2.2. Bacterial Growth Media

Luria Broth (LB)

Tryptone (10 g), Bacto yeast extract (5 g) and NaCl (10 g) were added to dH_2O to give a final volume of 1 litre. After autoclaving, filter sterilized solutions of thiamin and glucose were added to provide 35 mg l^{-1} and 1 g l^{-1}, respectively.

Luria Agar

Oxoid no. 3 (15 g) agar was added to 1 litre of Luria broth.

2.3. Molecular Biology Solutions

Tris-Cl

Tris base (tris (hydroxymethyl) aminomethane) was dissolved in dH_2O and the pH adjusted to the desired value by the addition of concentrated hydrochloric acid, with the final volume adjusted with dH_2O to 1 litre.

EDTA

EDTA (ethylenediaminetetra amino acid, disodium salt) was prepared as a 0.5 M stock solution in dH_2O. The pH was adjusted to pH 8.0 using 5 M NaOH.

CF11 Cellulose

CF11 cellulose was prepared as a 70 % (w/v) autoclaved solution in dH_2O

TB

TB was prepared by adding PIPES (Piperazine-N, N'-bis [2-ethanesulfonic acid]) (0.6048 g), $CaCl_2$ (2.205 g) and KCl (3.7275 g) to 100 ml dH_2O. The components were dissolved by adjusting the pH to pH 6.7 with KOH and then $MnCl_2$ (2.1769 g) was added and the volume adjusted to 200 ml. The solution was filter-sterilized and stored at 4°C.

TBE

TBE was prepared as a 10 fold stock solution and stored at room temperature. Tris base (108 g), boric acid (55 g) and 40 ml 0.5 M EDTA solution were used in a final volume of 1 litre dH_2O. The stock solution was diluted to the appropriate concentration prior to use.

SSC

SSC was made as a 20 fold stock solution by adding NaCl (175 g) and sodium citrate (88.2 g) to dH_2O to give a final volume of 1 litre. The stock solution was diluted to the appropriate concentration prior to use.

STE

STE was prepared as a 10 fold stock solution by adding NaCl (58.44 g), Tris (60.57 g) and EDTA (2.92 g) to dH_2O to give a final volume of 1 litre. The stock solution was diluted to the appropriate concentration prior to use.

Denhardts solution

Denhardts solution was prepared as a 100 fold stock solution by adding BSA (bovine serum albumin) (20 g), Ficoll (20 g) and PVP (polyvinylpyrollidone) (20 g) to dH_2O to give a final volume of 1 litre.

Phenol-chloroform-isoamyl alcohol (25:24:1)

Water-saturated phenol was equilibrated to pH 8.0 by the addition of an equal volume of 1.0 M Tris-Cl (pH 8.0) plus 0.2 % (w/v) 8-hydroxyquinoline (which prevents oxidation of the phenol, inhibits RNase activity and chelates metal ions; Kirby, 1956). This was mixed by vigorous magnetic stirring for 15 minutes, followed by overnight equilibration at 4°C. The top phase was discarded and equilibration repeated twice with an equal volume of 0.1 M Tris-Cl (pH 8.0). Chloroform and isoamyl alcohol were added in the appropriate ratio and the mixture equilibrated twice with 0.1 M Tris-Cl (pH 8.0). The equilibrated mixture was stored under 0.01 M Tris-Cl (pH 8.0) at -20°C.

Potassium Acetate Buffer

Potassium acetate (3 M) was dissolved in half volume dH_2O then the pH adjusted to pH 5.2 with glacial acetic acid; dH_2O was added to give a final volume of 1 litre.

Ethidium Bromide

A stock solution was prepared by adding ethidium bromide (0.1 g) to 10 ml dH_2O and stored at room temperature.

Loading Buffer for Agarose Gel Electrophoresis

A 6 fold stock solution was prepared with 60 % (w/v) sucrose, 6 mM disodium EDTA and 0.025 % (w/v) bromophenol blue dissolved in 6 X TBE.

2.4. Fungal Methods

2.4.1. Isolation, Maintenance and Preservation of Strains

Origins of *Rhizoctonia solani* strains are indicated in Table 2. 1. Isolation of strains from potato tubers was achieved by transferring black scabs from the tuber surface onto *Rhizoctonia* selective agar (1 g K_2HPO_4, 0.5 g $MgSO_4.7H_2O$, 0.5g KCl, 10 mg $FeSO_4.7H_2O$, 0.2 g $NaNO_2$, 0.4 g gallic acid, 50 mg chloramphenicol, 50 mg streptomycin, 20 g Oxoid no. 3 agar in a final volume of 1 litre dH_2O). Their anastomosis grouping was confirmed by pairing strains with known tester strains using standard methods (Parmeter *et al.*, 1969). Strains were maintained on potato dextrose agar (PDA) and preserved at 4°C under sterile distilled water and in addition at -70°C under 10 % (w/v) glycerol. *Verticillium biguttatum* strains (M 73, M 92 to 100) were obtained from Dr. P.J.H.F. van den Boogert, Netherlands. These were maintained on PDA and preserved at -70°C under 10 % (w/v) glycerol. *Cryphonectria parasitica* Cp 38755 and Cp 52571 were obtained from Dr. V. Rubio, Spain and maintained on PDA.

2.4.2. Microscopy

Microscopy was carried out using a Leitz Orthoplan microscope, with Plano objectives (x 10, x 25, x 70, x 90) and Periplan (x 10) eyepieces.

Video microscopy was completed using a colour Panasonic video camera (VW CL-350) attached to the above microscope. The camera was attached to a Toshiba video recorder (DV80B), which was connected to a Sony Trinitron 14" colour monitor (1460 UB). Black and white images were obtained using a Mitsubishi P61B video copy processor.

2.4.3. Anastomosis Reactions

Anastomosis was observed using two different techniques.

Rhizoctonia Strain	Anastomosis Group (AG)	Source
01R01	1-1A	ATCC 76121
01R02	1-1B	ATCC 76122
01R03	1-1C	ATCC 76123
01R04	1	Carling, D.E., Alaska
*1 to *49	1	Julian, M.C., Spain
21R01	2-1	ATCC 66154
021-14	2-1	Rubio, V., Spain
21-14	2-1	Rubio, V., Spain
21-41	2-1	Rubio, V., Spain
21-F16L	2-1	Rubio, V., Spain
2tR105	2-tulip	Schneider, J.H.M., Netherlands
2tR118	2-tulip	Schneider, J.H.M., Netherlands
2tR144	2-tulip	Schneider, J.H.M., Netherlands
2t124	2-tulip	Rubio, V., Spain
2t101	2-tulip	Rubio, V., Spain
GR1	2-2	van den Boogert, P.H.J.F., Netherlands
22R02	2-2	ATCC 76125
VR5	2-2	van den Boogert, P.H.J.F., Netherlands
B12	2-2	van den Boogert, P.H.J.F., Netherlands
23R01	2-3	Naito, S., Japan
3R4	3	Jager, G., Netherlands
3R8	3	Jager, G., Netherlands
3R9	3	Jager, G., Netherlands
3R41	3	Jager, G., Netherlands
FT1-20	3	Potato tubers, Scotland
FT100-104	3	Potato tubers, Scotland
FT200-204	3	Potato tubers, Scotland
FT300-304	3	Potato tubers, Scotland
FT400-404	3	Potato tubers, Scotland
JP1-4	3	Potato tubers, Scotland
MK1-2	3	Potato tubers, Scotland
APA1-2	3	Potato tubers, Scotland
APB1-3	3	Potato tubers, Scotland
APC1-2	3	Potato tubers, Scotland
04R22	4	Doornik, Netherlands
4/51	4	Deacon, J.W., Scotland
4/41	4	Deacon, J.W., Scotland
Me8-2	4	INIA, Spain
521	4	ATCC 64643
PA1	4	Papavizas, G.C., USA
Papa	4	McCabe, P.M., Scotland
05R01	5	ATCC 76128
R470	5	Deacon, Scotland
06R01	6	ATCC 76129
07R01	7	Rubio, V., Spain
08R01	8	ATCC 76106
09R01	9	ATCC 62804
11R01	11	ATCC 90857

Table 2. 1 Origins and anastomosis groupings of *Rhizoctonia solani* strains.

1. **Water agar slides.** Sterile glass coverslips (44 x 64 mm.) were coated with a thin film of 2 % agar (Oxoid no. 3) and placed upon water agar plates. Slides were inoculated with two opposing blocks of fungi approximately 20 mm apart. Following 24 hours incubation at 23°C, the coverslips were inverted onto observation chambers consisting of glass capillaries stuck to microscope slides (51 x 86 mm) (Figure 2. 1) and examined microscopically, through the coverslip and thin layer of agar.

2. **Cellophane overlaid agar.** Sterile 7 cm diameter discs of transparent cellulose film (autoclaved in dH_2O) were placed upon 2 % water agar plates. Fungi were inoculated approximately 30 mm apart and incubated at 23°C for 4 to 5 days and subsequently examined microscopically.

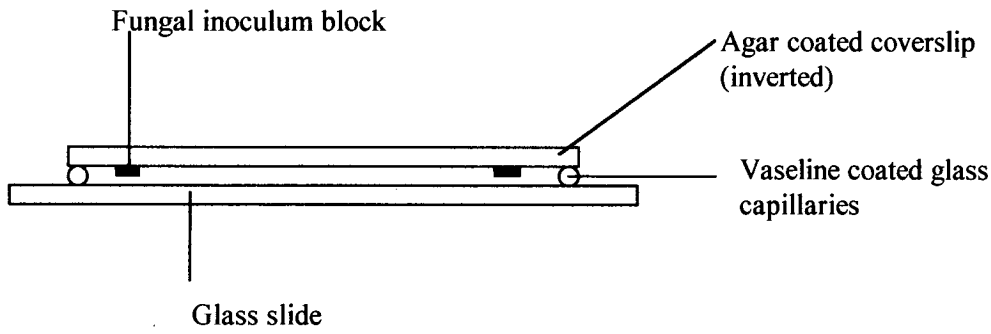


Figure 2. 1 Observation chamber for microscopic examination of anastomosis reactions.

2.4.4. Susceptibility of *Rhizoctonia solani* to *Verticillium biguttatum*

The method used was based on that of van den Boogert and Deacon (1994).

Preparation of *V. biguttatum* conidial suspension

Conidial suspensions were obtained by rinsing 14 day old colonies with sterile dH_2O and passing the washings through two layers of sterile cheesecloth. This was repeated a further two times, with the collected eluate being centrifuged for 15 minutes at 3000 *g*. The conidia were finally resuspended in sterile dH_2O and their concentration determined using a Neubaeur counting chamber; levels were adjusted to approximately 7 x 10^7 conidia per ml.

Interactions on cellulose agar

Agar discs of *R. solani* (6 mm diameter) were inoculated upon glass coverslips which were centrally located on plates of cellulose agar ($MnSO_4.7H_2O$ (0.0004 g), $ZnSO_4.7H_2O$ (0.0009 g), $FeCl_3.6H_2O$ (0.001 g), $MgSO_4.7H_2O$ (0.5 g), KCl (0.5 g), KH_2PO_4 (1.23 g), Oxoid agar no. 3 (20 g), $NaNO_3$ (2 g), cellulose powder (5 g) per litre dH_2O). 40 µl of *V. biguttatum* conidial suspension was inoculated in a straight line (2 cm long) at the periphery of the plate (Figure 2. 2). Plates were incubated for 14 days at 18°C, with the number of sclerotia being recorded at 2 day intervals. Each interaction was examined in triplicate.

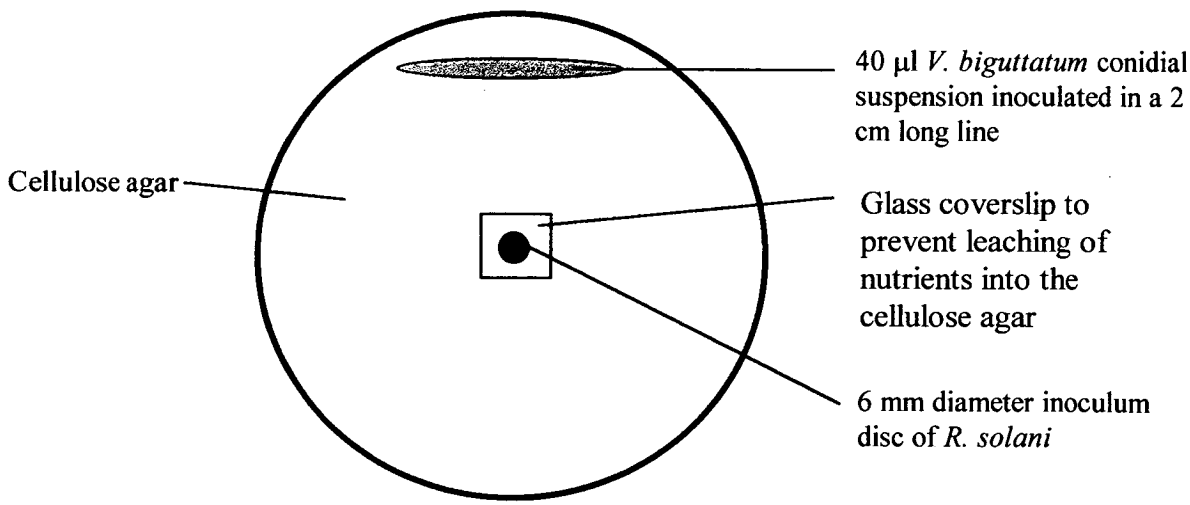


Figure 2. 2 Inoculum positions of *Rhizoctonia solani* and *Verticillium biguttatum* when studying interactions between the two fungi.

Interactions on starch agar

Interactions were also observed on starch agar (as for cellulose agar, but with cellulose replaced with 3.88 g starch) using the method described above. Due to the increased abundance of sclerotia on these plates, sclerotial area was estimated using a PC-based image analysis package with Optimas version 5.2 and Excel version 5.0. Images were obtained using a Nikon 55 millimetre macro lens connected to a Sony CCD camera.

2.4.5. Growth rates of *Rhizoctonia solani*

Discs (6 mm diameter) were excised from 7 day old *R. solani* cultures and centrally inoculated on either; potato dextrose agar, water agar, or water agar supplemented with either 2 % (w/v) cellulose or 2 % (w/v) citrus pectin. Linear extension rates were recorded as daily increments in colony diameter at 23°C. Three replicate plates per strain were inoculated and extension rates were recorded on two axes.

2.4.6. Plant pathogenicity tests

Plant pathogenicity tests were based on the method of Ichielevich-Auster *et al.* (1985). Pathogenicity was observed using the following host plants: tomato (*Lycopersion esculentum*), radish *(Raphanus sativus*), carrot (*Daucus carota*), lettuce (*Lactuca sativa*), cress (*Barbarea praecox*) and onion (*Allium cepa*). Discs (6 mm diameter) were excised from 7 day old cultures and centrally inoculated on water agar plates and incubated for two days at room temperature. Ten replicate plates were inoculated per strain. Five seeds (disinfected in 1 % sodium hypochlorite for 10 minutes) were placed around the periphery of each colony. Subsequent incubation was at room temperature for 10 days, following which disease severity was recorded on a scale of 0 to 5 based on the relative size of necrotic area on the shoots or roots as follows: 0 = no disease; 1 = 1 - 10 %; 2 = 11 - 30 %; 3 = 31 - 50 %; 4 = 51 - 80 % and 5 = entire hypocotyl infected. Isolates with a mean infectivity between 0 and 1 were considered non-pathogenic.

2.4.7. Pectic zymograms

Detection of pectic enzymes on pectin-containing polyacrylamide gels was based on the method of Cruickshank and Wade (1980) and Sweetingham *et al.* (1986).

Preparation of enzyme sample

A 10 mm diameter agar disc of *R. solani* was inoculated into 5 ml of pectic enzyme broth (2.64 g $(NH_4)_2SO_4$, 0.34 g K_2HPO_4, 0.14 g $MgSO_4.7H_2O$, 10 g citrus pectin per litre dH_2O) and incubated at 25°C for 10 days. One ml of culture supernatant was removed and Sephadex G25-150 was added to a concentration of 50 mg ml^{-1} to aid sample loading. In addition, bromophenol blue was added to a final concentration of 0.05 % and 30 µl of sample loaded onto a polymerized pectin acrylamide gel.

Pectin acrylamide gel electrophoresis

Using a BioRad PROTEAN II xi 2-D vertical electrophoresis tank, a pectin acrylamide gel (10.25 % acrylamide, 2.5 % bis-acrylamide, 0.1 % citrus pectin), buffered to pH 8.7 with citric acid buffer (0.525 g citric acid, 4.598 g tris base per litre dH_2O, pH 8.7) was prepared. Polymerization of the gel was carried out by the addition of 0.35 µl ml^{-1} TEMED (N, N, N', N'- tetramethylethylenediamine) and 0.7 mg ml^{-1} ammonium persulphate. Electrophoresis was completed at 4°C using 70 V for 1 hour in the following tank buffer: 7.22 g boric acid and 15.75 g sodium tetraborate decahydrate per litre dH_2O.

Gel staining

Following electrophoresis, gels were incubated in 0.1 M D,L-malic acid (pH 2) for 2 hours at room temperature. During this period, malic acid slowly reduces the pH of the gel from approximately pH 8.0 to pH 3.0, which enables each pectic enzyme to act at its optimal pH (Cruickshank and Wade, 1980). Gels were subsequently stained in 0.02 % (w/v) ruthenium red overnight at 4°C and destained in SDW for 3 to 5 hours. Enzyme action was determined as follows: pectin esterase produced zones with darker staining than background, polygalacturonase produced colourless or pale zones, pectin lyase produced yellow zones or clear zones bordered by a yellow margin.

2.4.8. Pectic enzyme assays

Preparation of enzyme sample

A ¼ oz. bijoux bottle containing 2 ml of either pectin broth ($(NH_4)_2SO_4$ (2.64 g), K_2HPO_4 (0.34 g), $MgSO_4.7H_2O$ (0.14 g), citrus pectin (10 g) per litre dH_2O), polypectate broth (as pectin broth with 10 g sodium polypectate substituting 10 g citrus pectin) or glucose broth (10 g glucose substituting 10 g citrus pectin), was inoculated with an 8 mm diameter agar disc of *R. solani* and incubated at 25°C. Cultures in glucose broth were included as controls. Following 5 days incubation, 500 µl of culture supernatant was removed and added to an equal volume of either pectin lyase buffer (50 µM tris-Cl, pH 8.3, 0.01 M $CaCl_2$, 1 % citrus pectin) (Pitt, 1988) or polygalacturonase buffer (45 mM sodium acetate buffer containing 1 % sodium polypectate) (Ayers *et al.*, 1966). Samples were mixed and 100 µl removed, which was boiled to inactivate the enzymes. Enzyme activities were measured using the thiobarbituric acid assay.

Samples were prepared on days 5 to 9 of culture incubation.

Thiobarbituric acid assay

The thiobarbituric acid assay was based on the method of Warren (1960). The assay measures the production of 4,5 unsaturated oligogalacturonides from the breakdown of pectin and the increase in reducing groups with dinitrosalicyclic acid from the substrate sodium polypectate.

100 µl of enzyme and buffer mixture was boiled for 5 minutes to inactivate the enzymes. Reagent A (125 µl, prepared from 200 µl 18 M sulphuric acid and 0.535 g periodic acid per 100 ml dH_2O) was added and the mixture incubated for 10 minutes at room temperature. Reagent B (250 µl, prepared from 5 ml 11.6 M HCl, 2 g sodium arsenite made up to 100 ml in dH_2O) was added and incubation continued for a further 2 minutes. Subsequently 1 ml of Reagent C (0.3 g thiobarbituric acid in 100 ml dH_2O) was added and the resultant mixture was boiled for 10 minutes and then held at 50°C until spectrometry. The production of 4,5 unsaturated oligogalacturonides was measured at a wavelength of 549 nm, whereas the increase in dinitrosalicyclic reducing groups was measured at a wavelength of 530 nm. Reference cuvettes in both cases contained the same reagents, except no fungal mycelia was added.

2.4.9. Phenol oxidase production

R. solani was centrally inoculated on agar containing 1.5 % malt extract, 2 % Oxoid agar no. 3 and either 0.5 % gallic acid or 0.5 % tannic acid (adjusted to pH 4.5 with NaOH) and incubated for 7 days at 25°C in the dark. Phenol oxidase production was indicated in both cases by a change in agar colour from light brown to dark brown. The diameter of the resulting colonies was recorded.

2.4.10. Cellulose degradation

Stacks of five filter papers (7 cm diameter, Whatman no. 3), weighed accurately to 4 decimal places, were placed in 250 ml Erlenmeyer flasks with 10 ml nutrient solution. The basal nutrient solution contained K_2HPO_4 (1 g), $MgSO_4.7H_2O$ (0.5 g) and Oxoid yeast extract (0.1 g) per litre dH_2O. In addition either $NaNO_3$ (5 g) or asparagine (3.88 g) (equivalent N content) was added, and some experiments contained a supplement of 3.4 meq Ca^{2+}, as calcium chloride. Discs (10 mm diameter) were inoculated at the periphery of each stack of filter papers. Flasks were incubated at 27°C for 22 days, then their contents placed in pre-weighed tins, oven-dried for 5 days at 80°C and re-weighed. Four replicate flasks were inoculated per isolate, in addition to six control flasks (no fungal inoculum) were used in each experiment.

The mean loss in weight from control flasks represented the difference between the original air-dry weight and oven-dry weight of the filter papers. This weight was subtracted from the difference in weight recorded between original filter paper weight and dried filter paper weight of the individual treatments, the resultant value being the amount of cellulose respired to carbon dioxide and water.

2.4.11. Heterokaryon formation

Homokaryotic isolates were placed 6 cm apart on potato dextrose charcoal agar (PDCA) (39 g Oxoid potato dextrose agar supplemented with 0.5 % (w/v) activated charcoal per litre dH_2O). Following 14 days incubation at 23°C, the plates were examined for tuft formation at the boundary of the two colonies. Where tufts had formed, fragments of hyphae were removed from the tufts using sterile forceps and placed on water agar plates and then incubated at 23°C. As soon as colonies began to

form, hyphal tips were transferred onto potato dextrose agar. Once colonies were established, confirmation that heterokaryons were formed was obtained using the test proposed by Adams and Butler (1982). This involved the colonies generated being paired to both parental homokaryons on PDCA plates. Lack of tuft formation with both parental homokaryons, following 14 days incubation at 23°C, was considered to be adequate evidence that heterokaryons had been successfully established.

2.4.12. dsRNA isolation

Isolation of dsRNA was completed using a modification of the methods of Morris and Dodds (1979) and Hoch *et al.* (1985).

Rhizoctonia broth (300 ml, containing 5 % glucose, 5 % malt extract, 5 % yeast extract) was inoculated with multiple plugs of *R. solani* and incubated aerobically for 10-14 days at 23°C. Approximately 10 g mycelia (dry weight) was ground to a powder with liquid nitrogen and mixed with the following reagents: 40 ml of pH 8.0 Tris-equilibrated phenol-chloroform-isoamylalcohol, 40 ml GPS buffer (0.1 M Na_2HPO_4, pH 9.5; 0.6 M NaCl; 0.2 M glycine), 1 ml 10 % sodium dodecyl sulphate (SDS) and 200 µl β-mercaptoethanol. The mixture was homogenized for 3 minutes using an Ultra-Turrax T25 (Janke and Kunkel, IKA Labortechnik) and then incubated aerobically at 23°C for 1.5 hours. Cell debris was removed by centrifugation for 15 minutes at 10 000 rpm in a GSA rotor in a Sorvall Superspeed centrifuge (DuPont). The supernatant was phenol-chloroform extracted three times by mixing for 1 minute with an equal volume of phenol-chloroform-isoamylalcohol and subsequently centrifuging for 5 minutes at 10 000 rpm in a SA600 rotor in a Sorvall Superspeed centrifuge. The final supernatant phase was ethanol precipitated in silanized Corex tubes overnight at 4°C with 0.1 volume 3 M potassium acetate pH 5.2 and 2.5 volumes ethanol. Nucleic acids were precipitated by centrifugation for 15 minutes at 7 500 rpm in a SA600 rotor, with the resultant pellets resuspended in a total volume of 7.5 ml STE (0.1 M NaCl; 0.05 M tris; 0.001 M EDTA pH 7.0), containing 15 % ethanol (STE-OH). The sample was loaded onto a CF11 cellulose column pre-equilibrated with 2 volumes STE, followed by 3 volumes STE-OH. ssRNA and DNA were eluted with 3 volumes STE-OH, and dsRNA was eluted with 2 volumes STE. Nucleic acids were concentrated by ethanol

precipitation overnight at -20°C, with the final pellet being resuspended in 100 µl SDW. Nucleic acid content was visualized using either agarose gel electrophoresis (Section 2.5.3) or polyacrylamide gel electrophoresis (Section 2.5.10).

2.4.13. Partial curing of dsRNA

Partial curing of dsRNA was achieved by repeatedly excising hyphal tips from young colonies grown on potato dextrose agar (PDA) containing the RNA synthesis inhibitor cycloheximide (Bottacin *et al.*, 1994; Elias and Cotty, 1996) at concentrations of; 0, 10, 50, 100, 400 µg ml^{-1}. Hyphal tips were left to regenerate for 7-14 days on fresh PDA at 23°C.

2.5. Molecular Methods

2.5.1. Removal of proteins by phenol-chloroform extraction

Nucleic acid samples were thoroughly mixed with an equal volume of pH 8.0 Tris-equilibrated phenol-chloroform-isoamylalcohol and centrifuged for 2 minutes at 13 000 rpm in a MSE Micro Centaur microfuge. The upper nucleic acid containing phase was retained and nucleic acids concentrated by ethanol precipitation (Section 2.5.2).

2.5.2. Ethanol precipitation of nucleic acids

Nucleic acids were precipitated from solution by addition of 0.1 volume 3M potassium acetate pH 5.2 and 2.5 volumes ethanol. Following thorough mixing, samples were incubated at -70°C for 15 minutes or -20°C for 30 minutes. Nucleic acids were pelleted by centrifugation at 13 000 rpm in a MSE Micro Centaur microfuge for 15 minutes. The resulting pellet was washed in 70 % ethanol and centrifuged for 5 minutes at 13 000 rpm and resuspended in a small volume of SDW.

2.5.3. Agarose gel electrophoresis

Prior to loading, DNA samples were mixed with one-sixth volume of loading buffer (60 % (w/v) sucrose, 6 mM disodium EDTA, 0.025 % (w/v) bromophenol blue in 6 x TBE). DNA fragments were separated in a 0.7 to 1.0 % (w/v in TBE) agarose gel (electrophoresis grade) containing 0.5 μg ml^{-1} ethidium bromide, with 1 X TBE (0.089 M tris, 0.089 M boric acid, 0.02 M EDTA) as running buffer. Molecular size markers used were *Hin*dIII digested bacteriophage lambda. DNA was visualized using a UV transilluminator (UV Products Inc., California) and photographed with a UVP camera and black and white pictures were obtained with a Mitsibushi P65B video copy processor.

2.5.4. Restriction digests

Restriction digests were completed with commercially available enzymes in their supplied buffers. Approximately 15 μl of DNA was digested in a total volume of 20 μl consisting of: 1 X restriction enzyme buffer, 10 units of restriction enzyme and SDW, for 1 to 16 hours at 37°C.

2.5.5. Production of competent *Escherichia coli* cells

Escherichia coli strain TG 1 or DL-5 was incubated on Luria broth (LB) agar overnight at 37°C, and from here a single colony was inoculated into 5 ml Luria Broth (LB). This was incubated overnight at 37°C with vigorous shaking and subsequently inoculated into 200 ml of LB, which was incubated at 18 to 23°C until the optical density at 600 nm reached 0.6. Cells were placed on ice for 10 minutes and then pelleted by centrifugation for 10 minutes at 4°C at 4250 rpm in a GSA rotor in a Sorvall Superspeed centrifuge (DuPont). The pelleted cells were resuspended in 20 ml of ice cold TB (10 mM PIPES, 55 mM $MnCl_2$, 15 mM $CaCl_2$, 250 mM KCl) and DMSO (dimethylsulfoxide) added to a final concentration of 7 %. Cells were left on ice for 10 minutes, aliquoted and frozen in liquid nitrogen. The resulting competent cells were stored at -80°C until required.

2.5.6. Transformation of competent *Escherichia coli* cells

Suspensions (200 µl) of competent *E. coli* cells plus up to 10 µl of DNA were added to a chilled eppendorf tube and incubated on ice for 30 minutes. The cells were heat shocked at 42°C for 30 seconds and returned to ice for 5 minutes. SOC (800 µl consisting of 2 % tryptone, 0.5 % yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgSO_4$, 10 mM $MgCl_2$, 20 mM glucose) was added and the mixture incubated aerobically at 37°C for 1 hour, before being spread on LB agar supplemented with 5 µg ml^{-1} ampicillin.

2.5.7. Small scale plasmid preparation

Five ml of an overnight culture grown in LB broth supplemented with ampicillin (5 µg ml^{-1}) was harvested by centrifugation at 3 000 rpm in a MSE Centaur 2 benchtop centrifuge, and the pelleted cells resuspended in 100 µl TEG (25 mM tris-Cl pH 8.0, 10 mM EDTA, 50 mM glucose), then 0.2 ml 0.2 M NaOH and 1 % SDS were added. Cells were vortexed and left on ice for 5 minutes, then 0.15 ml 3 M sodium acetate pH 5.0 added. Following 5 minutes incubation on ice, cell debris was removed by centrifugation in a MSE MicroCentaur microfuge at 13 000 rpm, 4°C for 10 minutes. The supernatant was extracted with 0.5 ml phenol-chloroform-isoamylalcohol, and then with 0.5 ml chloroform. DNA was precipitated with 0.9 ml ethanol for 5 minutes at room temperature, followed by centrifugation for 10 minutes at 4°C at 13 000 rpm. Pelleted DNA was dried and resuspended in 50 µl SDW.

2.5.8. Purification of DNA from agarose gels

DNA fragments were purified from agarose gels using the Geneclean II kit (BIO101 Inc., La Jolla, USA). DNA was excised from the gel while being illuminated by long-wave UV light (302 nm). The excised fragment was mixed with 4.5 volumes of 6 M sodium iodide and 0.5 volumes of TBE modifier, then heated at 45°C to 55°C for 5 minutes to dissolve the agarose. GLASSMILK (5 µl) was added and the solution incubated on ice for 5 minutes to allow binding of the DNA to the silica matrix. Bound DNA was pelleted by centrifugation at 13 000 rpm for 5 seconds and subsequently

washed three times with 500 µl of New Wash, centrifuging for 5 seconds each time. DNA was eluted from the GLASSMILK by incubating the pellet at 45°C to 55°C for 5 minutes with 5 to 20 µl SDW. GLASSMILK was removed by centrifugation at 13 000 rpm for 30 seconds and the DNA-containing supernatant retained.

2.5.9. Purification of DNA from acrylamide gels

To visualize DNA the gel was stained for 5 to 10 minutes with 0.2 % (w/v in dH_2O) methylene blue and destained in SDW. DNA was excised from the gel and placed upon silanized glass wool contained in a sealed blue Gilson pipette tip. Elution buffer (300 µl, consisting of 0.5 M ammonium acetate, 1 mM EDTA) was added and the apparatus incubated overnight at 30°C with agitation. Acrylamide was removed by cutting the sealed part off the blue tip, placing the tip in an eppendorf tube and placing this apparatus in a 50 ml Falcon tube, which was centrifuged at 3 000 rpm for 5 minutes in a MSE Centaur 2 benchtop centrifuge. A further 200 µl of elution buffer was added to the tip and the centrifugation repeated. The eluted nucleic acid was phenol-chloroform extracted (Section 2.5.1) and then extracted with ether by vortexing for 1 minute, followed by centrifugation at 13 000 rpm for 3 minutes. The upper ether phase was removed and the lower nucleic acid-containing phase was left at room temperature for 5 minutes to enable evaporation of traces of ether. DNA was concentrated by ethanol precipitation at -80°C for 30 minutes with 45 µl of 3 M sodium acetate pH 5.2, 4.5 µl of 1 M magnesium chloride and 1 ml of ethanol, with the final pellet resuspended in 5-20 µl SDW.

2.5.10. Polyacrylamide gel electrophoresis (PAGE)

PAGE was undertaken using a BioRad PROTEAN II xi 2-D vertical electrophoresis tank, with 15 x 15 cm glass plates separated by 1 mm spacers. A 5 % polyacrylamide gel was prepared using a mixture of; 16.6 ml Easi-Gel (30 % (w/v) acrylamide/ 1.034 % (w/v) bisacrylamide), 20 ml 5 X TBE, 0.7 ml 10 % ammonium persulfate and 62.7 ml dH_2O. Polymerization was achieved by adding 35 µl of TEMED (N, N, N', N'-tetramethylethylenediamine) immediately prior to pouring the gel. Following polymerization (1 hour), the electrophoresis tank was filled with 1 X TBE

and the nucleic acid samples loaded (mixed with one-sixth volume of 6 X loading buffer). Electrophoresis was completed at 100 V for 4-5 hours. Nucleic acids were detected by staining the gel in 0.5 $\mu g\ ml^{-1}$ ethidium bromide in 1 X TBE for 45 minutes at room temperature and visualized using UV light.

2.5.11. Nucleic acid hybridization

Nucleic acid hybridization was completed using a modification of the original method described by Southern (1975).

Nucleic acid preparation

Nucleic acids were separated by electrophoresis on a 0.7 to 1.0 % (w/v) agarose gel in 1 X TBE (Section 2.5.3). Nucleic acids were visualized and photographed using UV light and then denatured by placing the gel in denaturing solution (0.5 M NaOH, 1.5 M NaCl) for 40 minutes at room temperature with gentle shaking. The gel was rinsed and placed in neutralizing solution (0.5 M Tris-Cl pH 7.5, 3 M NaCl) for 40 minutes at room temperature with gentle shaking. Nucleic acids were then transferred to Hybond-N nylon membranes by capillary transfer.

Capillary transfer of nucleic acids to membranes

To enable capillary transfer an inverted, shallow tray was placed in a second tray containing a reservoir of 20 X SSC (3 M NaCl, 0.3 M sodium citrate). A wick was formed on the inverted tray using two sheets of Whatman 3MM filter paper soaked in 20 X SSC, with their ends resting in the reservoir. The inverted gel was placed on the wick (ensuring no trapped air bubbles) and a piece of Hybond-N nylon membrane matching the gel dimensions was placed on top. Two sheets of Whatman 3MM filter paper, cut to gel dimensions, were placed upon the nylon membrane. A 7 cm high stack of absorbent paper towels was rested upon the filter paper and pressed down by a 2 kg weight (Figure 2. 3). Capillary transfer proceeded overnight, following which the nylon membrane was removed and nucleic acids fixed to the membrane, either by baking for 2 hours at 80°C or by UV crosslinking for 2 minutes on a UV Stratalinker 1800 (Stratagene) at 254 nm.

Low stringency hybridization

Fixed membranes were placed in rollers containing 25 ml of pre-hybridization solution (7.5 ml 20 X SSC, 1.25 ml Denhardts solution, 1.25 ml 10 % SDS, 0.5 ml of 1

mg ml^{-1} denatured salmon sperm, 14.5 ml dH_2O) and incubated for 1 hour at 42°C in a Techne Hybridization HB-1 oven. The pre-hybridization solution was replaced with 25 ml of fresh pre-hybridization solution and the radiolabelled probe (Section 2.5.12) added. Hybridization was completed at low stringency (42°C) overnight. Membranes were then washed firstly in 2 X SSC, 0.1 % SDS for 10 minutes at room temperature, and secondly in 1 X SSC, 0.1 % SDS for 10 minutes at 42°C. Membranes were removed and wrapped in Saran wrap prior to autoradiography.

Autoradiography

Membranes, wrapped in Saran wrap, were placed within autorad cassettes, with a sheet of Agfa Curix X-ray film. Cassettes were placed at -70°C, with intensifying screens, for 4 to 48 hours. Films were developed using a Compact X2 automatic developer (X-Ograph Ltd., Wilts,UK).

2.5.12. Nucleic acid radioloabelling

Random primed labeling of DNA

This method was first described by Feinberg and Vogelstein (1983;1984). Radiolabelling was completed using the Boehringer Mannheim "High Prime DNA Labelling Kit". Contaminating RNA and proteins were cleaned from the DNA solutions as described in section 2.5.8, prior to radiolabelling. DNA (8 µl) to be labeled was denatured at 100°C for 10 minutes and chilled quickly on ice , then mixed with 4 µl high prime reaction mixture (random primers, Klenow polymerase, 5X reaction buffer in glycerol), 1 µl dATP, 1 µl dGTP, 1 µl dTTP and 5 µl α-^{32}P dCTP (50 µCi; 3 000mCi $mmol^{-1}$). The reaction was incubated at 37°C for 10 minutes and stopped by addition of 2 µl 0.2 M EDTA pH 8.0. Sterile dH_2O (180 µl) was added prior to purification of radiolabelled DNA.

Purification of radiolabelled nucleic acid

Radiolabelled DNA was separated from unincorporated radio-nucleotides using a Sephadex G-50 (Pharmacia) matrix size exclusion column. This was prepared by plugging a 1.0 ml syringe with siliconized glass wool and filling the syringe with Sephadex G-50 in TE buffer (1 M tris-Cl, 0.5 M EDTA adjusted to pH 7.5). The syringe was placed through a hole in the lid of a 50 ml Falcon tube and an eppendorf

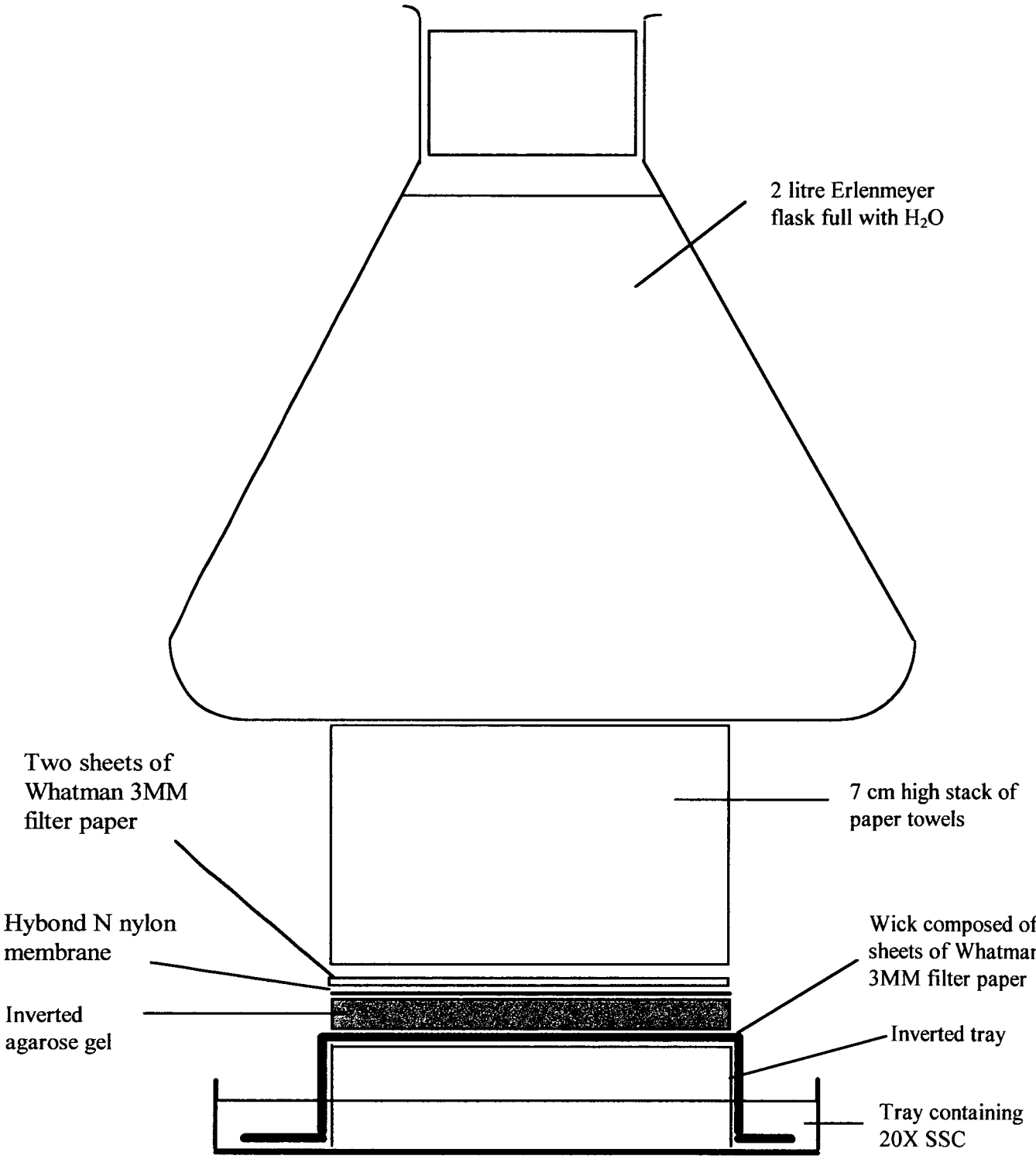


Figure 2. 3 Assembly of capillary blot apparatus.

tube placed at the bottom of the syringe. The unpurified radiolabelled probe was added to the column, and centrifuged at 3 000 rpm for 4 minutes in a MSE Centaur 2 benchtop centrifuge. The radiolabelled probe was contained in the eluate collected in the eppendorf tube. To check that labeling was successful, the radioactivity was checked using a Geiger counter (> 1 x 10^8 cpm μg^{-1}).

2.5.13. Preparation of cDNA from *Rhizoctonia solani* dsRNA

First and second strand synthesis

dsRNA (1 µg) was purified from a polyacrylamide gel (Section 2.5.9) and denatured by incubation for 20 minutes at 37°C with 4 µl 100 mM methyl mercuric hydroxide and 6 µl DEPC-treated SDW. Subsequently 1.5 µl RNasin (RNase inhibitor) was added and the methyl mercuric hydroxide was inactivated by incubation at room temperature for 15 minutes with 2 µl 100 mM β-mercaptoethanol. First-strand synthesis was completed at 37°C for 1 hour using 1 µl BRL random primers, 30 µl DEPC-treated SDW, 10 µl 5 X first strand buffer, 2.5 µl 10 mM dNTPs and 2.5 µl MMLV (Moloney murine leukemia virus) reverse transcriptase. Second strand synthesis was completed at 15.5°C for 2 hours using 25 µl of first strand reaction, 20 µl polymerase I buffer, 146.75 µl DEPC-treated SDW, 3.25 µl dNTP mix, 5 µl polymerase I.

Purification of cDNA

Nucleic acids were purified using the Gene Clean II kit (BIO101 Inc., La Jolla, USA). Samples from the second strand synthesis were mixed with 100 µl of 6 M sodium iodide and 5 µl GLASSMILK then incubated for 5 minutes at room temperature to allow binding of the DNA to the silica matrix. Bound nucleic acid was pelleted by centrifugation for 5 seconds at 13 000 rpm and subsequently washed three times with 400 µl New Wash, centrifuging for 5 seconds between washes. DNA was eluted from the GLASSMILK by incubating the pellet at 45 to 55°C with 20 µl DEPC-treated SDW. GLASSMILK was removed by centrifugation at 13 000 rpm for 30 seconds, and the nucleic acid containing supernatant retained. This was mixed with 20 µl 'One Phor All' buffer, 59 µl DEPC-treated SDW and 1 µl RNase H and incubated for 20 minutes at 37°C and the nucleic acid purified by phenol extraction (Section 2.5.1) and ethanol precipitation (Section 2.5.2) before being used in ligation reactions.

Blunt ended ligation

pUC18 was used as a vector for the cDNA insert. pUC18 (2 µl) was prepared for ligation by digesting with *Sma*I for 1 hour at 30°C in the following: 23 µl SDW, 3 µl 10 X buffer A, 2 µl *Sma*I. The reaction was stopped by heating at 60°C for 10 minutes. Ligation was completed by mixing 3 µl SDW, 10 µl cDNA insert, 3 µl *Sma*I digested pUC18, 2 µl 10 X ligation buffer and 2 µl T4 DNA ligase. Reactions were stopped by heating at 70°C for 10 minutes. Resulting plasmids were transformed (Section 2.5.6) into competent DL-5 *E. coli* cells and spread on LB agar supplemented with 40 µl X-Gal (20 mg ml^{-1}), 4 µl IPTG (100 mM) and 25 µl ampicillin (10 mg ml^{-1}).

2.5.14. Nuclease sensitivity tests

To confirm that the extracted nucleic acids were dsRNA, nuclease sensitivity tests were completed. Sample aliquots were incubated in each of the following. (i) SAM buffer (50 mM sodium acetate, 5mM magnesium sulphate, pH 5.0) with 20 µg/ ml RNase-free pancreatic DNase I. (ii) High salt buffer (10 mM Tris-Cl, 0.3 M NaCl, pH 7.2) with 20 µg/ ml pancreatic RNase A. (iii) Low salt buffer (10 mM Tris-Cl pH 7.2). Each sample was incubated at 30°C for 30 minutes, then phenol extracted (Section 2.5.1) and ethanol precipitated (Section 2.5.2), prior to agarose gel electrophoresis (Section 2.5.3). To confirm the dsRNA nature, the nucleic acid should be resistant to both DNase activity and RNase activity at high salt concentration. However, incubation with RNase at low salt concentration should result in degradation of dsRNA.

CHAPTER 3

CELLULOSE DEGRADATION BY *RHIZOCTONIA SOLANI*

3.1. Introduction

Cellulose forms a major constituent of the plant cell wall (Figure 3. 1), so cellulolytic enzymes are likely to have a crucial role in the pathogenicity of *R. solani* (Barker and Walker, 1962; Bateman, 1963a). Despite this, few studies on the cellulolytic activities of *R. solani* have been completed. The first of these studies by Blair (1943) examined eleven isolates, all of which failed to grow on cellulose filter paper moistened with a complete solution of nitrogen and mineral salts. This was attributed by Garrett (1956) to be due to a low 'inoculum potential'. Several further studies have demonstrated cellulolytic activity of *R. solani* either in soil or in pure culture; Tribe (1960) showed that *R. solani* could colonize cellophane film buried in cultivated soil; Bateman (1964b) demonstrated decomposition of cotton fibres; Garrett (1962) showed that *R. solani* could decompose filter paper and Chung *et al.* (1988) showed that hardwood bark compost did not support *R. solani* growth, whereas bark compost supplemented with 20 % (w/w) cellulose did support growth. Although these studies demonstrate that *R. solani* possesses cellulolytic activity, the individual studies utilized only a few isolates; for example Garrett (1962) used only three isolates (from swede, potato and lettuce), whereas Bateman (1964b) used only one isolate. However, *R. solani* is a diverse species complex comprised of twelve anastomosis groups (AGs) (Cubeta and Vilgalys, 1997), with each AG being equivalent to a separate biological species (Anderson, 1982; Ogoshi, 1987; Vilgalys and Cubeta, 1994; Adams, 1996), many of which preferentially infect different crops (Ogoshi, 1987). The few isolates for which cellulolytic activity has been examined are not categorized into anastomosis groups, despite the anastomosis group concept being introduced in 1937 by Schultz (1937). The isolates were from a diverse array of crops e.g. bean, potato and swede, and thus are likely to represent several AGs, possibly AG 1, 3 and 2-1 respectively. Since cellulolytic enzymes probably have a role in the early stages of plant infection, there may be variations in cellulolytic activity between anastomosis groups with differing host ranges.

In view of the potential variation in phenotype, both between and within individual AGs, cellulolytic activity of a wide range of isolates from differing AGs was assessed to determine whether cellulolytic activity is consistently associated with AG or

host range of the isolates. In addition, the cellulolytic activity of homokaryons, compared with their parental heterokaryotic isolate was examined.

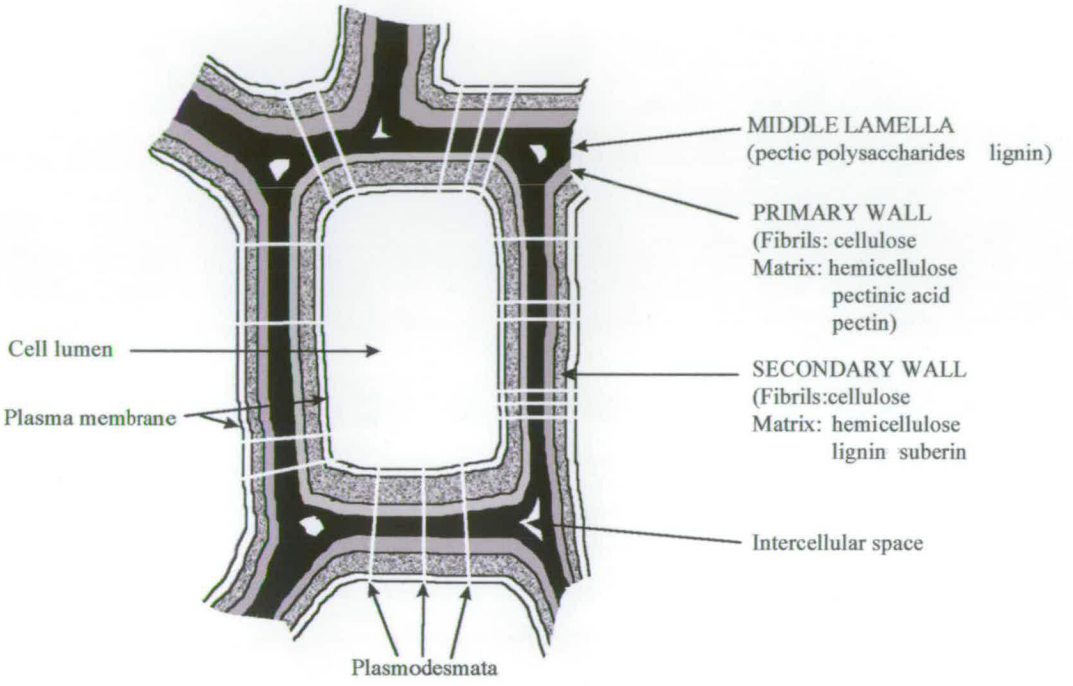


Figure 3. 1 Diagrammatic representation of the plant cell wall, indicating basic composition of different layers.

3.2. Materials and Methods

Cellulolytic activity was assessed as described in Section 2.4.10 for diverse *R. solani* isolates. Stacks of five filter paper discs were inoculated in the presence of various mineral nutrients containing different nitrogen-sources and levels of calcium. Isolates examined were from a range of AGs and sources, as indicated in Table 2.1. In addition, 29 homokaryotic strains denoted *1 to *30 were assessed for cellulolytic activity. These isolates were derived from single basidiospores of the heterokaryotic *Thanatephorus cucumeris* (*R. solani*) isolate 1R4 (AG-1), and were provided by M.C. Julian (CSIC, Madrid). Homokaryons were used to generate several heterokaryotic isolates as indicated in Section 2.4.11.

3.3. Results

3.3.1. Effect of Ca^{2+} on cellulose degradation

Calcium was found not to be essential for *R. solani* growth (Tyner and Sanford, 1935); however, since calcium accumulates at plant surface lesions sites (Bateman and Lumsden, 1965), the presence of calcium may either enhance or decrease the cellulolytic activity of *R. solani*. For three isolates, representing AG 2-2, 4 and 5, cellulose degradation was examined with either nitrate or asparagine as nitrogen source, in the presence or absence of 3.4 meq Ca^{2+} (Section 2.4.10). The addition of calcium to asparagine-containing medium had no significant effect on the cellulolytic activity of strains 04R22 (AG 4), 05R01 (AG 5) and GR1 (AG 2-2) (Table 3. 1). The presence of calcium in nitrate-containing media significantly ($P < 0.02$) enhanced cellulolysis by isolate 05R01, but had no effect on the cellulolytic activity of isolates 04R22 and GR1. Calcium was not included in further studies of cellulolytic activity.

	Cellulolytic activity (mg cellulose respired over 22 days) on various supplementary nutrients*			
Strain	**NO_3**	**$NO_3 + Ca^{2+}$**	**Asn**	**Asn + Ca^{2+}**
04R22	152.2 ± 19.6	171.5 ± 15.1	229.9 ± 18.6	233.0 ± 17.8
05R01	109.2 ± 11.4	158.3 ± 8.7	146.0 ± 7.8	155.0 ± 10.8
GR1	37.1 ± 3.9	38.8 ± 1.2	133.6 ± 38.3	140.1 ± 39.1

* Means of 4 replicates ± SEM

Table 3. 1 Cellulolytic activity (mg cellulose respired over 22 days) of three *Rhizoctonia solani* strains incubated on filter paper with nitrate (NO_3) or asparagine (Asn) in the presence or absence of 3.4 meq Ca^{2+}.

3.3.2. Effect of nitrogen source on cellulose degradation

Cellulose degradation was examined in the presence of either asparagine or nitrate as nitrogen source for strains 04R22, 05R01 and GR1 (Table 3. 1). All three isolates degraded significantly ($P < 0.05$) more cellulose in the presence of asparagine than of nitrate. The largest response was that of isolate GR1 (AG 2-2) which grew poorly on nitrate nitrogen, respiring 37.1 ± 3.9 mg cellulose over 22 days compared with 133.6 ± 38.3 mg cellulose respired over 22 days on asparagine as the nitrogen

source. Further studies encompassing isolates from ten anastomosis groups indicate that overall there was a highly significant ($P < 0.001$) increase in cellulose degradation in the presence of asparagine, compared with nitrate, as nitrogen source (Table 3. 2). However, the responses of individual isolates varied considerably: some isolates showed much higher activity with asparagine than nitrate (e.g. isolates 01R01, VR5, 3R41, 09R01), whereas others showed no response (e.g. 01R03) and occasionally the activity was less in the presence of asparagine than nitrate (e.g. 01R02, 2tR105). Within anastomosis groups, all four isolates of AG 2-2 showed more cellulolytic activity in the presence of asparagine compared with nitrate, with overall means of 150.5 and 32.1 mg cellulose respired over 22 days respectively. The same trend was observed for all four AG 3 isolates. In contrast, all four isolates of AG 2-1 showed no significant increase in cellulose degradation on asparagine compared with nitrate, with the mean levels of degradation for the four isolates being 121.5 and 142.7 mg on asparagine compared with nitrate.

The heterokaryotic strain 1R4 showed no significant difference in cellulose degradation in the presence of asparagine as opposed to nitrate (Table 3. 3). The thirty homokaryotic isolates (*1 to 30) derived from strain 1R4 can be split into three categories with respect to cellulose degradation in the presence of nitrate compared with asparagine (Table 3. 4). (i) Some showed enhanced cellulose degradation in the presence of asparagine compared with nitrate. (ii) Some resembled the parental strain 1R4 and showed no significant difference between asparagine and nitrate. (iii) Some isolates showed significantly decreased activity in the presence of asparagine as opposed to nitrate. The majority of isolates (*1-3, 5, 7, 9-18, 22-24, 26) fell into the first category, showing a highly significant increase ($P < 0.001$) in cellulose degradation with asparagine as opposed to nitrate. Of the remaining isolates, eight (*6, 8, 19, 20, 25, 29, 30) fell into the second category showing no significant difference between nitrate and asparagine. The remaining three isolates (*21, 27, 28) fell into the third category of displaying a significant reduction in cellulose degradation in the presence of asparagine compared with nitrate.

Strain	Anastomosis group	Cellulolytic activity (mg cellulose respired over 22 days)*	
		NO_3 as nitrogen source	Asparagine as nitrogen source
01R01	1A	69.5 ± 17.1	168.3 ± 50.1
01R02	1B	191.6 ± 30.1	110.1 ± 33.3
01R03	1C	207.3 ± 14.2	228.0 ± 70.0
21R01	2-1	69.2 ± 17.8	82.0 ± 27.8
21-14	2-1	135.1 ± 16.7	179.4 ± 21.8
21-41	2-1	154.0 ± 8.3	138.4 ± 14.3
21-F16L	2-1	127.8 ± 13.2	171.0 ± 24.3
2tR105	2t	49.8 ± 9.4	14.5 ± 7.1
2tR118	2t	56.0 ± 19.0	90.7 ± 26.3
2tR144	2t	89.0 ± 16.3	158.5 ± 6.5
2t124	2t	56.2 ± 18.5	75.3 ± 16.9
2t101	2t	81.0 ± 8.4	146.6 ± 30.9
GR1	2-2	29.7 ± 4.9	152.4 ± 36.5
22R02	2-2	23.5 ± 8.5	54.3 ± 6.6
VR5	2-2	30.6 ± 10.4	211.0 ± 37.6
BI2	2-2	44.6 ± 11.7	184.4 ± 11.7
23R01	2-3	30.1 ±8.7	88.2 ± 25.4
3R04	3	32.4 ± 13.1	68.6 ± 20.7
3R08	3	133.2 ± 14.2	160.3 ± 15.2
3R09	3	43.8 ± 8.7	94.7 ± 28.7
3R41	3	57.8 ± 10.0	278.4 ± 36.8
04R22	4	144.2 ± 23.5	277.3 ± 51.3
4/51	4	245.1 ± 6.0	288.8 ± 18.2
4/41	4	168.2 ± 35.2	197.8 ± 46.1
Me8-2	4	157.8 ± 8.1	107.3 ± 10.1
521	4	216.4 ± 23.0	255.5 ± 30.9
05R01	5	142.4 ± 8.5	185.9 ± 7.1
06R01	6	87.9 ± 7.0	54.2 ± 4.5
07R01	7	136.7 ± 16.1	178.4 ± 15.1
08R01	8	31.4 ± 3.7	75.2 ± 9.8
09R01	9	122.5 ± 16.4	335.4 ± 41.8
11R01	11	24.4 ± 9.9	52.7 ± 3.6

* Means of 4 replicates ± SEM

Table 3. 2 Cellulolytic activity (mg cellulose respired over 22 days) of *Rhizoctonia solani* strains incubated on filter paper with nitrate or asparagine, as nitrogen source, in the absence of a calcium supplement

	Cellulolytic activity (mg cellulose respired over 22 days) of strain 1R4	
Replicate	**NO_3 as nitrogen source**	**Asparagine as nitrogen source**
1	158.6	224.3
2	177.7	200.2
3	150.8	215.1
4	233.4	200.9

Table 3. 3 Cellulolytic activity (mg cellulose respired over 22 days) of the *Rhizoctonia solani* heterokaryotic strain 1R4 when incubated on cellulose filter paper plus either sodium nitrate or asparagine and mineral nutrients.

3.3.3. Variation of cellulose degradation between and within AGs

Examination of cellulolytic activity from differing anastomosis groups indicates that isolates from all the anastomosis groups tested, i.e. AG 1, 2-1, 2t, 2-2, 2-3, 3 to 9 and 11, are able to degrade cellulose, in the presence of either sodium nitrate or asparagine as sole nitrogen source (Table 3. 2). The level of cellulose degradation showed a significant ($P < 0.001$) degree of variation, both in the presence of asparagine and nitrate, ranging from 14.5 ± 7.1 to 335.4 ± 41.8 and from 23.5 ± 8.5 to 245.1 ± 6.0 mg cellulose respired over 22 days respectively. Within each anastomosis group where more than one isolate was studied (i.e. AG 1, 2-1, 2t, 2-2, 2-3, 3 and 4) the individual isolates showed no consistent level of cellulose degradation when asparagine was present as nitrogen source. The variation was greatest in AG 2t, where cellulolytic activity for isolate 2tR105 was less than a tenth of that for isolate 2tR144, and not so pronounced in anastomosis groups 1 and 2-1, where only a two-fold difference between isolates was observed. When sodium nitrate was incorporated as nitrogen source, with the exception of AG 2t and AG 2-2, again variability was displayed between isolates of the same anastomosis group. Within AG 2t and AG 2-2, the isolates show a consistently low level of cellulolytic activity, with mean levels of 66.4 and 32.1 mg cellulose respired over 22 days respectively. Sodium nitrate therefore does not enable efficient cellulose breakdown in AG 2t and AG 2-2.

Strain	Cellulolytic activity (mg cellulose respired over 22 days)†	
	NO_3 as nitrogen source	Asparagine as nitrogen source
Group 1		
*1	97.0 ± 3.7	182.1 ± 37.4
*2	113.6 ± 5.4	167.8 ± 42.7
*3	100.0 ± 8.6	158.6 ± 4.2
*5	53.7 ± 4.6	116.4 ± 12.0
*7	65.4 ± 3.6	121.2 ± 6.7
*9	121.5 ± 15.9	230.6 ± 5.6
*10	108.1 ± 2.8	224.4 ± 32.8
*11	146.6 ± 12.6	225.0 ± 11.6
*12	158.4 ± 1.3	227.0 ± 5.2
*13	99.0 ± 12.4	207.5 ± 47.8
*14	138.1 ± 4.3	301.0 ± 3.2
*15	77.4 ± 8.4	189.8 ± 28.0
*16	144.6 ± 15.2	192.3 ± 21.8
*17	118.9 ± 11.4	188.1 ± 38.6
*18	116.6 ± 15.8	217.9 ± 35.2
*22	169.1 ± 6.6	252.8 ± 12.4
*23	174.7 ± 11.9	230.2 ± 8.3
*24	131.6 ± 8.4	198.2 ± 21.8
*26	162.8 ± 20.5	204.4 ± 33.4
Group 2		
*6	57.8 ± 3.4	61.0 ± 4.8
*8	107.3 ± 9.3	123.4 ± 24.4
*19	264.5 ± 39.6	247.1 ± 32.5
*20	179.9 ± 20.5	199.8 ± 13.8
*25	150.7 ± 17.7	176.2 ± 36.2
*29	259.9 ± 32.2	253.9 ± 64.2
*30	112.1 ± 6.7	122.2 ± 2.9
Group 3		
*21	163.1 ± 23.6	117.3 ± 16.0
*27	123.3 ± 8.5	13.0 ± 7.3
*28	218.6 ± 21.5	145.4 ± 53.7

† Means of 4 replicates ± SEM

Table 3. 4 Cellulolytic activity (mg cellulose respired over 22 days) of *Rhizoctonia solani* homokaryotic strains, derived from the parental strain 1R4, incubated on cellulose filter paper plus either sodium nitrate (NO_3) or asparagine (Asn) and mineral nutrients. Isolates are split into three groups: Group 1 showed enhanced cellulose degradation in the presence of asparagine as opposed to nitrate, group 2 isolates showed no significant difference in cellulose degradation between asparagine and nitrate mineral nutrients and group 3 showed significantly decreased activity in the presence of asparagine as opposed to nitrate.

3.3.4. Variation of cellulose breakdown between homokaryons derived from a single heterokaryotic isolate

Many field isolates of *R. solani* are assumed to be heterokaryons (Flentje and Stretton, 1964; Flentje *et al.*, 1970; Anderson *et al.*, 1972; Bolkan and Butler, 1974), with the basidiospores formed by many isolates being binucleate, so they are able to maintain a heterokaryotic condition (Flentje *et al.*, 1963). Given appropriate environmental conditions, heterokaryons can be induced to produce ellipsoidal, uninucleate basidiospores, which germinate to form colonies displaying diversity of colour and patterns of sclerotium formation (Whitney and Parmeter, 1963; Bolkan and Butler, 1974). Cellulolytic activity between homokaryotic strains *1 to *30, generated from a single heterokaryotic strain, was examined to determine their variability in cellulolytic activity. The homokaryotic isolates displayed a diverse array of phenotypes, with respect to sclerotia colour and distribution (Figure 3. 2).

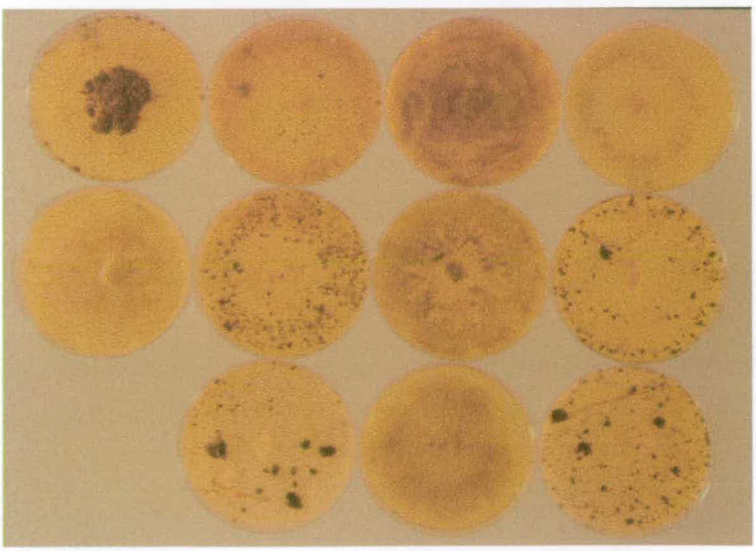


Figure 3. 2 Phenotypic appearance of homokaryotic isolates derived from the heterokaryotic isolate 1R4, following 28 days incubation at 23°C.

Each homokaryotic isolate derived from the heterokaryotic strain 1R4 was able to degrade cellulose (Table 3. 4), both in the presence of asparagine and of nitrate. Cellulolytic activity varied significantly ($P < 0.001$) between isolates, ranging from 13.0 ± 7.3 to 301.0 ± 3.2 on asparagine and from 53.7 ± 4.6 to 264.5 ± 39.6 mg cellulose respired over 22 days on nitrate.

3.3.5. Cellulose degradation by reconstituted heterokaryotic strains

Homokaryotic isolates *14 and *19 displayed high cellulolytic activity on asparagine (Table 3. 4) at 301 ± 3.2 and 247 ± 32.5 mg cellulose respired over 22 days respectively, whereas isolates *21 and *7 displayed low cellulolytic activity on asparagine, with 117.3 ± 16.0 and 121.2 ± 6.7 mg cellulose respired. Isolates *7, *14, *19 and *21 were paired together in all possible combinations on potato dextrose charcoal agar (PDCA) to generate heterokaryons (Section 2.4.11). Heterokaryon formation is characterized by tuft formation at the boundaries of opposing colonies on PDCA (Figure 3. 3). This is controlled, in AG 1 and AG 4, by two closely linked genetic loci termed the H-factor for heterokaryon incompatibility factor (Anderson *et al.*, 1972). Isolates which carry H factors differing at one or both loci are able to form heterokaryons, whereas isolates that have the same H-factors are unable to form heterokaryons. Of the isolates examined, heterokaryons were formed by the following combinations (Table 3. 5): *21 with either *19 or *14; *7 with either *19 or *14. Thus *19 and *14 evidently contain the same H-factors, which differ from the H factors of *7 and *21. Isolates *7 and *21 both contain the same H-factors. The reconstituted heterokaryons were then tested for cellulolytic activity, as described in Section 2.4.10. Heterokaryon formation was confirmed using the test proposed by Adams and Butler (1982). The heterokaryons generated fell into two broad categories: high and low, with respect to their cellulolytic activity (Table 3. 6). Heterokaryons 21*19 (1), 21*19 (2) and 7*19 displayed high cellulolytic activity of more than 286 mg cellulose respired over

22 days, whereas heterokaryons 21*14, 7*14 (1) and 7*14 (2) displayed low cellulolytic activity of less than 80 mg cellulose respired over 22 days.

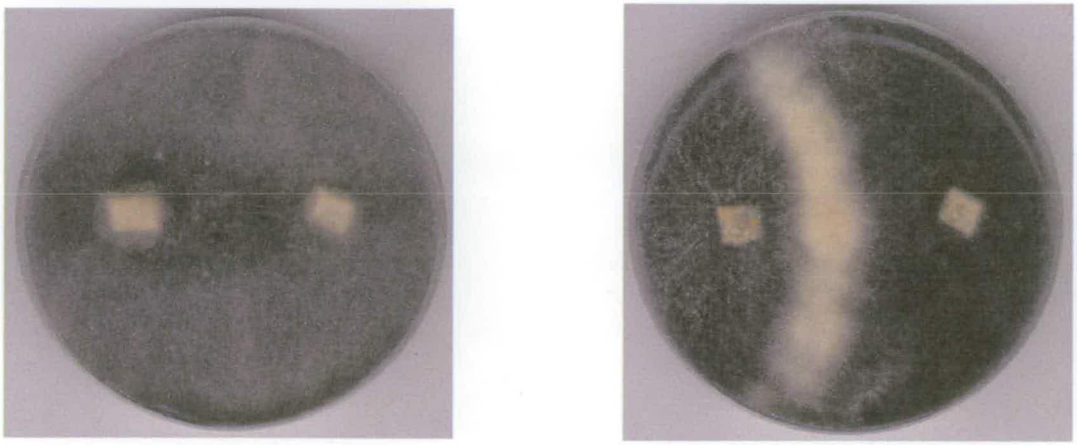


Figure 3. 3 Heterokaryon formation on potato dextrose charcoal agar (PDCA). (a) No heterokaryon formation, as indicated by the lack of tuft formation, following pairing of two identical homokaryons (*14 with *14) from the same sexual compatibility group and (b) heterokaryon formation indicated by tuft formation at colony boundaries, following pairing of two isolates (*7 with *14) from opposing sexual compatibility groupings.

Homokaryon strains	Celluloytic activity†	Heterokaryon formation
*21 x *14	L x H	Yes
*7 x *14	L x H	Yes
*7 x *19	L x H	Yes
*21 x *19	L x H	Yes
*19 x *19	H x H	No
*14 x *14	H x H	No
*19 x *14	H x H	No
*7 x *7	L x L	No
*21 x *21	L x L	No
*7 x *21	L x L	No

† H = High cellulolytic activity (*14 = 301.0 and *19 = 247.1 mg cellulose degraded over 22 days)
L = Low cellulolytic activity (*21 = 117.3 and *7 = 121.7 mg cellulose degraded over 22 days)

Table 3. 5 Combinations of homokaryons used to generate heterokaryons on potato dextrose charcoal agar (PDCA), with an indication of their cellulolytic activity in the presence of asparagine; high activity displayed by isolates *14 and *19 and low activity by isolates *21 and *7.

Heterokaryon strain	Cellulolytic activity (mg cellulose degraded over 22 days)*
HIGH	
21*19 (1)	286.4 ± 40.4
21*19 (2)	288.9 ± 17.1
7*19	314.6 ± 25.6
LOW	
21*14	63.0 ± 2.7
7*14 (1)	65.9 ± 9.8
7*14 (2)	79.2 ± 10.8

* Mean of 4 replicates ± SEM

Table 3. 6 Cellulolytic activity of reconstituted heterokaryotic strains on asparagine-containing medium (mg cellulose degraded over 22 days).

3.4. Discussion

Cellulose is composed of chains of β (1,4) linked glucose molecules which form crystalline fibres held together by hydrogen bonds. These are generally embedded within a matrix of lignin and hemicelluloses within the plant cell wall (Figure 3. 1). Degradation of cellulose requires a complex of enzymes, collectively termed "cellulase", which, in fungi, are categorized into three major groups (Beguin, 1990): (i) cellobiohydrolases which degrade cellulose stepwise from the non-reducing end, generating cellobiose subunits, (ii) endoglucanases which randomly cleave internal β-glucosidic bonds of cellulose molecules and (iii) β-glucosidases which hydrolyze cellobiose subunits and other low molecular weight cellodextrins. The overall cellulose degradation pathway is summarized in Figure 3. 4. Cellulose degradation is repressed in most organisms, including *R. solani*, by the presence of high concentrations of readily metabolized carbon sources such as glucose (Weinhold and Bowman, 1974; Merivuori *et al.*, 1984; Johnson *et al.*, 1985). A secondary level of control often exists. For example, in *Trichoderma* sp. cellulase synthesis is induced by cellulose degradation compounds, such as cellobiose or sophorose (2-0-β-D-glucopyranosyl-D-glucose). These are generated from cellulose by low levels of cellulases and β-glucosidases which are produced constitutively (Stewart and Leatherwood, 1976; Kubicek, 1987).

Previous reports (Tribe, 1960; Barker and Walker, 1962; Garrett, 1962; Bateman, 1964a; Chung *et al.*, 1988) indicate that *R. solani* can be induced to degrade cellulose, although individual studies examined only one or two isolates. The work of this chapter examined isolates from all the major anastomosis groups of *R. solani*, except for AG 10 and the bridging isolate AG B1. It was shown that all isolates degrade filter paper cellulose to a certain extent. However, the degree of cellulolytic activity varied considerably between the isolates. In the presence of sodium nitrate as sole nitrogen source, activity ranged from 29.7 to 245.1 mg cellulose respired over 22 days, and substituting sodium nitrate with asparagine resulted in cellulolytic activity ranging from 14.5 to 335.4 mg cellulose respired over 22 days.

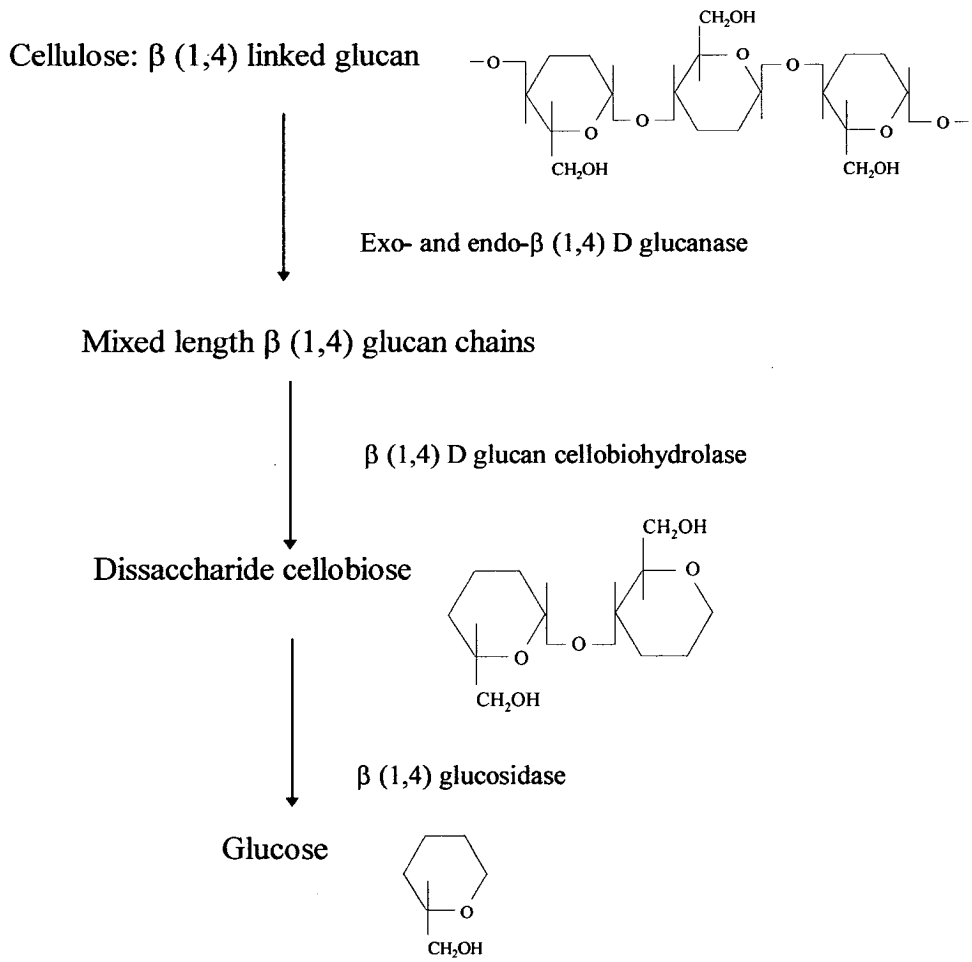


Figure 3. 4 Cellulose degradation by fungi.

Within any single anastomosis group, there was considerable variation in cellulolytic activity (with either nitrate or asparagine). For example, there was more than two fold variation between strains in AG 2-1 or in AG 4, more than three fold variation between AG 2 strains from tulip plants, almost four fold variation within AG 2-2 and more than four fold within AG 3. This level of variation within AGs was at least as great as the variation between AGs. The levels of cellulolytic activity and the degree of variation are consistent with previous studies. Daniels (1963) examined 14 isolates for cellulolytic activity on cellulose filter paper, cellulose film and flax fibres and found a high degree of variation between isolates as well as in ability to decompose different cellulose sources. Matsumoto (1921) examined six isolates and found considerable variation in celluloytic activity.

The levels of cellulolytic activity obtained are, however, fairly low for cellulolytic organisms. In comparison, *Rhizoctonia oryzae* degraded approximately 375 mg cellulose over the same time interval (Mulligan, 1993). Although this is higher than the levels obtained for the majority of *R. solani* isolates, it is low in comparison to many thermophilic organisms such as *Chaetomium thermophile* which shows a weight loss of approximately 1200 mg cellulose over a 3 week period (Deacon, 1985). The levels obtained for *R. solani* are consistent with low-activity cellulose degraders such as *Humicola grisea* and *Fusarium* sp. which degrade approximately 150 mg cellulose over a 22 day period (Mulligan, 1993). The *Rhizoctonia* isolates which are endophytes of orchids may be expected to have high cellulolytic activity. These isolates degrade cellulose and translocate the soluble carbon released into orchid seedlings enabling them to grow (Anderson and Rasmussen, 1996). However, no endophytes were examined in the present study.

McCabe (1994) reported that isolates of *R. solani* show much higher levels of cellulolytic activity than those in the present study. Following four weeks incubation on nitrate-containing media, four isolates were reported to have cellulolytic activity of 1114.2 ± 161.2, 917.6 ± 52.4, 994.3 ± 25.4 and 1153.1 ± 166.5 mg cellulose respired, whereas two isolates respired 310.9 ± 26.6 and 382.1 ± 20.0 mg. McCabe attributed these differences to the presence or absence of dsRNA within the isolates. The two isolates showing low cellulolytic levels contained dsRNA, whereas the four isolates showing high cellulolytic activity were reportedly cured of dsRNA by serial subculturing

of hyphal tips. Several isolates included in the present study contain dsRNA elements; for example isolates 021-14, 021-41, 2tR118, 3R8, 3R9, Me8-2, 521, 04R22, 05R01 (V. Rubio, pers. comm.; p. 91, chapter 4). The isolates displayed cellulolytic activity which varied five fold, ranging from 43.8 to 216.4 mg cellulose respired. The levels obtained were much lower than those reported by McCabe (1994) for dsRNA-free isolates. No isolates reported as being dsRNA-free were included in the present study, so no comparison with the high cellulolytic levels obtained by McCabe (1994) can be made.

The fluctuations in cellulolytic activity between isolates may be the result of alterations in mycelial branching patterns. Using interference-microscopy, Isaac (1964) reported that cellulose degradation occurs around short, branched hyphae in the older parts of the mycelium, but not in the trunk hyphae, nor in the younger hyphae. Similar results were obtained by Daniels (1963). The short branch hyphae bear some resemblance to the highly branched infection cushions formed during plant attack (Dodman and Flentje, 1970). Thus differences in cellulolytic activity may be due to variations in the control of branching by different isolates. The degree of hyphal branching is partially affected by temperature. Some isolates show higher degrees of branching with increasing temperatures, whereas others show decreased branching with increased temperature. Since the temperature was maintained at 27°C throughout the present study, differing isolates may show varying degrees of branching depending on their optimal growth temperatures. In addition, since the short, branched hyphae resemble infection cushions, their formation might be triggered by plant host exudates. These exudates also enhance cellulase release from the fungal cell wall (Lisker *et al.*, 1975a,b). Thus the cellulolytic activities obtained in these *in vitro* studies may be much lower than levels induced *in vivo*.

The different nitrogen sources supplied in this study led to different levels of cellulolytic activity of the isolates examined. In general, nitrogen sources greatly influence utilization of a given carbon source, such that fungi can be divided into three, or possibly four, categories with respect to nitrogen utilization (Lilly, 1965): Class 1 fungi utilize organic nitrogen, ammonium, nitrite, nitrate and were suggested to fix atmospheric nitrogen, although no unequivocal evidence has been reported regarding

dinitrogen fixation by fungi (Garraway and Evans, 1984); class 2 fungi utilize nitrate, nitrite, ammonium and organic nitrogen; class 3 fungi utilize only ammonium and organic nitrogen and class 4 fungi only grow using organic nitrogen. The majority of fungi, including *R. solani*, belong to class 2 and can grow efficiently using either organic or inorganic sources of nitrogen (Sherwood, 1970; Garraway and Evans, 1984). The *Rhizoctonia* endophytes of the orchid *Arundia chinensis* seem to be one exception in that they can utilize ammonium and organic nitrogen, but not nitrates, and thus belong to the class 3 category (Stephen and Fung, 1971). The lack of nitrate utilization usually results from an inability to synthesize nitrate reductase (Whitaker, 1976). *R. solani*, as a species, utilizes both inorganic and organic sources of nitrogen; however, the majority of isolates examined in this study showed varying degrees of cellulolytic activity depending on the nitrogen source present. The AG 2-2 isolates grew poorly on sodium nitrate compared with asparagine. This may be a specific response to sodium nitrate in terms of cellulose utilization or may be an indication of a general inability of AG 2-2 isolates to utilize nitrates effectively. In most isolates examined (27 out of 32) organic nitrogen supplied as asparagine increased cellulose degradation compared with degradation in the presence of nitrate nitrogen. Variation in nutrient assimilation depending on the nitrogen source is widespread in fungi, with no individual nitrogen source being better or worse for every isolate (Akai *et al.*, 1960). For example, Deshpande (1959) examined the ability of *R. solani* to utilize various nitrogen sources in minimal media containing $MgSO_4$, KH_2PO_4 and glucose as a carbon source. Nitrogen sources included peptone, asparagine, ammonium nitrate, ammonium sulphate, ammonium chloride, sodium nitrate and calcium nitrate. Growth of *R. solani* was obtained in all cases, with the greatest yield on peptone (172 mg dry weight) and the least on ammonium chloride (36 mg dry weight). Greater growth occurred on media containing asparagine (150 mg dry weight) as nitrogen source than on sodium nitrate (99 mg dry weight), as was observed for the majority of isolates in this study where cellulose was the sole carbon source. In *Leucostoma persoonii*, a similar trend is observed where asparagine enhances growth compared to sodium nitrate, with mean dry weights obtained of 339.9 ± 26.5 mg and 167.7 ± 19.8 mg respectively (Jensen and Adams, 1995).

The differences in nutrient assimilation depending on the nitrogen source can sometimes be explained by alterations in pH level during growth. Nitrogen sources such

as ammonium, whose uptake results in increased acidity of the medium can inhibit growth unless the medium is well buffered (Deshpande, 1959), whereas nitrates usually serve as a "sponge", mopping up free protons and reducing equivalents, such that the pH rises.

The ability to metabolize a given nitrogen source can affect the pathogenicity of fungal isolates, with specific amino acids having an inhibitory or stimulatory effect (van Andel, 1966; Weinhold *et al.*, 1969; Huber and Watson, 1974). For example, ripening blueberry fruits contain an increased content of histidine, which reportedly makes them more susceptible to infection by *Glomerella cingulata* (Stretch and Cappelini, 1965), whereas potato tubers grown at low potassium levels become more resistant to infection by *Phytophthora infestans* due to a reportedly increased arginine content of the tubers (Alten and Orth, 1941). Several reports on *R. solani* indicate that pathogenicity is increased in response to greater asparagine and ammonia levels, compared with pathogenicity when nitrate is present (Glynne, 1951; Papavizas and Davey, 1960; Huber and Watson, 1970). This present study shows that the majority of isolates have increased cellulolytic activity in the presence of asparagine compared to nitrate, which may, in part, explain the increased pathogenicity reported by the above authors for *R. solani*. Pectolytic enzyme activity may also be enhanced by levels of amino acids, thus acting synergistically with cellulolytic enzymes to enhance pathogenicity.

The response of pathogenicity-related enzymes to varying levels of amino acids may be crucial in host specificity of fungal pathogenicity. In response to stress and pathogen attack, nitrogen levels within plants undergo large changes. Many plants show large increases in asparagine levels (e.g. Steward *et al.* (1959); Nowakowski and Byers (1972)). However many other alterations can occur. For example, rice plants attacked by leaf hoppers (*Nilaparrata lugons* Stal.) show great increases in asparagine, arginine, lysine, proline and tryptophan content (Cagampang *et al.*, 1974), whereas proline accumulates in chlorine-deficient plants, showing a fifty-fold increase in cauliflower (Freney *et al.*, 1959). Thus the host specificity of *R. solani* may be related, in part, to differences in inhibitory/ stimulatory amino acids released by plants during infection, and these may in turn affect cellulolytic/ pectolytic enzyme activities. In *Rhizopus* fruit rot, cellulolytic and pectolytic enzymes have been demonstrated to be inhibited by a specific form of nitrogen (Spalding, 1969). In *Rhizoctonia*-incited diseases, isolates of AG 2-2 characteristically infect Chenopodaceae, which contain high levels of glycine betaine

(Storey *et al.*, 1977) which may trigger enhanced cellulolytic activity and account for the specificity. In the present study, nitrates supported particularly poor cellulolytic activity in AG 2-2 isolates, compared with isolates from other AGs. This may partially explain the results obtained by Elmer (1997), who reported decreased pathogenicity of *R. solani* AG 2-2 on table beets (*Beta vulgaris*), following the addition of calcium nitrate. A further example of infection being related to amino acids is reported for tomato varieties and infection by differing races of *Cladosporium* (Lowther, 1964). In the tomato variety Potentate, high levels of glutamine and sucrose are present, making Potentate highly susceptible to *Cladosporium*, whereas other tomato varieties contain low levels of sucrose and glutamine and are resistant to infection.

Many fungi show enhanced growth in the presence of calcium (Garraway and Evans, 1984). For example, calcium at 500 ng per litre results in a three-fold increase in the growth of *Saccharomyces carlsbergensis* (Lotan *et al.*, 1976). Reports on calcium requirements of *R. solani* are conflicting. Tyner and Sanford (1935) concluded that calcium was not essential for growth of *R. solani*, whereas Young and Bennet (1922) reported calcium ions to be indispensable for growth. However, since calcium is highly abundant at infection sites (Bateman, 1963a; Bateman, 1964b), calcium ions were included in initial trials to determine their effect on cellulolytic activity. The addition of calcium to asparagine-containing medium had no significant effect on the cellulolytic activity of the three isolates (04R22, 05R01 and GR1) examined. The presence of calcium in nitrate-containing media enhanced cellulolytic activity by isolate 05R01, but had no effect on cellulolytic activity of isolates 04R22 and GR1. In some species, the extent to which calcium is required has been shown to be dependent on the composition of the growth medium. For example, in studies on *Phytophthora parasitica*, calcium is absolutely required for growth when nitrates are the sole nitrogen source, but in the presence of L-asparagine as nitrogen source, calcium is not required, even though its presence enhances growth (Hendrix and Guttman, 1970). Thus since calcium was not an absolute requirement, it was not incorporated in further studies. In the presence of sodium nitrate, calcium enhances growth by some isolates. This may potentially be due to alterations in the branching patterns of some isolates in response to calcium. Mycelium of *Fusarium graminearum* growing in the presence of 14 nM calcium were highly branched in comparison to isolates grown at other calcium concentrations

(Robson *et al.*, 1991). Since Isaac (1964) reported that cellulolytic activity was localized around terminal, short branched hyphae when *R. solani* is grown on cellulose filter paper, it may be that induction of additional branching in response to calcium is responsible for the increase in cellulolytic activity observed by some isolates. The short, branched hyphae observed by Isaac (1964) were similar to the branching formation observed to form during infection cushion formation, where calcium levels are high.

In order to further investigate the variation in cellulolysis by *R. solani*, a heterokayotic field strain (1R4) of AG 1 was examined in addition to 29 homokaryotic, single spore isolates (SSI) of strain 1R4. These SSI showed diverse variation in phenotype with respect to colour, growth rate, abundance of aerial mycelia and sclerotial morphology. Similar morphological variations in SSI have been reported by several authors (Sims, 1960; Whitney and Parmeter, 1963; Flentje and Stretton, 1964; Papavizas, 1965). For example Hawn and Vanterpool (1953) reported that growth rates of SSI varied from a trace to 12.5 mm day^{-1}, compared with 13 mm day^{-1} for the heterokaryotic parent. Papavizas (1965) obtained similar results for the growth rates of sixty SSI from one isolate of *Pellicularia praticola*, with rates varying from 0.03 to 0.71 mm $hour^{-1}$. In the present study, the cellulolytic activity of the heterokaryotic parent isolate was good on both nitrate and asparagine. The levels of activity of the SSI ranged from 54 to 247 and from 13 to 254 mg cellulose respired on nitrate and asparagine, respectively. The SSI could be divided into three categories regarding their cellulolytic activity: (i) those which showed enhanced cellulose degradation in the presence of asparagine compared with nitrate (ii) those which resembled the parental strain 1R4 and showed no significant difference between asparagine and nitrate and (iii) isolates showing significantly decreased activity in the presence of asparagine as opposed to nitrate. Papavizas and Ayers (1965) reported similar variation in pectic enzyme activity amongst SSI. For example, pectin methylesterase activity of isolate R118-42 was tenfold that of R118-43 and four fold that of the parental isolate R118. Virulence of SSI was also highly variable, but no relation between enzyme activity and virulence was observed. This immense variation in morphology, cellulolytic activity and pectolytic activity amongst SSI is likely to arise during basidiospore formation. *R. solani* cells are multinucleate, containing 2 to 18 nuclei (Domsch *et al.*, 1980). Prior to basidia formation, the nuclei in the vegetative cells pair, and the pairs become separated by

septa. These binucleate cells form the basidia. Within the basidium, the nuclei fuse, forming a diploid, which undergoes meiosis to form haploid nuclei, which separate to form four uninucleate basidiospores (Flentje *et al.*, 1970). These basidiospores show variation in their phenotype. In addition, aberrations can occur during basidiospore formation. For example recombination frequently occurs during meiosis, which leads to increased variation in the phenotype of the basidiospores. Mutation and the presence of any transposable genetic elements further increase the degree of variation observed in basiodiospore progeny.

Once basidiospores are formed, they can be paired to reform heterokaryotic isolates. Heterokaryon formation is controlled by two closely linked loci termed the H-factor (Anderson *et al.*, 1972). Isolates which carry H factors differing at one or both loci are able to form heterokaryons, whereas isolates that have the same H factors are unable to form heterokaryons. The present study, which examined homokaryotic isolates *7, *14, *19 and *21 found two groups; isolates *19 and *14 contained one type of H factors, whereas isolates *7 and *21 contained a second set of H factors. Heterokaryons were formed in the following combinations: 21*14, 7*14, 7*19 and 21*19. Heterokaryon formation was confirmed using the test proposed by Adams and Butler (1982). Cellulolytic activity, when determined for the reconstituted heterokaryons, led to the recognition of two groups: (a) high cellulolytic activity of isolates 21*19(1), 21*19(2), 7*19 and (b) low cellulolytic activity of isolates 21*14, 7*14(1), 7*14(2). Thus for cellulolytic activity the heterokaryons tend to resemble the phenotype of one of the contributing SSI. This is in contrast to results obtained by Whitney and Parmeter (1963) who found that the re-formed heterokaryons were culturally distinct from those of the contributing SSI. Cubeta *et al.* (1993) similarly obtained re-formed heterokaryons that were morphologically distinct from either SSI. In addition Whitney and Parmeter (1963) found that their re-formed heterokarons displayed characteristics resembling those of the original parental heterokaryon. The work in this study contradicts this, in that the re-formed heterokaryons differed in their cellulolytic activity not only from each other, but also from the original parental heterokaryon. Field isolates are assumed to be heterokaryotic (Flentje and Stretton, 1964; Flentje *et al.*, 1970; Anderson *et al.*, 1972; Bolkan and Butler, 1974). If re-formed heterokaryons resemble the original parental isolates as reported by Whitney and Parmeter (1963), then this would tend to decrease the variation in field isolates, and thus

their survival ability. However, the present study indicates that re-formed heterokaryons can differ from parental heterokaryons in their cellulolytic activity and this may in part account for the enormous variation in cellulolytic activity of field isolates.

R. solani has been demonstrated by several authors to grow successfully as a saprotroph in soil (Blair, 1943; Tribe, 1960; Garrett, 1962; Chung *et al.*, 1988), and can increase its biomass and production of sclerotia at the expense of soil organic matter (Christias and Lockwood, 1973). Cellulose is the most abundant utilisable organic compound in soil (Alexander, 1964) and *R. solani* utilizes this for saprotrophic growth, translocating the obtained nutrients throughout the mycelial network. This saprotrophic growth in soil and over the host surface forms an important precursor to infection by *R. solani* (Hayman, 1969). In stunting diseases of cereals, by *R. solani* AG 8, the extensive mycelial network is thought to facilitate infection, giving rise to the characteristic "bare patch" symptoms in crops. Since *R. solani* utilizes cellulose for saprotrophic growth, these *in vitro* cellulolytic trials may give some indication of the saprotrophic ability of isolates. For example, AG 8 isolates may potentially show high cellulolytic activity, to enable extensive growth upon decaying cereal residues prior to infection. However, the AG 8 isolate examined in this study had poor cellulolytic activity on both asparagine and nitrate. Drawing any assumptions about the relationship between cellulolytic activity and saprotrophic ability from this study should be done with caution, as the saprotrophic ability of *R. solani in vivo* is regulated by several factors. The saprotrophic ability of *R. solani* depends on a variety of factors including non-biotic soil determinants such as temperature, moisture, soil aeration etc. and the ability to outcompete other soil micro-organisms, which is in turn dependent upon resistance to toxins and antibiotics produced by other organisms. Rao (1959) reported that *R. solani* shows poor competitive saprotrophic abilities compared with other root parasites, partially due to low tolerance of antifungal agents. In the absence of competition, *R. solani* is a highly efficient saprotroph. Garrett (1956) indicates the saprotrophic ability is positively correlated to growth rate. This is supported by Papavizas (1964) who examined several SSI and found that the saprotrophic ability was correlated to the growth rate. The SSI examined were highly variable in their saprotrophic ability and Papavizas hypothesized that "cellulose decomposition may vary from very feeble to very strong". This indeed has been shown to be the case for the SSI examined in the present study, with cellulolytic

activity ranging from 13.0 to 253.9 mg cellulose respired over 22 days on asparagine mineral nutrients.

In vivo saprotrophic activity decreases in the presence of abundant cellulose, due to poor competitive 'saprotrophic' ability of *R. solani* (Chung *et al.*, 1988), with other highly active cellulolytic organisms out-competing *R. solani*. Once cellulose levels decline, then *R. solani* shows good saprotrophic growth due to decreased competition. Several other saprotrophic organisms show similar growth phases. For example mushroom spawn will not 'run' satisfactorily until composts have undergone the initial stages of decomposition in which saprotrophic sugar fungi are present and highly active (Garrett, 1962).

Papavizas and Davey (1961) found that saprotrophic growth was slow when cellulose was the sole organic carbon source. This activity, though, could be enhanced by the addition of sodium nitrate and calcium. In the present *in vitro* studies, cellulolytic activity was generally less in the presence of sodium nitrate compared with asparagine, and calcium did not significantly increase the cellulolytic activity.

CHAPTER 4

CHARACTERIZATION OF dsRNA-CONTAINING ISOLATES

4.1. Introduction

Mycoviruses (dsRNA elements) are present within several phytopathogenic fungal species and are subdivided into four groups (Ghabrial, 1998): *Totiviridae*, *Partiviridae*, *Hypoviridae* and those remaining unclassified. In many cases, the presence of dsRNA alters the fungal phenotype. For example, in *Ophiostoma ulmi* the presence of specific dsRNA elements have been correlated with a "diseased" phenotype of the host. These isolates display reduced growth rate, poor virulence, abnormal "amoeboid" morphology, impaired sexual reproduction, reduced production of cerato-ulmin and alteration in pigmentation (Brasier, 1983; Sutherland and Brasier, 1997). In *Cryphonectria parasitica*, dsRNA alters the phenotype to a weakly virulent (hypovirulent) state, characterized by lower pigmentation, sporulation and virulence, enabling dsRNA to be used successfully as a biocontrol agent (reviewed by Nuss, 1992). *R. solani* has been reported by several authors to harbour dsRNA (Castanho *et al.*, 1978; Zanzinger *et al.*, 1984; Finkler *et al.*, 1985; Bharathan and Tavantzis, 1990; Bharathan and Tavantzis, 1991; Kousik *et al.*, 1994; Jian *et al.*, 1997; Jian *et al.*, 1998). However, there is much controversy over whether dsRNA is consistently associated with increased or decreased virulence. Castanho *et al.* (1978) reported dsRNA to be associated with hypovirulence, whereas Finkler *et al.* (1985) associated dsRNA with increased virulence. Several other studies found no consistent correlation between the degree of virulence and the presence of dsRNA (Zanzinger *et al.*, 1984; Bharathan and Tavantzis, 1990; Kousik *et al.*, 1994). It is likely that certain dsRNA segments lead to increased virulence, whereas other segments result in the hypovirulent phenotype. Thus, for dsRNA to be used in a biocontrol strategy against *R. solani*, the segments potentially resulting in hypovirulence must be determined. In addition, for biocontrol to be effective, the dsRNA must be transmissible throughout field populations. Transmission occurs via compatible hyphal anastomosis reactions (Finkler *et al.*, 1988). This transmission may conceivably be enhanced by the mycoparasitic activities of *Verticillium biguttatum*, which forms a "nutrient sink" within the mycelium of *R. solani* (van den Boogert and Deacon, 1994) strong enough to reverse the direction of net cytoplasmic flow (Deacon, 1996). Thus, it may be possible to use the nutrient mobilization effect of

V. biguttatum to "pull" dsRNA through adjoining vegetatively compatible colonies, within a *R. solani* mycelial network.

The work of this chapter examines the dsRNA content of isolates from a single field site and characterizes them with respect to virulence, growth rate, pectic and cellulolytic enzyme activities, phenol oxidase activity and vegetative compatibility reactions. In addition, their susceptibility to mycoparasitism by *V. biguttatum* was examined.

4.2. Results

4.2.1. dsRNA in *Rhizoctonia solani*

4.2.1.1. Isolation of dsRNA

dsRNA was isolated from *R. solani* using a modification of the methods of Morris and Dodds (1979), Hoch *et al.* (1985) and McCabe (1994), as indicated in Section 2.4.12. Total nucleic acid was extracted by homogenization with liquid nitrogen, followed by phenol-chloroform extraction. Nucleic acids were separated using CF 11 cellulose chromatography, in the presence or absence of 15 % ethanol: DNA and ssRNA were eluted in the presence of 15 % ethanol and dsRNA was eluted in the absence of 15 % ethanol. Initial studies lacked liquid nitrogen in the extraction procedure, which resulted in nucleic acid degradation, visualized as "smears" on agarose gels (Figure 4. 1). The visualization of large molecular weight segments was further improved by separation of nucleic acids using polyacrylamide gel electrophoresis (Section 2.5.10) as opposed to agarose gel electrophoresis (Section 2.5.3) (Figure 4. 2).

Isolated nucleic acid was shown to be dsRNA by nuclease sensitivity tests, as described by Kim *et al.* (1990) (Section 2.5.14). dsRNA was resistant to both DNase plus RNase A when used at high salt concentration, but sensitive to RNase A when used at low salt concentration, thus confirming the dsRNA nature (Figure 4. 3). As a further control to confirm that the extraction procedure was repeatable, dsRNA was extracted from *Aspergillus foetidus* IMI 041 871 (Figure 4. 3). Five segments of approximate sizes 5.0, 4.0, 3.5, 2.5 and 2.0 kb were visualized upon agarose gel electrophoresis, which correlated with those described by Banks *et al.* (1970). The dsRNA sizes are, however, approximations since DNA size standards were used throughout this study.

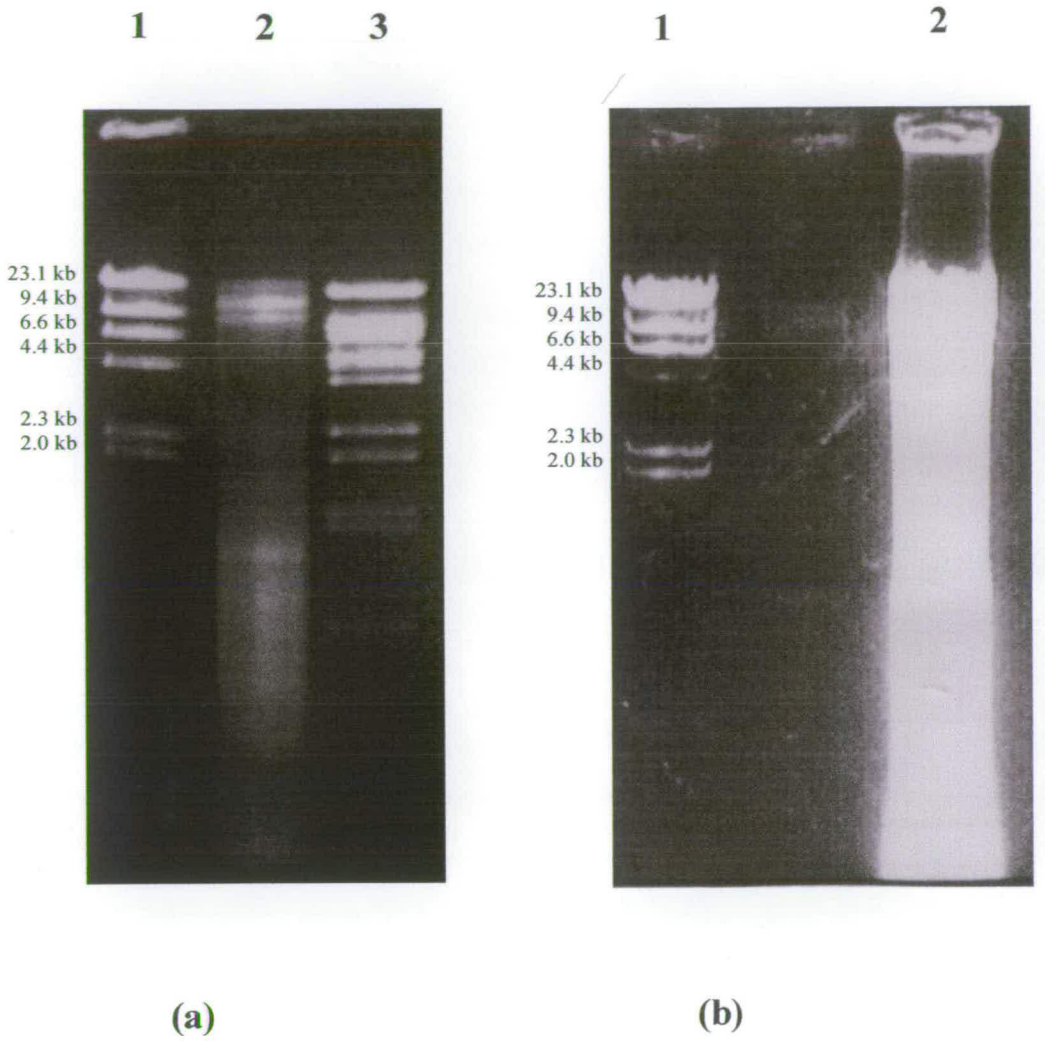


Figure 4. 1 Visualization of dsRNA extracted from *Rhizoctonia solani* and separated by 0.8 % (w/v) agarose gel electrophoresis (a) dsRNA extracted using liquid nitrogen prior to phenol extraction and CF11 cellulose chromatography. Lane 1 shows λ DNA digested with *Hin*dIII; sizes of respective bands are indicated on the left side of the photograph. Lane 2 shows multiple segments of dsRNA extracted from *R. solani* isolate FT8. Lane 3 shows λ DNA digested with *Bst*I. (b) Nucleic acids obtained by phenol extraction followed by CF11 cellulose chromatography, without the inclusion of liquid nitrogen. Lane 1 shows λ DNA digested with *Hin*dIII; sizes of respective bands are indicated on the left side of the photograph. Lane 2 shows degraded dsRNA extracted from *R. solani* following dsRNA extraction without using liquid nitrogen.

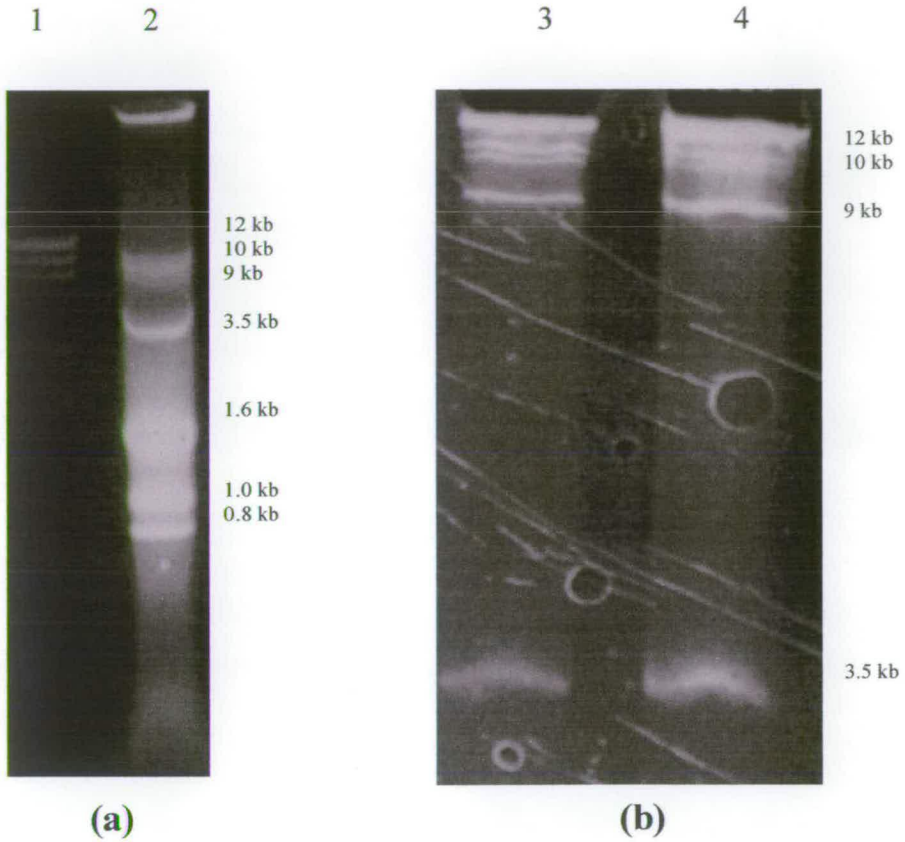


Figure 4. 2 Visualization of dsRNA extracted from *Rhizoctonia solani* isolates FT 203 and FT 204 following separation by (a) 0.8 % agarose gel electrophoresis and (b) polyacrylamide gel electrophoresis. Larger segments are shown with more clarity on polyacrylamide gel electrophoresis compared with agarose gel electrophoresis. Lane 1 contains λ DNA digested with *Hin*dIII. Lane 2 and 3 contain multiple segments of dsRNA extracted from isolate FT 203. Lane 4 shows dsRNA extracted from isolate FT 204. Approximate sizes of segments are indicated to the right of each photograph.

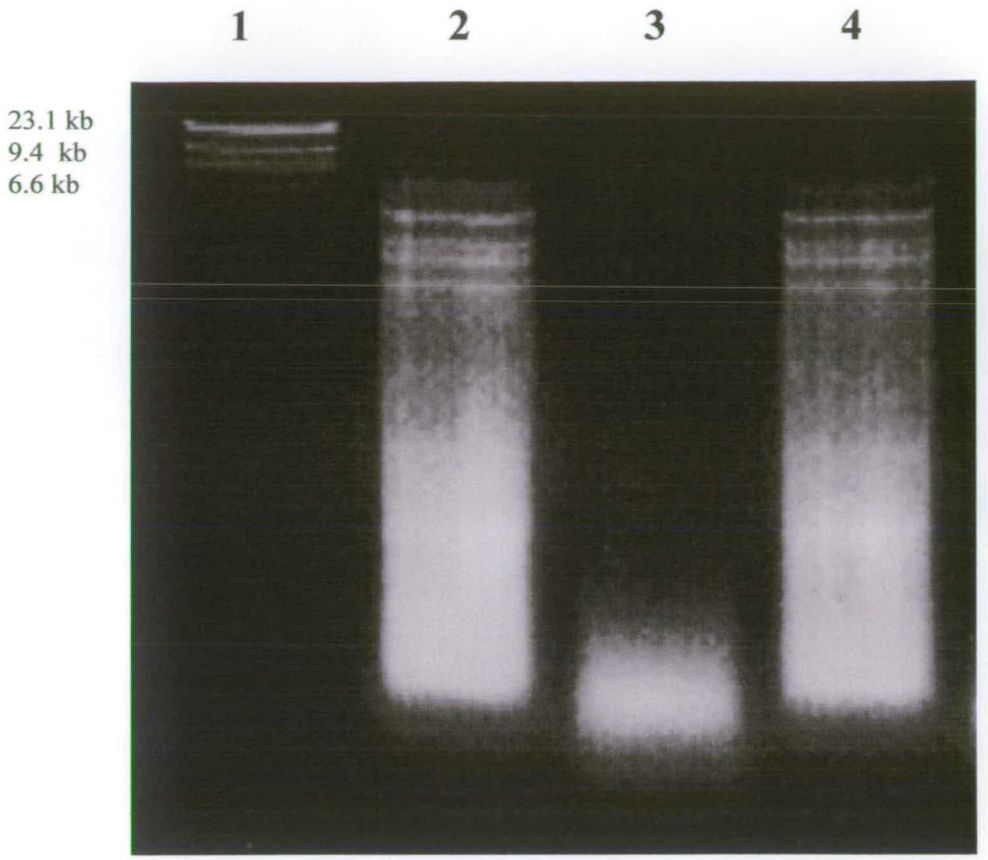


Figure 4. 3 Visualization of nucleic acids extracted from *Aspergillus foetidus* IMI 041 871. Lane 1 shows λ DNA digested with *Hin*dIII; sizes of visible bands are indicated on the left side of the photograph. Lane 2 shows extracted nucleic acids digested with DNase. Multiple segments of dsRNA are visible. Lane 3 shows nucleic acids following RNase A digestion at low salt concentration. DsRNA is susceptible to RNase A degradation at low salt concentration, thus no dsRNA segments are visible. Lane 4 shows nucleic acids following digestion with RNase A at high salt concentration. DsRNA is resistant to digestion with RNase A at high salt concentration, thus multiple dsRNA segments are visible.

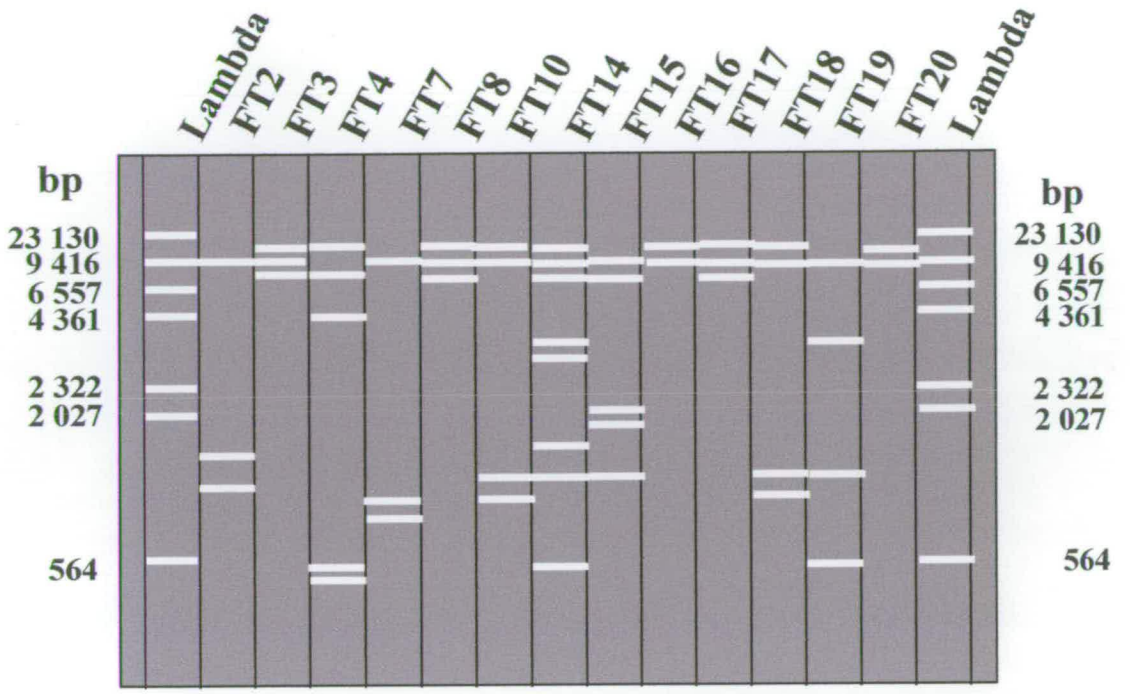


Figure 4. 4 dsRNA segments electrophoresed on a 0.8 % (w/v) agarose gel from *Rhizoctonia solani* AG 3 isolates obtained from a single field site. λ DNA digested with *Hin*dIII was used as a molecular marker, with sizes indicated to each side of the gel. DsRNA was extracted by homogenization with liquid nitrogen, phenol extraction and CF 11 cellulose chromatography. Segments were shown to be dsRNA by nuclease sensitivity.

4.2.1.2. dsRNA in *R. solani* isolates from a single field site

To determine the abundance of dsRNA in natural field isolates of AG 3, strains were isolated from potato tubers harvested from a single field site near Carnoustie, Scotland (Section 2.4.1). DsRNA was subsequently extracted using CF 11 cellulose chromatography (Section 2.4.12). Each isolate examined contained two or more segments of dsRNA (Figure 4. 4). Segments ranged in size from 0.3 to 15 kb, and can be classified into three broad size categories, as proposed by Bharathan and Tavantzis (1990): (i) large (L) being 4.6 kb or greater, (ii) medium (M) in the range 1.1 to 4.5 kb and (iii) small (S) being 0.5 to 1.0 kb. Each isolate contained at least one large (L) segment of dsRNA of a size comparable to the hypovirulence-encoding L-dsRNA of *C. parasitica* (12 712 bp).

4.2.1.3. dsRNA in *R. solani* isolates from a single potato tuber

dsRNA content was examined (Section 2.4.12) in three AG 3 isolates, FT 201, FT 203 and FT 204, harvested from a single potato tuber. All three isolates contained multiple segments of dsRNA (Figure 4. 5); however, isolates varied in the size of segments present. Isolates FT 203 and FT 204 contained segments of the same size, 9.5, 8.0, 6.5, 3.0, 1.6, 1.0 and 0.8 kb, whereas isolate FT 201 lacked the 8.0 and 0.8 kb segments, but contained an extra segment of 1.3 kb size. Again every isolate contained at least one L-dsRNA segment of a size comparable to the hypovirulence-encoding L-dsRNA of *C. parasitica*, previously reported by Shapira *et al.* (1991a).

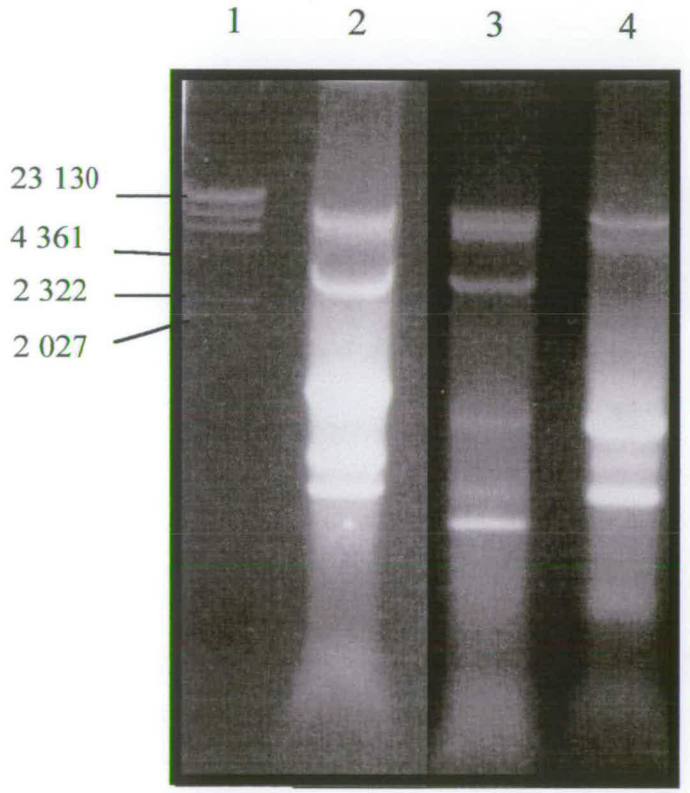


Figure 4. 5 dsRNA segments extracted from *Rhizoctonia solani* AG 3 isolates obtained from a single potato tuber. Ds RNA was separated by agarose (0.8%) gel electrophoresis. Lane 1 shows λ DNA digested with *Hin*dIII; sizes of respective bands are indicated on the left side of the photograph. Lanes 2 to 4 show multiple dsRNA segments extracted from isolates FT 203, FT 204 and FT 201 respectively.

4.2.1.4. dsRNA in *R. solani* isolates of differing anastomosis groups

R. solani isolates examined in Sections 4.2.1.2 and 4.2.1.3 all belonged to AG 3. Isolates of other anastomosis groups were therefore examined to determine whether dsRNA was present in the same abundance. Extraction of dsRNA from the following isolates 021-41 (AG 2-1), 04R22 (AG 4), PA1 (AG 4), Papa (AG 4) and 05R01 (AG 5), indicated that dsRNA was present in all isolates examined (Figure 4. 6). Isolates contained between one and four segments of sizes between 0.8 and 10.0 kb. Each isolate contained one segment of L-dsRNA, which presumably has coding capacity.

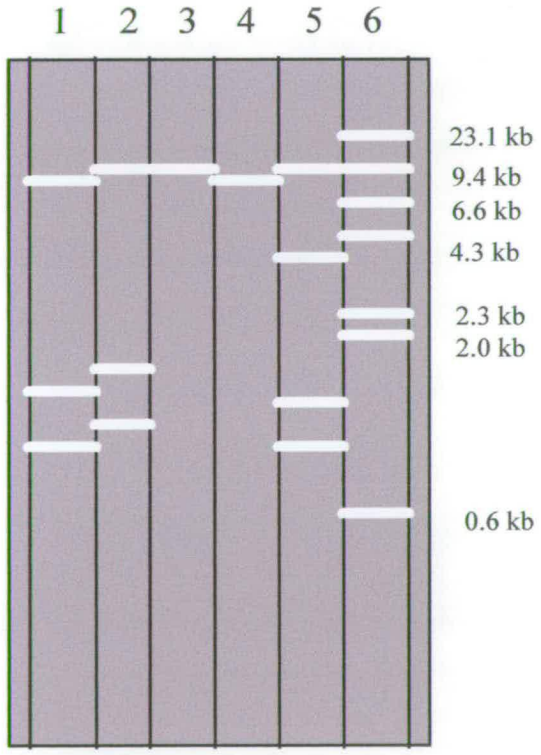


Figure 4. 6 dsRNA segments extracted from *Rhizoctonia solani* isolates of differing anastomosis grouping, visualized following electrophoresis on a 0.8% agarose gel. Lanes 1 to 5 show dsRNA extracted from isolates 04R22 (AG 4), PA1 (AG 4), Papa (AG 4), 05R01 (AG 5) and 021-41 (AG 2-1) respectively. Lane 6 shows λ DNA digested with *Hin*dIII, sizes of respective bands are indicated on the right side of the diagram.

4.2.2. Pathogenicity of dsRNA-containing isolates

Previous reports on dsRNA-containing phytopathogens have linked dsRNA to increased or decreased virulence in the fungal host (Nuss and Koltin, 1990; Castanho *et al.*, 1978). Plant pathogenicity trials were therefore completed on the AG 3 isolates from Carnoustie, to determine the variability in pathogenicity between dsRNA-containing isolates. Trials were completed as described in Section 2.4.6 using the following host plants: tomato (*Lycopersicon esculentum*), radish *(Raphanus sativus*), carrot (*Daucus carota*), lettuce (*Lactuca sativa*), cress (*Barbarea praecox*) and onion (*Allium cepa*). Following 10 days incubation pathogenicity was recorded, on a disease index scale from 0 to 5, based on the relative size of the necrotic area on the root as follows: 0 = no disease; 1 = 1 - 10 %; 2 = 11 - 30 %; 3 = 31 - 50 %; 4 = 51 - 80 % and 5 = entire root infected. Isolates with a mean infectivity between 0 and 1 were considered non-pathogenic.

Examination of roots indicated that for each strain the disease index was low, with the mean index for the six host crops ranging from 1.5 to 2.6, and with an overall mean of 2.0 (Table 4. 1). Analysis of variance indicated no significant differences between strains regarding overall disease severity. The susceptibility of individual hosts to the isolates examined was significantly different ($P < 0.001$). Radish was highly susceptible to infection (mean disease index = 4.5), whereas, overall, isolates were non-pathogenic towards cress seedlings (mean disease index = 0.6).

Infection of shoots was much less severe (Table 4. 2). All isolates were, overall, non-pathogenic to the six host crops, with mean infection levels ranging from 0.1 to 0.5. Analysis of variance indicates no significant difference between strains regarding disease severity on shoots. A significant difference ($P < 0.001$) of susceptibility was observed between host crops; however, they were all scored resistant to *R. solani* infection in the 10 day assay.

	Disease index on host plants*						
Strain	**Carrot**	**Onion**	**Lettuce**	**Cress**	**Tomato**	**Radish**	**Mean**
FT 2	3.3 ± 0.3	0.6 ± 0.3	1.4 ± 0.2	0.4 ± 0.2	0.8 ± 0.2	4.6 ± 0.1	**1.9**
FT 3	1.8 ± 0.3	0.8 ± 0.2	0.8 ± 0.2	0.7 ± 0.2	1.1 ± 0.2	4.0 ± 0.2	**1.5**
FT 4	3.1 ± 0.3	1.2 ± 0.3	1.4 ± 0.2	0.5 ± 0.2	1.7 ± 0.3	3.5 ± 0.2	**1.9**
FT 7	2.4 ± 0.3	1.2 ± 0.3	1.0 ± 0.2	0.4 ± 0.1	0.9 ± 0.2	4.2 ± 0.2	**1.7**
FT 8	2.0 ± 0.3	1.4 ± 0.3	1.2 ± 0.2	0.1 ± 0.1	1.2 ± 0.2	4.7 ± 0.1	**1.8**
FT 10	2.2 ± 0.3	1.5 ± 0.4	1.0 ± 0.2	0.4 ± 0.2	1.7 ± 0.3	4.3 ± 0.2	**1.8**
FT 14	2.6 ± 0.3	1.3 ± 0.4	2.1 ± 0.2	1.6 ± 0.3	1.8 ± 0.3	4.7 ± 0.1	**2.4**
FT 15	2.7 ± 0.3	1.2 ± 0.3	2.4 ± 0.2	0.8 ± 0.2	3.8 ± 0.2	4.8 ± 0.1	**2.6**
FT 17	3.1 ± 0.3	1.5 ± 0.3	1.0 ± 0.2	0.6 ± 0.2	1.4 ± 0.2	4.8 ± 0.1	**2.0**
FT 18	3.6 ± 0.3	1.8 ± 0.4	2.2 ± 0.2	1.5 ± 0.3	1.4 ± 0.3	4.8 ± 0.1	**2.6**
FT 19	2.6 ± 0.3	0.9 ± 0.3	2.6 ± 0.2	0.3 ± 0.1	0.6 ± 0.2	4.5 ± 0.1	**1.9**
FT 20	2.3 ± 0.3	0.5 ± 0.2	1.9 ± 0.2	0.5 ± 0.2	0.7 ± 0.2	4.0 ± 0.2	**1.7**
FT 201	1.9 ± 0.3	0.7 ± 0.2	1.3 ± 0.2	1.0 ± 0.3	1.5 ± 0.3	4.6 ± 0.1	**1.8**
FT 203	3.1 ± 0.3	1.3 ± 0.3	1.2 ± 0.2	0.6 ± 0.2	0.8 ± 0.2	4.8 ± 0.1	**2.0**
FT 204	2.6 ± 0.3	1.2 ± 0.4	1.4 ± 0.2	0.1 ± 0.1	1.2 ± 0.3	4.6 ± 0.1	**1.9**
Mean	**2.6**	**1.2**	**1.5**	**0.6**	**1.4**	**4.5**	**2.0**

* Means of 50 replicates ± SEM

Table 4. 1 Disease index for *Rhizoctonia solani* (AG 3) on roots over six host crops. Disease index was recorded for 50 seedlings after 10 days incubation at 22°C. Disease index was recorded on a scale of 0 to 5; 0 = no disease; 1 = 1-10 %; 2 = 11-30 %; 3 = 31-50 %; 4 = 51- 80 % and 5 = entire root infected. Isolates with a mean infectivity between 0 and 1 were considered non-pathogenic.

	Disease index on host plants*						
Strain	**Carrot**	**Onion**	**Lettuce**	**Cress**	**Tomato**	**Radish**	**Mean**
FT 2	0	0.3 ± 0.2	0.5 ± 0.2	0	0	0.1 ± 0.1	**0.2**
FT 3	0	0.6 ± 0.2	0.1 ± 0.1	0	0.1 ± 0.1	0.4 ± 0.1	**0.2**
FT 4	0	1.4 ± 0.4	0.7 ± 0.2	0	0	0.1 ± 0.1	**0.4**
FT 7	0	0.6 ± 0.3	0.4 ± 0.2	0	0	0.2 ± 0.1	**0.2**
FT 8	0	0.4 ± 0.2	0.6 ± 0.2	0	0	0.2 ± 0.1	**0.2**
FT 10	0	0.8 ± 0.4	0.3 ± 0.1	0	0	0.5 ± 0.2	**0.3**
FT 14	0	1.3 ± 0.4	0.9 ± 0.2	0	0.1 ± 0.1	0.3 ± 0.15	**0.4**
FT 15	0	0.1 ± 0.1	0.6 ± 0.2	0	0.3 ± 0.2	0	**0.2**
FT 17	0	0.2 ± 0.2	1.4 ± 0.3	0	0	0.8 ± 0.2	**0.4**
FT 18	0	0.7 ± 0.3	1.0 ± 0.2	0	0.1 ± 0.1	0.1 ± 0.1	**0.3**
FT 19	0	0.4 ± 0.3	0.9 ± 0.2	0	0	0.5 ± 0.2	**0.3**
FT 20	0	0.3 ± 0.2	2.4 ± 0.3	0	0.1 ± 0.1	0.3 ± 0.2	**0.5**
FT 201	0	0	1.0 ± 0.2	0	0.1 ± 0.1	0.2 ± 0.1	**0.2**
FT 203	0	0.9 ± 0.3	0.3 ± 0.1	0	0	0.3 ± 0.1	**0.2**
FT 204	0	0.4 ± 0.2	0.1 ± 0.0	0	0	0.1 ± 0.1	**0.1**
Mean	**0**	**0.5**	**0.7**	**0**	**0.1**	**0.2**	**0.3**

* Means of 50 replicates ± SEM

Table 4. 2 Disease index of *Rhizoctonia solani* infection on shoots of six potential host species. Disease index was recorded for 50 seedlings after 10 days incubation at 22°C. Disease index was recorded on a scale of 0 to 5; 0 = no disease; 1 = 1-10 %; 2 = 11-30 %; 3 = 31-50 %; 4 = 51- 80 % and 5 = entire shoot infected. Isolates with a mean infectivity between 0 and 1 were considered non-pathogenic.

4.2.3. Pathogenicity-related enzymes of dsRNA-containing isolates

As noted in Section 4.2.2, pathogenicity is reported to be linked to the presence or absence of dsRNA in certain fungal hosts (reviewed by Nuss and Koltin (1990)). Enzymes that have been implicated in pathogenicity include cellulases, pectic lyase, polygalacturonase and phenol oxidases, as described in Section 1.3.2. These were examined for a range of isolates containing multiple dsRNA segments (as described in Section 4.2.1), to seek any consistent associations.

4.2.3.1. Cellulolytic activity

Cellulase is a secondary enzyme in the pathogenic response of *R. solani*, being released following polygalacturonase and pectic lyase (Ayers *et al*, 1966; Bateman, 1970). In *C. parasitica*, cellulase activity is suppressed in the presence of dsRNA (Elliston, 1985; Rigling *et al.*, 1989; Hillman *et al.*, 1990), so cellulolytic activity was examined for twelve of the AG 3 isolates which contained dsRNA. Cellulolytic activity was assayed by inoculating stacks of cellulose filter paper with *R. solani* and subsequently recording the amount of cellulose respired over a 22 day period (Section 2.4.10). All isolates were capable of cellulose degradation (Table 4. 3). This was significantly greater ($P < 0.001$) in mineral nutrients containing asparagine as opposed to nitrates as the sole nitrogen source. Between isolates, in the presence of either asparagine or nitrate there were significant differences in cellulolytic activity at $P < 0.01$ and $P < 0.001$, respectively.

4.2.3.2. Phenol Oxidase Activity

Phenol oxidases are widely distributed in fungi (Matsubara and Iwasaki,1972; Kojima *et al.*, 1990), including *R. solani* (Tolmshoff, 1970), and include the copper-containing laccases (Lerch *et al.*, 1978). These enzymes oxidize a large variety of organic substrates (Bollag *et al.*, 1978; 1988), such as tannic acid and gallic acid, and this can be observed using Bavendamms' tests (Bavendamm, 1928 a, b). Phenol

oxidases are reported to be suppressed in the presence of dsRNA in species such as *C. parasitica* (Rigling *et al.*, 1989) and *Diaporthe ambigua* (Smit *et al.*, 1996), thus dsRNA-containing isolates of *R. solani* were examined for their production of this enzyme. Assays involved inoculating isolates on malt extract agar containing either tannic acid or gallic acid (Section 2.4.9). Production of phenol oxidases on gallic acid agar is indicated by a change in colour of the agar from orange/ brown to dark brown/ black (Figure 4. 7). All fifteen isolates examined grew, and produced a strong colour reaction, on gallic acid agar following four days incubation at 27°C (Table 4. 4). On tannic acid agar, no change in agar colouration occurred (Figure 4. 8) for any isolate examined (Table 4. 4). Isolates grew poorly or not at all on tannic acid agar (Table 4. 4).

	Cellulolytic activity (mg cellulose respired over 22 days)*	
Strain	**NO_3 as nitrogen source**	**Asparagine as nitrogen source**
FT 2	123.3 ± 7.6	187.6 ± 4.1
FT 3	51.4 ± 8.1	119.9 ± 15.9
FT 4	60.9 ± 9.5	160.3 ± 7.2
FT 7	94.8 ± 17.8	144.6 ± 24.7
FT 14	63.3 ± 7.8	106.6 ± 5.1
FT 17	120.9 ± 28.1	152.9 ± 35.9
FT 18	118.2 ± 31.9	239.4 ± 19.9
FT 19	112.4 ± 15.3	181.0 ± 4.4
FT 20	63.0 ± 22.1	159.6 ± 10.9
FT 201	72.2 ± 5.6	150.2 ± 12.8
FT 203	44.9 ± 7.5	130.7 ± 24.5
FT 204	40.9 ± 4.7	134.0 ± 19.1

* Means of four replicates ± SEM

Table 4. 3 Cellulolytic activity (mg cellulose respired over 22 days) of *Rhizoctonia solani* (AG 3) strains incubated on filter paper with nitrate (NO_3) or asparagine (Asn) as nitrogen source.

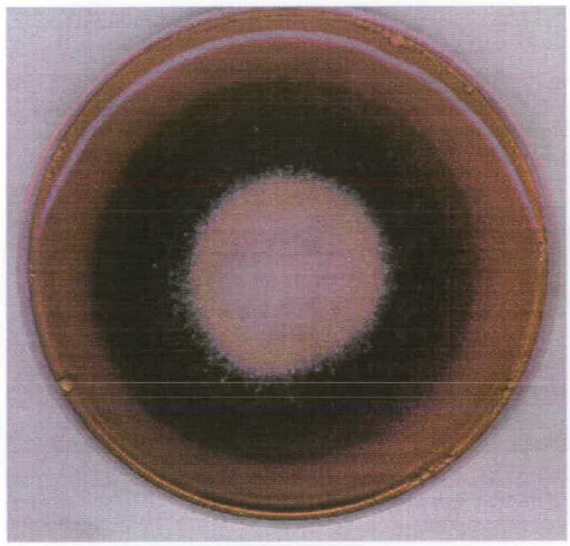


Figure 4. 7 Growth of a *Rhizoctonia solani* AG 3 isolate on gallic acid agar for four days at 27°C. The change in agar colour from orange/ brown to dark brown indicates phenol oxidase production.

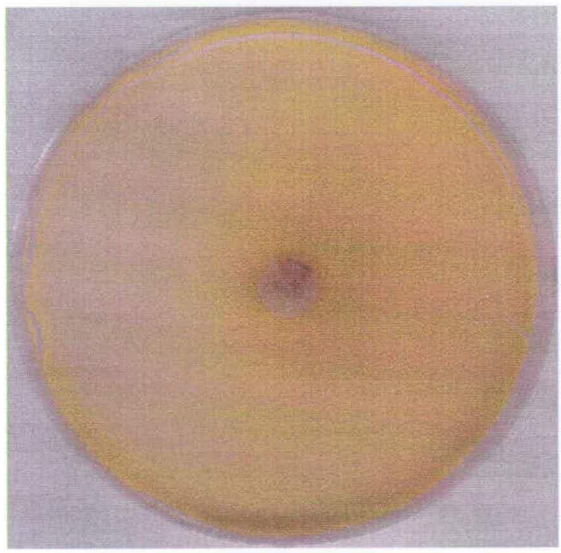


Figure 4. 8 Inoculation of *Rhizoctonia solani* AG 3 isolates on tannic acid agar resulted in little or no growth following 14 days incubation at 27°C. Alteration of agar colour, which would indicate phenol oxidase production, was not observed.

Strain	Inoculation on gallic acid agar		Inoculation on tannic acid agar	
	Colony diameter (mm) after 4 days incubation	Colour reaction	Radial growth (mm) after 14 days incubation	Colour reaction
FT 2	60	+	4	0
FT 3	42	+	4	0
FT 4	8	+	0	0
FT 7	44	+	2	0
FT 8	55	+	0	0
FT 10	57	+	4	0
FT 14	60	+	4	0
FT 15	45	+	2	0
FT 17	36	+	0	0
FT 18	48	+	2	0
FT 19	55	+	4	0
FT 20	8	+	0	0
FT 201	48	+	6	0
FT 203	57	+	4	0
FT 204	27	+	0	0

Table 4. 4 Colony diameter of *Rhizoctonia solani* AG 3 isolates on gallic acid and tannic acid agar following 4 and 14 days incubation, at 27°C, respectively. A colour change in the agar indicated the production of phenol oxidases in response to gallic acid or tannic acid.

4.2.3.3. Pectic enzyme production

Pectic enzymes are the first enzymes produced during pathogenic attack by *R. solani* and are responsible for "tissue maceration" (Bateman, 1963a). Their production *in vitro* is readily observed using pectic zymograms. This technique detects polygalacturonase, pectin lyase and pectin esterase by differential staining of pectin-containing polyacrylamide gels with ruthenium red. Polygalacturonase activity is detected as clear zones in the gel, pectin lyase activity is observed as yellow or clear zones surrounded by a yellow fringe, and pectin esterase is observed as zones which stain darker than the background colour of the gel.

Cultures were incubated for 10 days in broth containing 1 % citrus pectin to induce pectic enzymes. Culture filtrates, in addition to pure enzymes as controls, were loaded onto zymograms containing 1 % citrus pectin (Section 2.4.8). On these zymograms, only polygalacturonase could be detected, with minor differences observed between isolates (Figure 4. 9). The presence of 1 % citrus pectin in the culture broth resulted in the residual pectin reducing the resolution of the zymograms (Cruikshank and

Wade, 1980), so the concentration of citrus pectin was reduced from 1.0 % to 0.1 % in the culture media. This resulted in greater resolution, with the isolates being split into two groups regarding pectic enzyme production (Figure 4. 10). Filtrates of isolates FT 7, FT 20 and FT 203 formed two bands of polygalacturonase/ pectin lyase activity and one band of pectin esterase activity. The remaining isolates (FT 2, FT 3, FT 4, FT 8, FT 10, FT 14, FT 15, FT17, FT 18, FT 19, FT 201 and FT 204) produced three bands of polygalacturonase/ pectin lyase activity and a pectin esterase band. Control reactions could not distinguish between polygalacturonase and pectin lyase activity, as no yellow fringe was observed around the zone of clearing for pectin lyase activity.

4.2.3.4. Pectic enzyme activity

The zymograms of Section 4.2.3.3 indicated the presence or absence of pectic enzymes, but did not distinguish between pectin lyase and polygalacturonase production, so enzyme assays were completed. Polygalacturonase activity was determined using spectrophotometry to measure the increase in reducing groups with dinitrosalicyclic acid (Urbanek *et al.*, 1975; Marcus and Shejter, 1983) following degradation of sodium polypectate to oligogalacturonic acid and galacturonic acid. Pectin-lyase activity was determined by following the appearance of Δ 4,5-unsaturated bonds following the cleavage of α-1,4 glycosidic linkages of citrus pectin (Byrde and Fielding, 1968). Cultures were incubated for five to nine days in broth containing either sodium polypectate or citrus pectin, to measure polygalacturonase or pectin lyase activity respectively (Section 2.4.8). Control reactions consisted of cultures incubated in broth containing glucose, which represses pectic enzyme activity (Weinhold and Bowman, 1974). Each day, an aliquot of culture supernatant was removed, and tested for production of polygalacturonase and pectin lyase using the thiobarbituric acid assay. All isolates produced both pectin lyase (Table 4. 5) and polygalacturonase (Table 4. 6). Pectin lyase activity was generally low, being significantly ($P < 0.001$) less than polygalacturonase activity. No significant difference was observed between isolates, for either pectin lyase or polygalacturonase activity over the five to nine day period. Pectic enzyme activity was totally suppressed in the glucose controls.

4.2.4. Linear extension rates of dsRNA-containing isolates

Linear growth after 72 hours incubation was examined upon a range of agars (Section 2.4.5): potato dextrose agar (PDA), water agar, cellulose agar and pectic agar. Growth of isolates, assessed as colony diameter, was significantly different ($P < 0.001$) on each type of agar (Table 4. 7), with PDA supporting the greatest growth. The addition of cellulose to water agar (cellulose agar) had no significant effect on the growth of the isolates. Growth was significantly reduced ($P < 0.001$) by the addition of citrus pectin to water agar (pectic agar), with an, overall, mean colony diameter of 12.7 mm growth compared with 35.0 mm growth for water agar. Between isolates no significant difference was detected in growth.

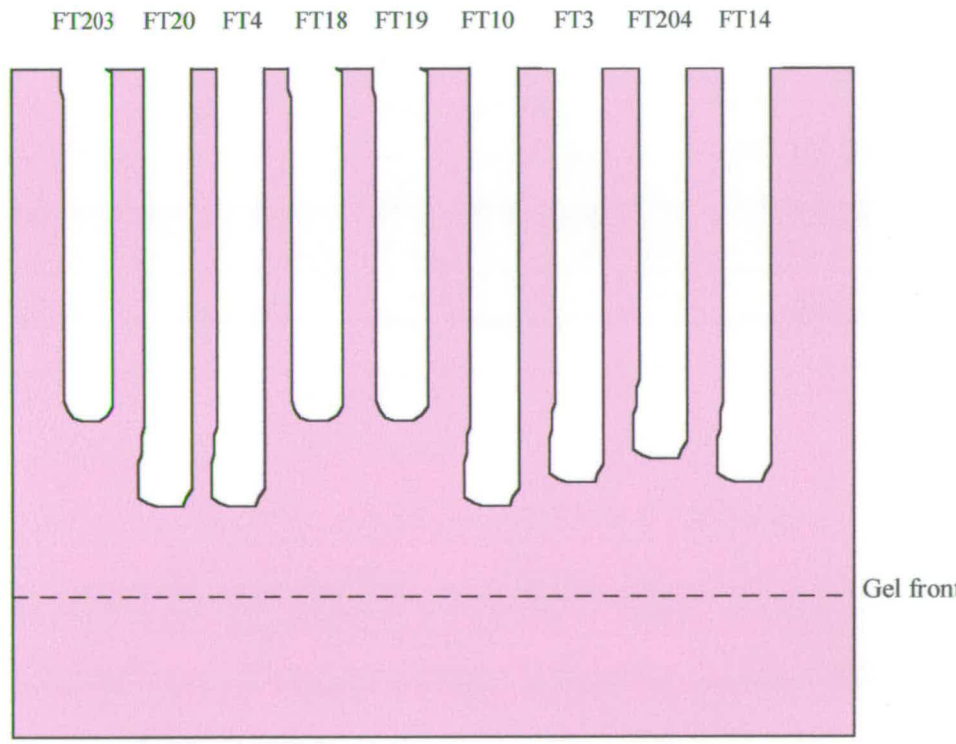


Figure 4. 9 Diagram of a pectic zymogram, loaded with culture supernatant (containing 1 % citrus pectin) from 10 day incubations with the *Rhizoctonia solani* isolates shown above. Residual pectin in the culture supernatant was high, resulting in poor resolution of gels visualized as "smears", as opposed to distinct bands. Polygalacturonase should be observed as clear zones, pectin lyase as clear zones surrounded by a yellow fringe, and pectin esterase as zones which stain darker than background.

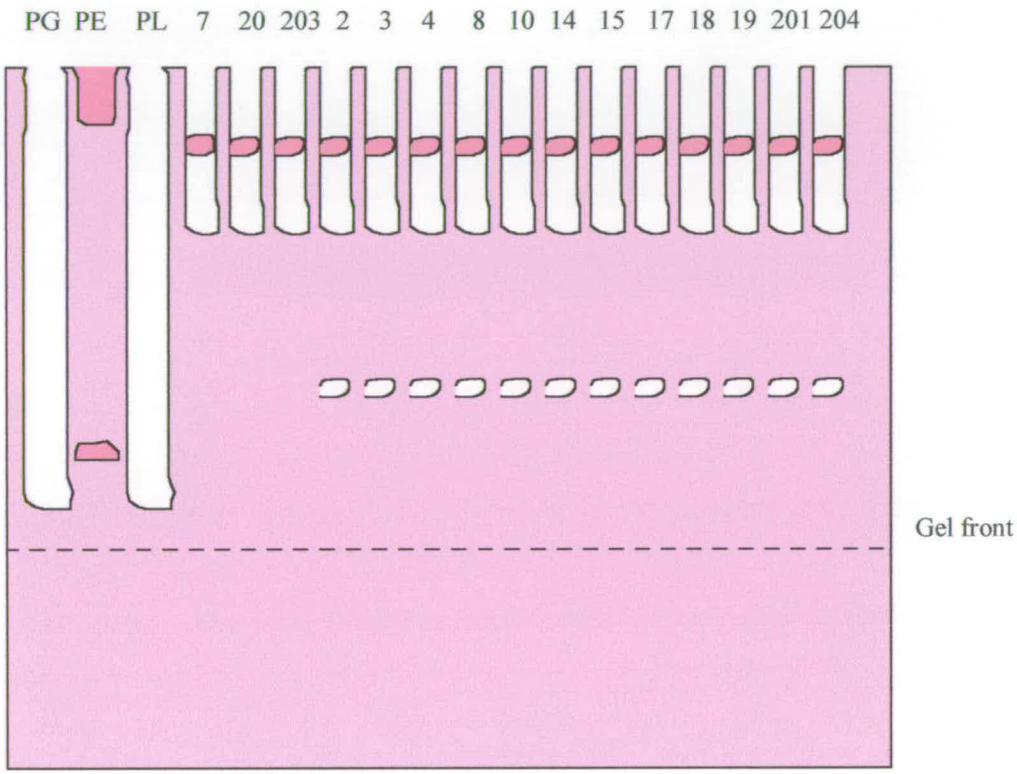


Figure 4. 10 Diagram of a pectic zymogram, loaded with culture supernatant (containing 0.1 % citrus pectin) from 10 day incubations with the *Rhizoctonia solani* FT isolates indicated. PL, PG and PE indicates control lanes for pectin lyase, polygalacturonase and pectin esterase activity, respectively. Polygalacturonase should be observed as clear zones and pectin lyase as clear zones surrounded by a yellow fringe; however the two activities could not be differentiated. Pectin esterase stains as zones a darker pink than the background colour.

	Pectin lyase activity*				
	Days of incubation in citrus pectin broth at 27°C				
Strain	**5**	**6**	**7**	**8**	**9**
FT 2	4.7 ± 1.8	0	42.3 ± 2.0	2.3 ± 1.2	0
FT 3	9.3 ± 3.0	13.7 ± 1.9	27.3 ± 2.7	4.0 ± 2.3	0
FT 4	21.3 ± 0.9	0.7 ± 0.7	4.0 ± 2.0	0	3.7 ± 0.9
FT 7	44.3 ± 2.0	0	3.0 ± 1.0	0	0.7 ± 0.7
FT 8	16.3 ± 1.7	60.7 ± 1.9	2.3 ± 2.3	22.3 ± 0.3	0
FT 10	9.3 ± 1.2	52.3 ± 4.3	8.0 ± 2.1	3.7 ± 2.7	15.0 ± 4.0
FT 14	48.7 ± 1.8	0	69.3 ± 3.0	12.7 ± 1.8	34.7 ± 1.4
FT 15	14.0 ± 2.6	25.3 ± 4.4	0	12.0 ± 1.5	11.3 ± 0.9
FT 17	7.3 ± 1.8	5.3 ± 3.3	7.0 ± 1.5	0	0
FT 18	18.7 ± 2.3	31.3 ± 2.0	0.7 ± 0.7	34.0 ± 3.2	0
FT 19	17.0 ± 2.6	5.3 ± 3.5	0	40.3 ± 4.1	0
FT 20	24.3 ± 5.2	0	18.7 ± 1.9	21.3 ± 3.3	0
FT 201	12.7 ± 1.8	38.3 ± 1.7	0	0	0
FT 203	14.3 ± 2.7	13.3 ± 2.7	19.7 ± 2.3	33.7 ± 1.9	0
FT 204	19.3 ± 0.7	52.0 ± 4.0	25.0 ± 1.5	34.3 ± 0.3	0

*Means of three replicates ± SEM

Table 4. 5 Pectin lyase activity of *Rhizoctonia solani* isolates following five to nine days incubation in broth containing citrus pectin. Culture supernatant was removed and substrate degradation assessed using the thiobarbituric acid assay. One unit indicates an increase in absorbance of 0.01 at 549 nm.

	Polygalacturonase activity*				
	Days of incubation in sodium polypectate broth at 27°C				
Strain	**5**	**6**	**7**	**8**	**9**
FT 2	45.0 ± 2.9	26.7 ± 1.2	0.7 ± 0.3	26.0 ± 4.2	18.0 ± 3.6
FT 3	44.3 ± 2.9	15.0 ± 1.7	97.7 ± 1.4	67.7 ± 2.9	0
FT 4	20.3 ± 0.3	21.7 ± 2.0	14.7 ± 1.4	35.7 ± 0.9	21.0 ± 3.5
FT 7	31.3 ± 2.3	63.3 ± 6.1	73.7 ± 0.7	80.0 ± 4.3	96.7 ± 2.3
FT 8	25.0 ± 3.2	47.7 ± 9.5	29.0 ± 3.6	45.0 ± 3.2	111.0 ± 4.9
FT 10	73.7 ± 2.9	15.0 ± 3.8	42.7 ± 3.5	0	125.7 ± 3.0
FT 14	20.7 ± 2.7	15.7 ± 2.3	61.7 ± 2.4	61.3 ± 4.1	45.0 ± 6.1
FT 15	9.3 ± 2.3	23.7 ± 0.7	55.3 ± 3.8	39.3 ± 5.2	37.3 ± 5.2
FT 17	78.3 ± 7.0	48.3 ± 0.7	155.3 ± 4.3	3.0 ± 1.7	0
FT 18	26.3 ± 4.7	47.7 ± 4.1	2.0 ± 2.0	3.0 ± 3.0	16.7 ± 1.8
FT 19	69.3 ± 3.5	56.0 ± 3.5	83.0 ± 4.0	39.3 ± 6.7	65.0 ± 2.1
FT 20	4.3 ± 1.8	48.3 ± 1.8	45.7 ± 1.2	16.0 ± 2.3	57.3 ± 3.5
FT 201	31.3 ± 2.2	76.3 ± 2.0	118.3 ± 6.1	59.0 ± 9.2	84.7 ± 4.4
FT 203	27.7 ± 6.2	68.3 ± 7.3	39.7 ± 3.0	0.7 ± 0.7	0
FT 204	39.3 ± 2.9	53.0 ± 4.6	109.3 ± 2.4	38.0 ± 4.7	0

* Means of three replicates ± SEM

Table 4. 6 Polygalacturonase activity of *Rhizoctonia solani* isolates following five to nine days incubation in broth containing sodium polypectate. Culture supernatant was removed and substrate degradation assessed using the thiobarbituric acid assay. One unit indicates an increase in absorbance of 0.01 at 530 nm.

	Colony diameter (mm) on various agars after 72 hours incubation*			
Strain	**PDA**	**Water**	**Cellulose**	**Pectic**
FT 2	48.7 ± 0.5	20.3 ± 0.3	20.7 ± 0.9	10.3 ± 0.6
FT 3	60.0 ± 1.7	37.8 ± 2.1	33.0 ± 2.0	18.0 ± 0.3
FT 4	63.5 ± 0.6	23.5 ± 1.3	22.7 ± 1.4	7.3 ± 0.6
FT 7	64.2 ± 1.0	40.8 ± 1.2	37.5 ± 1.3	21.2 ± 1.0
FT 8	64.7 ± 0.8	33.3 ± 2.8	31.8 ± 1.3	10.2 ± 0.2
FT 10	64.5 ± 0.4	33.8 ± 1.4	31.0 ± 1.0	15.7 ± 0.3
FT 14	45.8 ± 0.9	40.2 ± 1.1	39.0 ± 1.2	9.0 ± 1.0
FT 15	62.2 ± 2.6	35.8 ± 1.0	28.5 ± 0.5	8.8 ± 1.2
FT 17	55.7 ± 1.0	24.7 ± 1.3	23.3 ± 0.3	9.3 ± 0.8
FT 18	60.5 ± 1.1	38.8 ± 2.0	35.7 ± 1.7	11.7 ± 0.4
FT 19	66.8 ± 0.9	36.0 ± 1.1	31.0 ± 1.1	16.8 ± 0.3
FT 20	45.3 ± 2.1	20.5 ± 0.3	20.0 ± 0.6	7.3 ± 0.3
FT 201	55.5 ± 1.2	33.8 ± 0.8	30.2 ± 2.0	12.8 ± 0.3
FT 203	55.5 ± 1.5	39.0 ± 1.0	33.0 ± 1.5	15.8 ± 0.4
FT 204	54.7 ± 0.9	36.0 ± 1.3	32.2 ± 1.3	16.3 ± 1.1
Mean	57.8	35.0	30.0	12.7

* Means of four replicates ± SEM

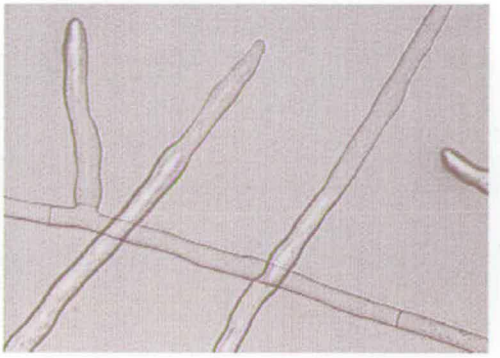
Table 4. 7 Colony diameters (mm), following 72 hours incubation at 23°C, of dsRNA-containing *Rhizoctonia solani* (AG 3) isolates on four agar types; potato dextrose agar (PDA), water agar (2 % Oxoid no. 3) and water agar supplemented with either 2 % cellulose (cellulose agar) or 2 % citrus pectin (pectic agar).

4.2.5. Anastomosis of field isolates

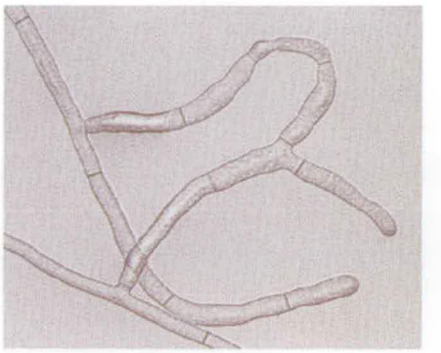
4.2.5.1. Anastomosis reactions

Anastomosis is observed as hyphal fusion between opposing isolates. Pairing of colonies led to three types of interactions: hyphae of isolates of different anastomosis groups grew up to or past each other without contact (Figure 4. 11a); isolates of the same anastomosis group underwent hyphal fusion followed by a compatible reaction where cytoplasmic fusion occurs (Figure 4. 11b); isolates of the same anastomosis group underwent hyphal fusion followed by an incompatible reaction, where cell death to each side of the fusion point occurs (Figure 4. 11c). This killing reaction was variable: in some reactions only one cell to each side of the fusion point died, whereas in other reactions death was more extensive, with up to twelve or more cells on each side of the

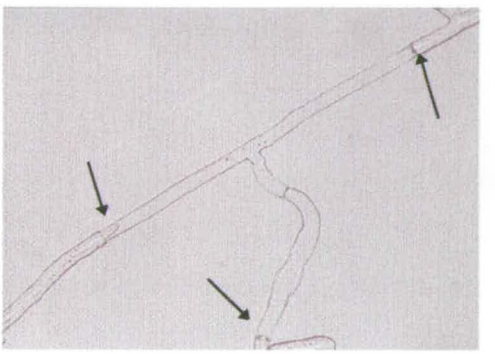
fusion point dying. Hyphal anastomosis reactions were limited to hyphal tip cells of side branches. Main hyphae were never observed to undergo hyphal fusion.



(a)



(b)



(c)

Figure 4. 11 Anastomosis reactions of *Rhizoctonia solani*: (a) No reaction where isolates of different anastomosis groups show no hyphal attraction (b) Vegetatively compatible reaction indicated by cytoplasmic continuity following hyphal fusion and (c) Vegetatively incompatible reaction where hyphal fusion was followed by death of hyphal cells to each side of the fusion point. Death extended to the septa indicated by the arrows.

Anastomosis reactions were observed on both water agar-coated slides and on cellophane-overlaid water agar (Section 2.4.3). Water agar-coated slides were generally less suitable for observation of anastomosis reactions, as incubation periods of more than 60 hours generally led to widespread vacuolation and death of hyphae, sometimes prior to contact of the two colonies. Growth on cellophane-overlaid water agar was more stable and anastomosis reactions could be readily observed for a longer period (up to 3 weeks). In addition, both macroscopic and microscopic manifestations of vegetative compatibility could be observed on cellophane-overlaid plates. Macroscopic

reactions were visualized as a barrage reaction, at the point where two opposing incompatible colonies meet, compared with a compatible reaction, where no barrage reaction is observed.

4.2.5.2. Compatibility of isolates from single potato tubers

dsRNA can be transmitted during vegetatively compatible anastomosis reactions; however, in vegetatively incompatible reactions, a transmission frequency of 4 % has been reported for *Ophiostoma novo-ulmi* (Brasier, 1984; 1986). Dissemination of dsRNA thus requires high levels of vegetative compatibility in natural field populations, before a dsRNA-based biocontrol strategy would be effective. Initial studies examined the vegetative compatibility reactions (Section 2.4.3) of isolates harvested from individual potato tubers. Five isolates from each of three tubers were paired in all possible combinations, and the reactions examined microscopically. Triplicate plates were used for each pairwise combination. Isolates harvested from individual tubers were found to be fully compatible with one another (Figure 4. 12), indicating that over a small 'geographical' distance, vegetative compatibility should not pose a barrier to dissemination of dsRNA.

4.2.5.3. Compatibility of isolates from a single field site

Following from the previous tests (Section 4.2.5.2), eighteen isolates collected from within a single field site were examined, in pair-wise combinations, for their vegetative compatibility to determine the degree of vegetative compatibility over a larger area. Reactions were determined microscopically in triplicate (Section 2.4.3). Of the total 153 pairings of strains (excluding self reactions), 31 showed incompatible reactions in all three replicates and 44 showed incompatible reactions in 2 of 3 replicates. Overall, a high proportion (44 %) of the total reactions were incompatible reactions (Figure 4. 13), which may be limiting to the dissemination of dsRNA. Reactions were completed in triplicate and within these, a high degree of variability occurred, with many pair-wise combinations showing two compatible and one incompatible reaction and vice versa.

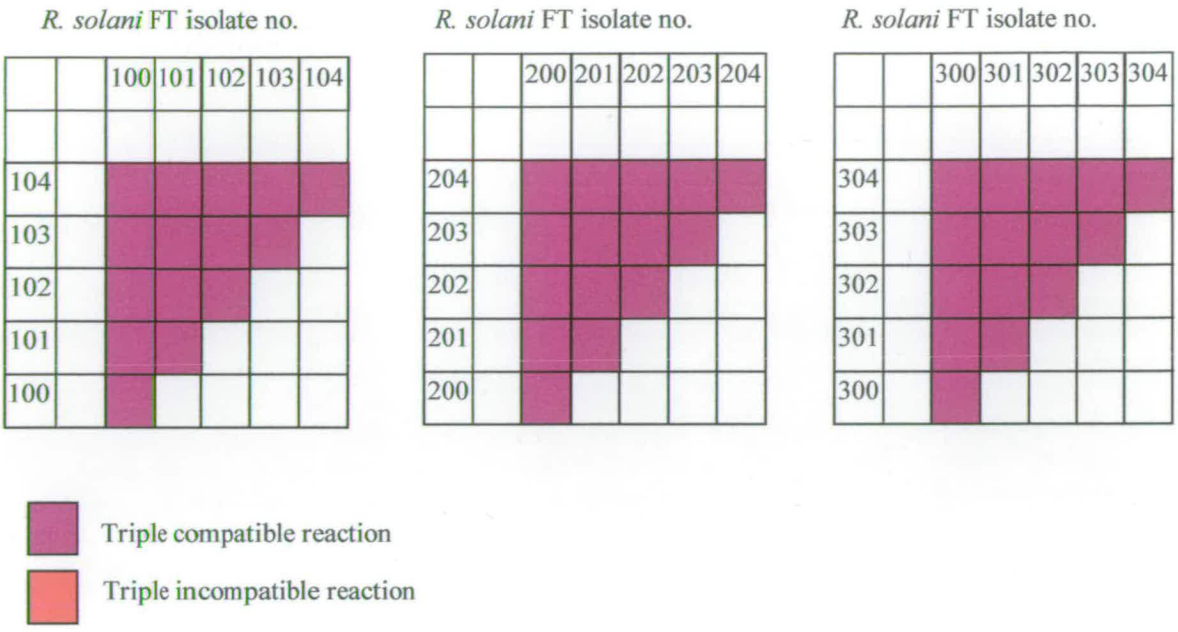


Figure 4. 12 Vegetative compatibility groupings of *Rhizoctonia solani* AG 3 isolates harvested from three different potato tubers within a single field site. Five isolates were harvested per tuber, and paired against one another and their compatibility reaction recorded in triplicate.

4.2.5.4. Compatibility of isolates from diverse locations

Isolates harvested from a single field site showed a high degree of vegetative incompatibility with each other, so isolates were examined from diverse locations within Scotland to determine the degree of vegetative compatibility. All pair-wise combinations examined were vegetatively incompatible (Figure 4. 14). Scottish isolates were then paired with four isolates from the Netherlands (03R04, 03R08, 03R09 and 03R41), to determine vegetative compatibility over a larger geographical distance. All isolates were found to be incompatible with one another (Figure 4. 15).

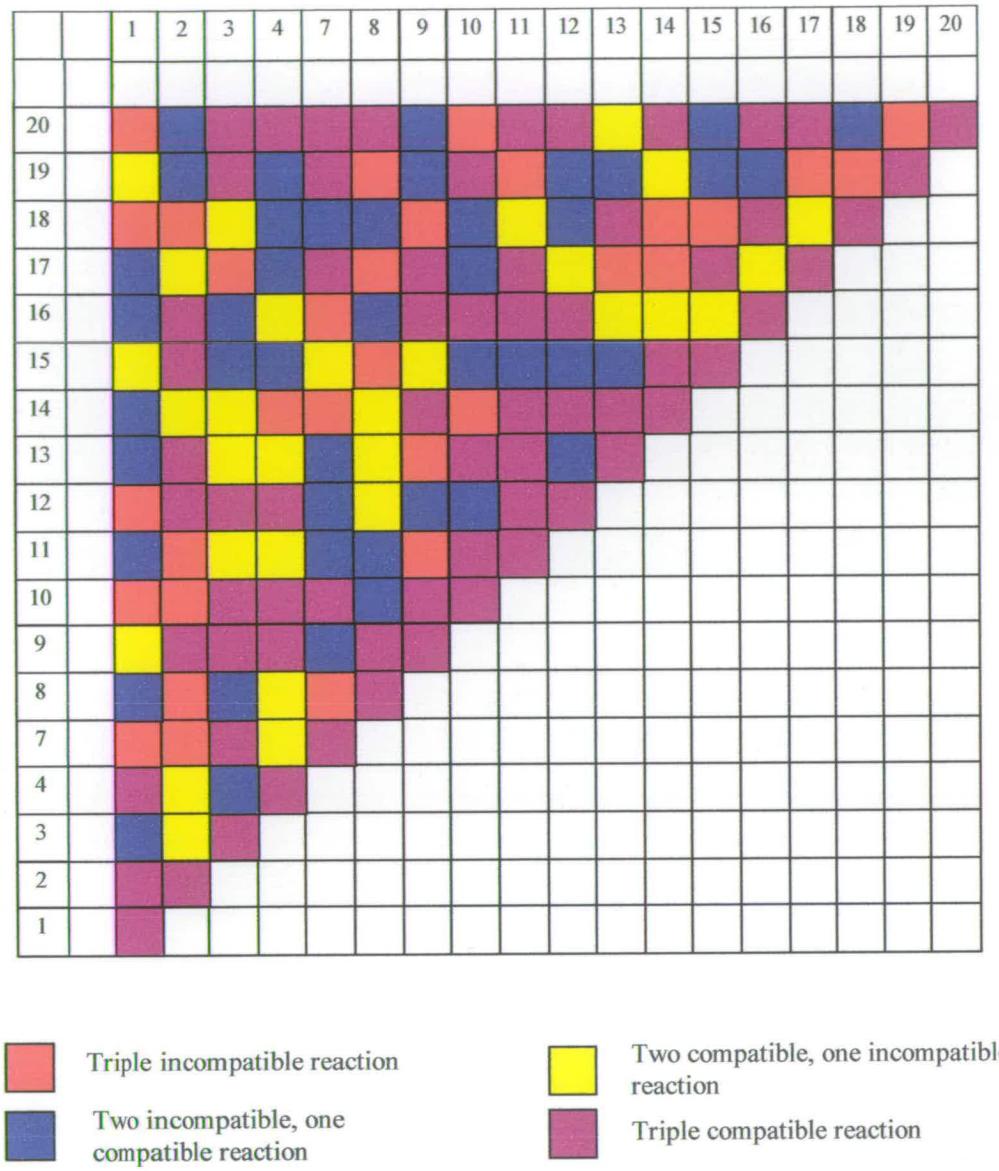


Figure 4. 13 Vegetative compatibility reactions of *Rhizoctonia solani* AG 3 isolates harvested from different potato tubers within a single field site. One isolate was harvested per tuber, and these were paired against one another and their compatibility reaction recorded in triplicate.

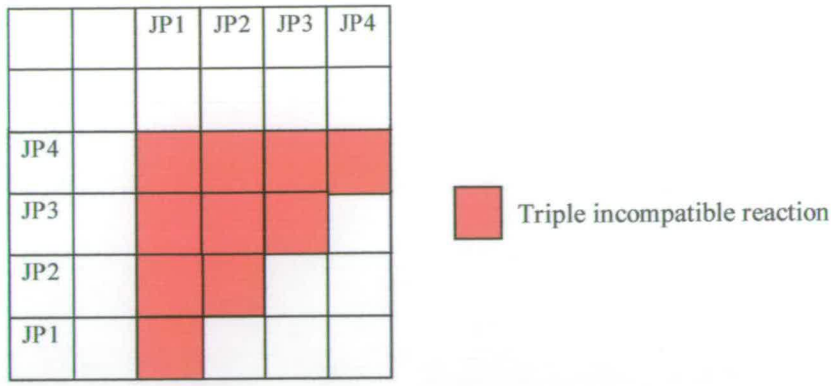


Figure 4. 14 Vegetative compatibility groupings of *Rhizoctonia solani* AG 3 isolates harvested from different potato tubers grown throughout Scotland. One isolate was harvested per tuber, and paired against every other, with their compatibility reaction recorded in triplicate.

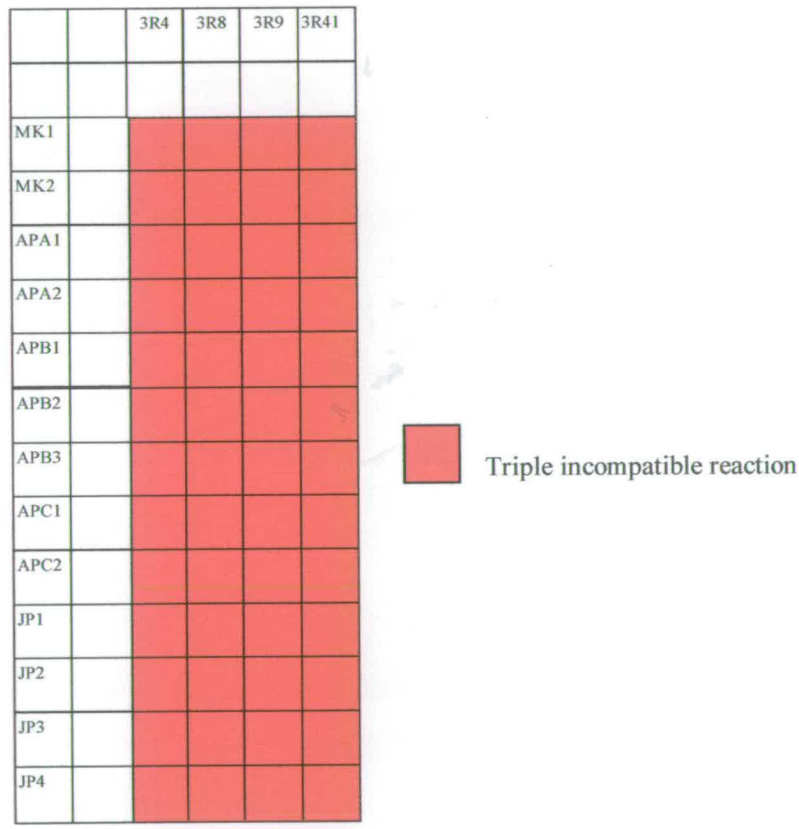


Figure 4. 15 Vegetative compatibility groupings of *Rhizoctonia solani* AG 3 isolates harvested from potato tubers grown throughout Scotland (JP 1-4, APC 1-2, APB1-3, APA 1-2, MK 1-2) paired against isolates from the Netherlands (3R4, 3R8, 3R9, 3R41). Compatibility reactions were recorded in triplicate.

4.2.6. Interaction of *Verticillium biguttatum* with *R. solani*

Verticillium biguttatum is a biotrophic mycoparasite of *R. solani* (van den Boogert and Deacon, 1994). *V. biguttatum* infection proceeds in four stages (van den Boogert *et al.*, 1989): (i) germination of the mycoparasite (ii) coiling and appressed growth along the host hyphae (iii) penetration of the host hyphae and (iv) internal growth and subsequent sporulation outside the host. The main macroscopic effect on *R. solani* of this interaction is a reduction in the number of sclerotia at least 75 mm away from the site of parasitism (van den Boogert and Deacon, 1994). In addition, adjoining vegetatively compatible colonies have sclerotial suppression when only one of the colonies is inoculated with *V. biguttatum*. Adjoining vegetatively incompatible colonies show no suppression in sclerotial numbers in the non-inoculated colony. Sclerotial suppression alone makes *V. biguttatum* a good prospective biocontrol agent especially for diseases such as black scurf of potato. A secondary level of biocontrol may also be potentially achieved. van den Boogert and Deacon (1994) hypothesize that *V. biguttatum* represents a nutrient sink, diverting nutrients throughout the *Rhizoctonia* colony and any adjoining vegetatively compatible colonies. This hypothesized diversion of cytoplasmic contents may potentially "pull" dsRNA from one colony of *R. solani* to other colonies upon anastomosis. This led to the susceptibility of *R. solani* to *V. biguttatum* being examined by determining the reduction in sclerotial numbers following mycoparasitism by *V. biguttatum*.

4.2.6.1. Effect of *V. biguttatum* isolates on *R. solani*

To determine which *V. biguttatum* isolate gave greatest suppression of sclerotia formation by *R. solani*, four *V. biguttatum* isolates (M 73, M 92, M 95 and M 98) were examined for sclerotia reduction of eight *R. solani* isolates. Interactions were examined on cellulose agar following 14 days incubation at 23°C (Section 2.4.4). No significant difference between *V. biguttatum* isolates in their degree of sclerotial suppression was observed (Table 4. 8). *V. biguttatum* isolate M 92 was selected for further study, as the overall mean for sclerotia suppression was marginally higher, being 69.6 %, compared

with 36.5 %, 56.2 % and 45.9 % suppression for isolates M 73, M 98 and M 95 respectively.

4.2.6.2. Effect of *V. biguttatum* on dsRNA-containing isolates

The effect of *V. biguttatum* isolate M 92 on dsRNA-containing AG 3 isolates was examined on cellulose agar, following 14 days incubation at 23°C (Section 2.4.4). *V. biguttatum* M 92 had a highly significant effect ($P < 0.001$) on sclerotia formation (Table 4. 9). Sclerotia formation was suppressed to levels between 96.8 % and 100 % in the presence of *V. biguttatum* M 92.

		% suppression of sclerotia formation by isolates of *V. biguttatum*			
***Rhizoctonia* strain**	**Anastomosis group**	**M 73**	**M 92**	**M 95**	**M 98**
01R03	1	25.0	54.0	0	31.8
2tR105	2 tulip	47.7	29.8	0	58.9
JP2	3	17.1	86.8	70.2	33.4
03R04	3	81.1	94.8	85.5	77.1
Sc222	3	61.3	100	69.9	72.9
03R09	3	46.3	90.7	65.0	82.1
AG-3	3	0	40.0	36.6	49.5
04R22	4	13.4	60.4	40.2	44.0
Mean		**36.5**	**69.6**	**45.9**	**56.2**

Table 4. 8 Percentage suppression of sclerotia formation of *Rhizoctonia solani* isolates when inoculated with *Verticillium biguttatum* isolates and incubated for 14 days on cellulose agar at 23°C. Mean of three replicates.

	Number of sclerotia		
***Rhizoctonia* strain**	***Rhizoctonia* alone**	***Rhizoctonia* plus *Verticillium***	**% Sclerotia suppression**
FT 2	76.7 ± 5.8	0	100
FT 3	85.7 ± 9.4	2.7 ± 2.7	96.8
FT 7	44.0 ± 6.5	0.3 ± 0.3	99.3
FT 8	74.0 ± 3.8	0	100
FT 10	90.0 ± 35.6	2.0 ± 0.6	97.8
FT 14	165.3 ± 37.5	0.7 ± 0.3	99.6
FT 17	127.3 ± 11.6	0.7 ± 0.3	99.5
FT 19	107.3 ± 13.9	0.3 ± 0.3	99.7
FT 20	32.7 ± 20.0	0.3 ± 0.3	99.1

Table 4. 9 Suppression of sclerotia formation of dsRNA-containing *Rhizoctonia solani* AG 3 isolates by *Verticillium biguttatum* M 92 following 14 days incubation on cellulose agar at 23°C. Mean of 3 replicates.

4.2.6.3. Effect of *V. biguttatum* on different anastomosis groups

Previous studies by Jager and Velvis (1983 a, b) indicate that *V. biguttatum* populations increase on *Rhizoctonia* infected potato tubers, whereas other crops do not permit multiplication of the *V. biguttatum* population to the same extent. Since Solanaceae are characteristically infected by AG 3 isolates (Ogoshi, 1987), this may indicate that the mycoparasite *V. biguttatum* does not interact in the same manner with the other anastomosis groups. *V. biguttatum* M 92 was shown to significantly ($P < 0.001$) suppress the formation of sclerotia of *R. solani* AG 3 isolates (Table 4. 10). Examining sclerotia suppression by *V. biguttatum* on other AGs indicated that the degree of suppression varied between AGs (Table 4. 11). AG 2-2 isolates showed no significant difference in sclerotia formation in the presence and absence of *V. biguttatum*. AGs 2-1, 2-3, 3, 5, 8 and 11 were shown to have significant ($P < 0.05$) sclerotia suppression (> 70 %) (Table 4. 11). AGs 1, 2t, 4, 6 and 9, showed no significant difference in sclerotia formation in the presence and absence of *V. biguttatum*.

Rhizoctonia strain	Number of sclerotia		% suppression
	Rhizoctonia alone	*Rhizoctonia* plus *Verticillium*	
03R04	134.7 ± 49.7	0	100
03R08	2.0 ± 2.0	0	100
03R09	313.7 ± 57.6	29.3 ± 4.3	90.7
03R41	136.0 ± 73.2	0	100
JP1	53.3 ± 30.9	9.7 ± 8.2	81.8
JP2	226.7 ± 25.5	30.0 ± 13.9	86.8
JP3	213.0 ± 50.5	53.7 ± 9.8	74.8
JP4	508.0 ± 54.4	3.7 ± 2.0	99.3
Sc222	150.7 ± 58.6	0	100
MK1	66.0 ± 27.8	0.3 ± 0.3	99.5
MK2	160.0 ± 60.0	0.7 ± 0.7	99.6
APB1	159.7 ± 24.0	9.3 ± 3.0	94.2
APB2	68.0 ± 49.8	0.3 ± 0.3	99.6
APB3	98.3 ± 5.8	0	100
APA1	97.7 ± 19.9	0	100
APA2	66.0 ± 23.6	0	100
APC1	48.0 ± 9.5	0	100
APC2	336.3 ± 63.0	0	100
FT11	136.3± 8.4	9.3 ± 2.9	93.2
FT13	125.3 ± 60.3	0	100

Table 4. 10 Suppression of sclerotia formation of *Rhizoctonia solani* AG 3 isolates by *Verticillium biguttatum* M 92 following 14 days incubation on cellulose agar at 23°C. Means of 3 replicates.

		Number of sclerotia		
Rhizoctonia strain	Anastomosis group	*Rhizoctonia* alone	*Rhizoctonia* plus *Verticillium*	% suppression
01R01	1	13.7 ± 8.4	1.7 ± 0.9	87.6
01R02	1	1385.0 ± 93.0	776.0 ± 57.9	43.8
01R03	1	189.0 ± 65.0	87.0 ± 21.1	54.0
21R01	2-1	342.0 ± 53.7	112.0 ± 35.7	76.8
2tR105	2t	239.0 ± 53.7	8.0 ± 4.2	96.7
2tR118	2t	169.3 ± 26.9	88.7 ± 17.6	47.6
2tR144	2t	147.3 ± 76.2	20.7 ± 3.8	86.0
VR5	2-2	102.3 ± 9.7	90.3 ± 10.0	10.7
BI2	2-2	66.3 ± 17.6	66.0 ± 14.4	0.5
GR1	2-2	21.0 ± 1.7	21.0 ± 7.1	0
22R02	2-2	119.0 ± 18.0	115.7 ± 45.9	3.4
23R01	2-3	32.3 ± 4.7	4.0 ± 4.0	87.6
2-3/ 144	2-3	280.7 ± 53.2	0	100
03R04	3	182.3 ± 75.7	9.5 ± 0.3	94.8
03R09	3	210.5 ± 33.8	15.3 ± 8.2	92.7
JP3	3	213.0 ± 50.5	53.7 ± 14.7	74.8
FT 13	3	125.3 ± 60.3	0	100
04R22	4	291.0 ± 47.4	115.3 ± 32.8	60.4
4/41	4	274.3 ± 31.3	57.3 ± 16.8	79.1
c-233	4	424.7 ± 53.5	0	100
05R01	5	413.7 ± 6.3	114.0 ± 15.0	72.4
06R01	6	441.0 ± 90.4	266.0 ± 22.2	39.7
08R01	8	76.0 ± 27.2	0	100
09R01	9	348.5 ± 144.0	178.0 ± 55.7	48.9
11R01	11	219.0 ± 31.8	37.3 ± 11.8	83.0

Table 4. 11 Suppression of sclerotia formation of *Rhizoctonia solani* isolates from differing anastomosis groups by *Verticillium biguttatum* M 92 following 14 days incubation on cellulose agar at 23°C. Means of 3 replicates.

4.2.6.4. Effect of *V. biguttatum* on starch agar plates

For three strains of *R. solani*, 03R04, 01R03 and 2tR144 the susceptibility to parasitism by *V. biguttatum* was examined on starch agar as opposed to cellulose agar (Section 2.4.4). Here sclerotia formation was more abundant, so the quantity of sclerotia was calculated using Optimas and Excel 5 computer packages (Table 4. 12). Greyscale values did not give an effective indication of differences between control and *V. biguttatum* inoculated plates, as the differences were small. The area covered by sclerotia gave a better indication. This showed a reduction in sclerotia area for the three strains in the presence of *V. biguttatum*; however, the difference was not significant. This method was validated by calculating the ratio of sclerotia numbers on *V. biguttatum* to control plates for strain 01R03 (the only plate where sclerotia numbers were countable) at 62.6 %, and the ratio of the areas covered by sclerotia was 67.2 % Thus the two methods appear to be consistent. Comparing these results with those obtained on cellulose, the degree of suppression is of a similar level. For example, isolate 01R03 showed 67.2 % suppression of sclerotia on starch and 46.0 % on cellulose, whereas isolate 03R04 showed 12.6 % suppression on starch and 5.2 % on cellulose.

	Area of sclerotia (cm^2)		Greyscale*	
***Rhizoctonia* strain**	***Rhizoctonia* alone**	***Rhizoctonia* plus *Verticillium***	***Rhizoctonia* alone**	***Rhizoctonia* plus *Verticillium***
03R04 (AG 3)	9.8 ± 2.5	1.2 ± 0.1	145.2 ± 2.4	153.5 ± 2.3
01R03 (AG 1)	0.7 ± 0.3	0.4 ± 0.1	167.5 ± 2.4	168.9 ± 1.8
2tR144 (AG 2t)	4.2 ± 0.6	1.6 ± 2.6	156.9 ± 0.8	156.2 ± 5.0

Table 4. 12 Area (cm^2) covered by *Rhizoctonia solani* sclerotia on starch agar plates and the corresponding greyscale values for the plates. Greyscale values range from 44 as black to 255 as white, and were calculated using Optimas and Excel 5. Measurements were taken following 14 days incubation at 23°C. Mean of three replicates.

4.3. Discussion

dsRNA mycoviruses are widespread in fungi, with many isolates harbouring multiple infections (Buck, 1986; Nuss and Koltin, 1990; Ghabrial, 1994). For example, isolates of *Saccharomyces cerevisiae* commonly harbour the L-A and L-BC totiviruses, in addition to the 20S(W) and 23S(T) RNA replicons (Wickner, 1996). These viruses can co-exist as they rely on different replication proteins (Buck, 1998). Although many fungal species harbour dsRNA, the distribution within isolates is highly variable. For example, 72 % of *Botrytis cinerea* isolates contained 1 to 8 segments of dsRNA (Howitt *et al.*, 1995), whereas only 7 % of *Aspergillus* (Section *nigri*) and *Aspergillus* (Section *flavi*) isolates contained dsRNA (Varga *et al.*, 1994; Elias and Cotty, 1996). The present study examined the abundance of dsRNA mycoviruses in *R. solani*, using CF11 cellulose chromatography, followed by agarose gel electrophoresis. Each AG 3 isolate harvested from separate tubers within a single field site contained multiple dsRNA segments, ranging in size from 0.3 to 15 kb. Every isolate examined from other anastomosis groups (AG 2-1, 4 and 5), contained dsRNA. This study indicates that dsRNA may be ubiquitous within *R. solani* field populations. However, previous studies indicated dsRNA distribution to be highly variable. Castanho *et al.* (1978) and McCabe (1994) reported dsRNA to be much less abundant, with only 23 % and 20 % of isolates containing dsRNA, respectively. Kousik *et al.* (1994) extracted dsRNA from 54 % of isolates and Zanzinger *et al.* (1984) found dsRNA in 98 % of isolates examined. These studies examined isolates collected from diverse geographic locations, as opposed to the single field site used in the present study. Similar variation has been reported in *C. parasitica* isolates, depending on the geographical distribution (Peever *et al.*, 1997). In a survey of 595 isolates, 28 % contained dsRNA. However, the incidence was highly variable, with all isolates from County Line in Michigan containing dsRNA, whereas isolates from New Hampshire and Ontario contained no dsRNA. In China, 172 isolates were examined, with only 2 % containing dsRNA. Similar results were obtained for *Phytophthora infestans* where 36 % of Mexican isolates contained dsRNA, but it was not detected in any of the European or North American isolates examined (Nuss and Koltin, 1990).

The number and sizes of dsRNA segments found within isolates is highly variable. Isolates in the present study contained 1 to 8 segments of dsRNA ranging in

size from 0.8 to 15 kb. These can be divided into three broad size categories, as proposed by Bharathan and Tavantzis (1990): (i) large (L) being 4.6 kb or greater (ii) medium (M) in the range 1.1 to 4.5 kb and (iii) small (S) being 0.5 to 1.0 kb. Each isolate contained at least one large (L) segment of dsRNA. Similar diversity in dsRNA distribution is observed in other species. For example *Cryptococcus hungaricus* CBS 6569 contained two segments of sizes 5.0 and 1.7 kb (Pfeiffer *et al.*, 1998), whereas in other species dsRNA is more abundant. *Ophiostoma novo-ulmi* isolate Ld contains 10 segments ranging in size from 0.33 to 3.49 (Rogers *et al.*, 1986; 1987). In many rust fungi, the number of segments present is much greater. *Puccinia sorghii* contains at least 10 segments of 4.0 to 6.5 kb, and at least 10 medium to small size segments (Zhang *et al.*, 1994).

The banding patterns of dsRNA were highly variable, with some isolates containing the same segmentation. Of the isolates harvested from separate tubers, FT 3, FT 8 and FT 17 contained three large segments with the same electrophoretic mobility, FT 10 and FT 18 contained four similar sized segments and FT 16 and FT 20 contained two segments of equal size. Strains FT 203 and FT 204 from the same tuber contained seven segments of identical size. Since the isolates were harvested from a small locality, and contained similar banding patterns, it is likely that some of the segments may be the same, possibly the result of horizontal transmission by vegetative anastomosis. To determine whether the segments are related, northern hybridization studies are required. These, however, have not been completed in the present study. Previous work indicates sequence homology between dsRNA segments extracted from *R. solani* isolates of the same anastomosis group, but not from different anastomosis groups (Bharathan and Tavantzis, 1987). This, though, is not unexpected since the differing anastomosis groups are genetically isolated, due to their lack of hyphal fusion (Vilgalys and Cubeta, 1994; Adams, 1996). Subsequent work, however, has indicated a limited degree of dsRNA homology between AGs. Bharathan and Tavantzis (1990) reported cross hybridization between three segments of size 1.8, 6.4 and 2.3 kb from isolates Rhs47 (AG 2), Rhs1A1 (AG 3) and Rhs (AG 5), which may indicate a common dsRNA progenitor. Bharathan and Tavantzis (1991) subsequently examined homology between isolates of the same AG that were geographically distant. No hybridization was observed between Japanese and American isolates from AG 1, 2, 4 and 5. Isolates located from within Maine, however, showed a certain degree of cross-hybridization,

both with each other and also towards isolates from Colorado. They concluded that the degree of homology decreased with increasing distance, so it is of interest to determine the level of homology at the field/ tuber level. A high degree of homology may be indicative of extensive transmission of dsRNA via hyphal anastomosis, which is a pre-requisite for any biocontrol strategy based on possible dsRNA-mediated hypovirulence. Within other species, dsRNA elements show varying degrees of homology. For example, in *Chalara elegans* Nag Raj and Kenchick, a 2.8 kb band was found to hybridize between eight isolates of differing geographic origin, but no hybridization was observed towards high molecular weight dsRNA (Bottacin *et al.*, 1994). In *C. parasitica*, the diversity of dsRNAs have been extensively characterized, with several types recognized: CHV1-EP713, CHV2-NB58 and CHV3-GH2 (Peever *et al.*, 1998). In addition to these, various others have been reported including reovirus-like dsRNAs from isolate C18 of West Virginia (Enebak *et al.*, 1994) and dsRNA within the mitochondria of isolate NB631 from New Jersey (Polashock and Hillman, 1994). Additionally, Peever *et al.* (1997) found further dsRNA segments from *C. parasitica* isolates of New Jersey that did not cross-hybridize with any known dsRNAs. Within *R. solani*, many hybridization groups can be expected, as each AG is essentially genetically isolated. Within each AG, several hybridization groups may be present due to the lack of homology between dsRNA segments from *R. solani* reported to date. Of the characterized *C. parasitica* dsRNAs, some are widespread geographically, while others are found in relatively localized regions. For example, CHV1-EP713 is only found in Europe (L'Hostis *et al.*, 1985; Paul and Fulbright, 1988; Heiniger and Rigling, 1994) and China (Liang *et al.*, 1992; Quan *et al.*, 1994). In eastern North America, dsRNA of 595 isolates was examined and found to belong to three hybridization groups (Peever *et al.*, 1997). Interestingly, none of the isolated dsRNA segments hybridized to European CHV1-EP713, despite CHV1-EP713 being previously released in numerous locations for biocontrol purposes. These debilitated isolates, with slow growth rate and decreased sporulation, are presumably unable to persist alongside their healthy counterparts.

The present study did not examine the localization and encapsidation of the dsRNA segments. Previous reports indicate dsRNA of *R. solani* to reside in the mitochondrial fraction (Tavantzis, 1994; Jian *et al.*, 1997). Similarly dsRNA of *C. parasitica* and *O. novo-ulmi* has been found in the mitochondria (Rogers *et al.*, 1987; Polashock *et al.*, 1994). Mycoviruses tend to either be encapsidated in protein capsids

or alternatively associated with lipid-rich cytoplasmic vesicles, e.g. *C. parasitica* hypovirus (Fahima *et al.*, 1993) and *Alternaria solani* (Zabalgogeazcoa *et al.*, 1997). Castanho *et al.* (1978) were unable to detect encapsidated viral particles in *R. solani*. Subsequent studies, however, reported isometric particles of 33 nm diameter (Finkler *et al.*, 1985; Finkler *et al.*, 1988; Tavantzis, 1994). However, dsRNA from all isolates may not necessarily be encapsidated. In *C. parasitica*, the majority of dsRNA elements are associated with lipid-rich vesicles, but some have recently been reported to be encapsidated in 60 nm isometric particles (Enebak *et al.*, 1994).

DsRNA elements are generally non-symptomatic, as in many rust fungi (Zhang *et al.*, 1994), but some have been reported to alter the fungal phenotype. For example, *Chalara elegans* shows reduced growth rate and virulence, and an increase in phialospore production in the presence of dsRNA (Bottacin *et al.*, 1994). *C. parasitica* isolates become hypovirulent, in addition to showing reduction of pigmentation, reduced conidiation, loss of female fertility, and down-regulation of several virulence-associated proteins including oxalate, laccase, cryparin, cellulase, cutinase, protease and polygalacturonase. Previous reports have tried to correlate dsRNA in *R. solani* with altered phenotype. Initial reports by Castanho and Butler (1978a) indicated that the presence of dsRNA resulted in a hypovirulent phenotype. However, Finkler *et al.* (1985) subsequently contradicted this by reporting dsRNA to be associated with virulent isolates of *R. solani*. Several subsequent reports indicate dsRNA to be ubiquitous within *R. solani*, with no apparent correlation between the presence of dsRNA and the degree of pathogenicity (Zanzinger *et al.*, 1984; Hyakumachi *et al.*, 1985; Bharathan and Tavantzis, 1990; Bharathan and Tavantzis, 1991; Washington and Martin, 1991; Kousik *et al.*, 1994). It is likely that specific dsRNA elements give rise to certain phenotypes. A similar situation occurs in *O. novo-ulmi* where many isolates contain multiple dsRNA segments, with three specific segments (2.43, 0.95 and 0.33 kb) being required to invoke the hypovirulent phenotype in the host (Rogers *et al.*, 1986; 1988). Likewise Stanway and Buck (1984) surveyed over a hundred *Gauemannomyces graminis* isolates, finding no correlation between the presence of dsRNA and virulence. They concluded that hypovirulence may be linked to the presence of specific dsRNAs. The present study examined the virulence of fifteen *R. solani* field isolates, in an attempt to correlate virulence with the presence/ absence of specific dsRNA elements. Each isolate contained multiple dsRNA segments, with some isolates containing segments

which were identical in electrophoretic mobility. Between the isolates, no significant difference in virulence upon six host crops was observed. Similar results were obtained by van den Boogert *et al.* (1998b) who reported that pathogenicity of AG 2-2IIIB field isolates showed little intrafield variation, but differed significantly between fields. This may indicate that the coding capacity of dsRNA contained within these isolates is similar, which would not be unexpected since the isolates were harvested from a small geographical locality. Northern hybridization studies, however, would be required for confirmation. The disease severity, obtained in the present study, was fairly low, ranging from 1.5 to 2.6, on a scale of 0 to 5. Mean levels between 0 and 1 are taken to indicate hypovirulence (Ichielevich-Auster *et al.*, 1985). Potentially, the low disease severity may be the result of dsRNA-encoded functions. However, to determine this, isolates without dsRNA in the same host genetic background are required. The work of chapter 5 examines approaches to curing isolates of their dsRNA, in an attempt to specifically correlate dsRNA with virulence.

The enzymes underlying pathogenicity are widely reported to vary in the presence of dsRNA (Buck, 1998). In *R. solani* several enzymes are released during tissue invasion, including polygalacturonase, trans-eliminases, cutinase, phenol oxidases, cellulases and hemicellulases (Bateman, 1963a, 1964; Ayers *et al.*, 1966; Bateman *et al.*, 1969; Baker and Bateman, 1978). The pectic enzymes, cellulases and phenol oxidases were examined to determine whether fluctuation exists between the dsRNA-containing isolates. No comparisons to isolates lacking dsRNA were made, due to the lack of availability of cured isolates.

Pectic enzymes include polygalacturonase and the trans-eliminases (pectin lyase and pectin esterase). Polygalacturonase is the first enzyme released during tissue invasion, being responsible for "tissue maceration" (Bateman, 1963a), a process which is aided by trans-eliminases (Ayers *et al.*, 1966). Previous reports on pectic enzymes indicate that they are produced by both highly virulent and avirulent strains of *R. solani* (Sherwood, 1966). Marcus *et al.* (1986) examined the individual enzymes and their relationship to virulence. They found polygalacturonase to be produced by both virulent and hypovirulent isolates, whereas pectin lyase was absent from hypovirulent isolates, and only produced by virulent isolates. Similar results were obtained in the present study, which indicated both polygalacturonase and pectin lyase to be produced by 15 weakly virulent isolates. The levels of pectin lyase produced were significantly lower

than that of polygalacturonase. The lack of pectin lyase production by hypovirulent isolates in the study of Marcus *et al.* (1986) may potentially be related to the presence or absence of dsRNA. The present study examined only isolates containing dsRNA, due to the lack of availability of dsRNA-free isolates. McCabe (1994) examined pectic enzymes of two isolates, PA1 and I13, both of which contained dsRNA. Removal of dsRNA from PA1 resulted in increased polygalacturonase and pectin lyase activity, whereas no alteration in enzyme activity was observed for strain I13 upon removal of dsRNA. This may indicate that the dsRNA elements present in PA1 (2.1 and 2.3 kb) and I13 (2.0 and 2.2 kb) encode different functions. The study by Marcus *et al.* (1986) made no attempt to correlate pectin lyase production with the presence of dsRNA. However, it is interesting to note that virulent isolate 82 produced pectin lyase, and has since been reported to harbour dsRNA (Finkler *et al.*, 1985), whereas hypovirulent isolate 521 contains no dsRNA (Finkler *et al.*, 1985) and does not produce pectin lyase. In further support that dsRNA may affect pectin lyase expression, Finkler *et al.* (1988) transferred dsRNA via hyphal anastomosis from a virulent to a hypovirulent *R. solani* isolate. The converted strain subsequently displayed endo-pectin lyase activity. This may be the result of a regulatory cascade triggering the production of several virulence-associated enzymes. Further investigation into the relationship of pectic enzymes and dsRNA content is warranted.

Pectic enzymes are the initial enzymes released during plant tissue invasion by *R. solani*. Subsequently cellulases are released which are involved in intracellular penetration and the ultimate collapse of the cells (Bateman, 1970). Cellulase secretion in *C. parasitica* is altered in the presence of dsRNA. Isolates containing dsRNA failed to produce cellulase when grown on cellulose as sole carbon source, whereas those lacking dsRNA readily secreted cellulases (Wang and Nuss, 1995). Initial studies by McCabe (1994) examined two *R. solani* isolates (PA1 and I13), which degraded 310.9 ± 26.6 and 382.1 ± 20.0 mg cellulose over 4 weeks, respectively. Curing these isolates of dsRNA resulted in a three to four fold increase in cellulolytic activity. Three derivatives of PA1 were examined giving 1114.2 ± 161.2, 917.6 ± 52.4 and 994.3 ± 25.4 mg cellulose respired over 4 weeks, whereas one derivative of I13 respired 1153.1 ± 166.5 mg cellulose over 4 weeks. The present study examined cellulolytic activity of dsRNA-containing isolates of AG 3, and found activity to vary significantly between the

twelve isolates examined. The high degree of variability was not unexpected, as studies presented in chapter 3 of this thesis found that cellulolytic activity was highly variable between isolates, and is likely to be more important in the saprotrophic phase rather than the pathogenic phase of *R. solani*. Again further investigation is warranted between dsRNA and dsRNA-free isolates, to confirm the initial results obtained by McCabe (1994). This work initially aimed to further examine the relationship, but was hampered by the lack of dsRNA-free isolates.

In other species further traits are reported to show altered expression in the presence of dsRNA. These traits include loss of phenol oxidase activity, alteration of cryparin levels, reduction in growth rate and reduction of gallic and tannic acid oxidation (Anagnostakis, 1987; Ghabrial, 1994). Again no significant differences were found between isolates in the present study regarding oxidation of both gallic acid and tannic acid. Linear growth rate was constant between the isolates examined. Prior studies by McCabe (1994) found no alteration in growth rate between strains containing dsRNA and those cured of dsRNA. This is in contradiction to the isolates examined by Castanho and Butler (1978b), which showed a ten-fold increase in linear extension upon curing the isolates of dsRNA. In further contradiction, Zanzinger *et al.* (1984) found that isolates cured of dsRNA showed an increase in growth rate. This again may be an indication that different segments of dsRNA have different effects on fungal virulence.

Of the phenotypic properties examined, excluding cellulase activity, there were no significant differences in expression between the isolates, despite the diversity in dsRNA banding patterns. This may indicate that either: (i) the dsRNA does not encode factors which alter the host phenotype with respect to pathogenic characteristics or (ii) the genetic information encoded by the dsRNA in the different hosts is the same. If this was the case, it may be that the virulence, and hence the pathogenic enzymes involved, are only being expressed at a fraction of their original levels, or indeed, that the isolates were previously avirulent, and the dsRNA has triggered a pathogenic response in the fungal isolates. To determine this, however, isolates cured of dsRNA are required. The work presented in chapter 5 examines the curing of isolates.

For a dsRNA-mediated biocontrol strategy to be successful, dsRNA transfer between isolates must occur. Transmission is generally limited to horizontal modes via hyphal anastomosis. The efficiency of this process is assisted by the high concentration of dsRNA near the hyphal tips, brought about by continual protoplasmic streaming from

the older regions of the hyphae towards the tip cells. In *R. solani*, direct microscopical evidence of dsRNA transfer between cells during hyphal anastomosis has not been observed. However, several circumstantial reports indicate that transfer does occur. Castanho and Butler (1978a) reported pairing an isolate containing dsRNA (189a) with a cured dsRNA-free isolate (189HT3) on Y-plates. Following anastomosis, all colonies re-isolated contained dsRNA, indicating successful transmission, plus dissemination of dsRNA throughout the mycelial network. Jian *et al.* (1997) conducted similar experiments to transfer either a 6.4 or 3.6 kb dsRNA segment to dsRNA-free genetic backgrounds. Finkler *et al.* (1985) used a different approach involving a virulent isolate containing dsRNA, which was benomyl resistant and sensitive to another compound, codenamed BTN. The hypovirulent isolate lacked dsRNA and was benomyl sensitive and resistant to BTN. Following anastomosis, virulent colonies were obtained which contained dsRNA, but were benomyl sensitive and BTN resistant. This was taken to indicate that dsRNA transfer had occurred into the previously hypovirulent isolate. In these studies, transfer has been demonstrated following compatible anastomosis reactions. However, anastomosis is frequently followed by an incompatible anastomosis reaction, where dsRNA transfer is less efficient. In *O. novo-ulmi* transfer occurs slowly at a negligible rate of 4 % following an incompatible anastomosis reaction (Brasier, 1984, 1986). Developing a dsRNA-mediated biocontrol strategy for *C. parasitica* was successful in Europe, but unsuccessful in the U.S.A. (reviewed by Nuss (1992)). This was due to a low number of VCGs in Europe, such that dsRNA dissemination via hyphal anastomosis was successful, whereas in the U.S.A., a greater number of VCGs were present such that the majority of hyphal anastomosis reactions were followed by incompatible reactions. This is exemplified by studies by Anagnostakis *et al.* (1986) who found only 2 VCGs throughout both Greece and Germany and 8 in Croatia. Within Modena in Italy, only 4 VCGs were present (Zambonelli and Zechini d'Aulerio, 1986). Within single field sites, only one VCG was usually found (Grente, 1981). In stark contrast; in West Virginia and Connecticut, 27 and 48 different VCGs, respectively, were reported in single small clear-cut plots (Anagnostakis and Kranz, 1987; Milgroom *et al.*, 1991). This greatly limited dissemination of dsRNA, as the majority of anastomoses between neighbouring mycelial networks were incompatible. It is, therefore, empirical for biocontrol to determine the VCGs of field populations of *R. solani*.

In the present work, at the tuber level, isolates of *R. solani* were fully compatible with one another, indicating that dsRNA dissemination should not be impeded. However, the degree of incompatibility increased greatly when isolates from differing tubers from within a single field site were examined, with 44 % of the total reactions being incompatible. This increased to 100 % of pair-wise combinations being incompatible when isolates were harvested from differing areas of Scotland, and indeed from differing countries. Similar results were obtained by van den Boogert *et al.* (1998a) who found that compatible reactions occurred at a frequency of < 1 % when pairing AG 2-2 IIIB isolates from distant plants. In contrast, isolates from single or neighbouring plants showed a high frequency of compatible fusions. Studies by MacNish *et al.* (1993) also showed similar results. AG 8 isolates from the same patch were fully compatible with one another, but incompatible with isolates from other patches. Additionally isolates from south Australia were incompatible with isolates from both America and western Australia. From these combined results, dissemination of dsRNA throughout natural field populations of *R. solani* is likely to be limited to small areas, with no widespread transmission beyond adjoining colonies. Potentially, this could be overcome by using isolates from multiple VCGs. However, due to the lack of compatibility between any isolates from diverse sources, this may not be entirely successful. This lack of vegetative compatibility is not observed to the same extent in other species. For example, in *O. novo-ulmi*, 90 % and 47 % of isolates belong to the same VCG in Portugal and Poland, respectively. In North American *C. parasitica* populations the VCG diversity is high. This led to the need to produce cDNA from hypovirus CHV1-713 (Choi and Nuss, 1992b), which was then successfully transformed and expressed in virulent *C. parasitica* isolates, converting them to the hypovirulent phenotype. These novel strains contain a chromosomally integrated copy of the viral RNA, in addition to cDNA-derived cytoplasmic dsRNA forms, which are fully transmissible via anastomosis. The presence of the chromosomally integrated copy of the viral RNA enables the potential additional transmission routes via conidia and via ascospores through nuclear inheritance, therefore overcoming the previous barrier to dissemination posed by vegetative compatibility. Further studies indicate that the CHV1-713 cDNA can be transformed into other species, which including *C. cubensis*, *C. havanensis*, *C. radicalis* and *Endothia gyrosa* (Chen *et al.*, 1994, 1996). Upon transformation, isolates displayed altered morphology, reduction in phenol oxidase

production (except *E. gyrosa*) and attenuation of fungal virulence. Subsequent studies indicated that transformed *C. cubensis* and *E. gyrosa* strains were unable to elicit infections, indicating that biocontrol could be successfully achieved in several other species using cDNA derived from CHV1-713. Indeed, it may be possible that the cDNA can be transformed into *R. solani* isolates, with subsequent expression of the hypovirulent phenotype. However, a suitable transformation system is first required. The work of chapter 6 involves development of a transformation protocol, and transformation with cDNA derived from CHV1-713.

An alternative means of circumventing the problems associated with VCGs would be to suppress the vegetative incompatibility reactions using antisense mRNA to block the *vic* gene expression (Benedetti *et al.*, 1987).

An interesting anomaly in the vegetative compatibility results of the present study is the lack of reproducibility between isolates from differing tubers, where one compatible result could be followed by two incompatible results and vice versa in triplicate tests. Similar inconsistencies were reported by Julian *et al.* (1996) when single spore isolates (SSI), derived from an AG 1 isolate, were paired either with their parent of other SSI from the group. Vegetative compatibility is essentially controlled by two factors. (i) Environmental factors such as nutrient stress leads to the majority of hyphal anastomoses, including self-reactions, being incompatible fusions. This, however, cannot account for the variability reported here, as the media and growth conditions used throughout were unaltered. Further environmental factors which may alter the vegetative compatibility response in *R. solani* include ultra-violet light, which has been shown to cause instability of VCGs in *C. parasitica* (Rizwana and Powell, 1992). (ii) Genetical events underlying vegetative compatibility are highly complex, and as yet uncharacterized in *R. solani*. In *Podospora anserina*, nine genetic loci control vegetative incompatibility (Esser, 1974; Labarere *et al.*, 1974); six of these loci have two alleles each, and three of them have three, four and five alleles. The total number of genotypes possible from such an arrangement would be 7680. For isolates to be fully compatible they must be identical at each genetic locus, with a difference at one locus being enough to produce an incompatible reaction. Due to the close similarity of phenotypic characteristics (e.g. virulence and pectic enzymes) of the FT field isolates used in this study, they are likely to have a similar genetic background, with isolates from a single potato tuber presumably being clonal, hence their compatible anastomosis

reaction. Assuming radial spread of *R. solani* from a point source, the longer growth has occurred, the greater is the probability of mutagenesis occurring within the vegetative compatibility loci, in addition to alteration in nuclear content. Indeed successive laboratory subculture may also result in modification, which may lead to variation in vegetative compatibility responses. However, reports indicate that many VCGs are stable throughout laboratory manipulations (Leslie, 1993). For example, Anagnostakis (1977) found no evidence for instability of VCGs in *C. parasitica*. In *Neurospora crassa*, alteration in VCG was associated with gross genomic re-organization as opposed to single loci mutations (Pittenger, 1964). In addition, since death during incompatibility responses is a gradual response, a single locus mutation could cause only a weak incompatible response, which may be observed following 48 hours, or in other cases after 200 hours. A difference in two loci, however, may cause a stronger reaction, such that death occurs quickly in every case. However, M.C. Julian (pers. comm.) found that both compatibility and incompatibility reactions were stable for up to 3 to 4 weeks incubation on cellophane-overlaid water agar plates.

A further source of variation which may influence vegetative compatibility gene expression includes epistatic genes, modifiers and suppressors (Julian *et al.*, 1996). Potentially the dsRNA viruses produce DNA copies which then insert into the host genome, possibly within the vegetative compatibility loci. This would then lead to gene disruption and hence an incompatibility reaction following anastomosis. Evidence of genomic incorporation of dsRNA into *R. solani* has been reported by Lakshman and Tavantzis (1994) and Tavantzis (1994). Similarly, sequence homology has been reported between dsRNA elements and nuclear DNA within various plant species.

With respect to using *V. biguttatum* as a dsRNA mobilizing agent, isolate M 92 gave a high degree of sclerotial suppression on AG 3 isolates, but not so high with isolates from other anastomosis groups. Since it is thought that *V. biguttatum* exerts its effect on *R. solani* by forming a continuing nutrient sink throughout the mycelial network (van den Boogert and Deacon, 1994), this would then prevent the formation of sclerotia by diverting nutrients away from sclerotial production to the *V. biguttatum* inoculum. Microscopic observations of the interaction between *R. solani* and *V. biguttatum* indicate that rapid cytoplasmic streaming of 8 to 11 $\mu m\ sec^{-1}$ occurs towards *V. biguttatum* (Deacon, 1996). Thus, the differences in sclerotial suppression between

the AGs could be due to a lower rate of *V. biguttatum*-induced cytoplasmic streaming, thus enabling differing proportions of nutrients to be re-diverted into the sclerotia. If this is the situation, then increased dissemination of dsRNA in the presence of *V. biguttatum* may be limited in some isolates (e.g. 2tR105, which shows 29.8 % sclerotial suppression), but greater in isolates showing greater suppression (e.g. 08R01 which shows 100 % sclerotial suppression in the presence of *V. biguttatum*).

CHAPTER 5

EFFECTS OF dsRNA ON FUNGAL PHENOTYPE

5.1. Introduction

dsRNA elements are present within *R. solani* in varying degrees of abundance. What effect these elements have on the host phenotype is still a cause of controversy. Initial reports indicated that the presence of dsRNA was associated with a hypovirulent phenotype (Castanho and Butler, 1978a). However, this was subsequently contradicted by Finkler *et al.* (1985), who reported that dsRNA was associated with virulent isolates of *R. solani*. Several subsequent reports indicated dsRNA to be ubiquitous within *R. solani*, with no apparent correlation between the presence of dsRNA and the degree of virulence (Zanzinger *et al.*, 1984; Hyakumachi *et al.*, 1985; Bharathan and Tavantzis, 1990; Bharathan and Tavantzis, 1991; Washington and Martin, 1991; Kousik *et al.*, 1994). It is likely that specific dsRNA elements give rise to certain phenotypes. A similar situation occurs in *Ophiostoma novo-ulmi* where many isolates contain multiple dsRNA segments, with three specific segments (2.43, 0.95 and 0.33 kb) being required to invoke the hypovirulent phenotype in the host (Rogers *et al.*, 1986; Rogers *et al.*, 1988). To try and correlate dsRNA with a certain phenotype, various approaches are available. Curing of isolates can be completed, such that the presence/ absence of dsRNA segments can be studied in the same genetic background. Alternatively, cDNA can be constructed and subsequently transformed into a dsRNA-free isolate. This, though, requires the development of a transformation procedure. In addition, cDNA could be used for sequence determination, to determine the coding capacity, and potential intracellular functions.

The work in this chapter aimed to cure isolates of dsRNA and subsequently correlate the alteration with virulence. Additionally, hybridization studies were completed to determine whether homology exists between dsRNA of *R. solani* and hypovirus CHV1-713 of *C. parasitica*.

5.2. Results

5.2.1. Curing by hyphal tip subculture

Initial attempts were made to cure isolates of dsRNA used hyphal tip subculture. This technique has been reported to give curing frequencies of 25 % (McCabe, 1994). Three AG 3 isolates, FT 10, FT 201 and FT 204, were grown on potato dextrose agar (PDA) and the young hyphal tips repeatedly excised to fresh PDA. Each generation formed was examined for dsRNA content (Section 2.4.12). The parental FT 10 isolate contained four dsRNA segments of sizes 10.0, 9.0, 1.0 and 0.8 kb. After the third successive hyphal tip subculture the progeny were unaltered in dsRNA content compared with the parental isolate (Table 5. 1). Isolate FT 204 contained seven dsRNA segments of 9.5, 8.0, 6.5, 3.0, 1.6, 1.0 and 0.8 kb. Ten successive generations were examined for dsRNA content and again all derivatives contained the same dsRNA segments as the parental isolate (Table 5. 1). A third isolate FT 201 was examined over ten successive generations from hyphal tips. The parental isolate contained five dsRNA segments of 9.5, 6.5, 1.6, 1.3 and 1.0 kb. The 1.3 kb segment was lost following the second hyphal tip subculturing (Table 5. 1), but there was no further alteration in dsRNA content up to the tenth generation.

	DsRNA segments (Kb) in isolates		
Generation number	**FT 201**	**FT 204**	**FT 10**
PARENT	**9.5, 6.5, 1.6, 1.3, 1.0**	**9.5, 8.0, 6.5, 3.0, 1.6, 1.0, 0.8**	**10.0, 9.0, 1.0, 0.8**
1	As parent	As parent	As parent
2	Lost 1.3 kb segment	As parent	As parent
3	Lost 1.3 kb segment	As parent	As parent
4	Lost 1.3 kb segment	As parent	
5	Lost 1.3 kb segment	As parent	
6	Lost 1.3 kb segment	As parent	
7	Lost 1.3 kb segment	As parent	
8	Lost 1.3 kb segment	As parent	
9	Lost 1.3 kb segment	As parent	
10	Lost 1.3 kb segment	As parent	

Table 5. 1 Attempted curing of dsRNA from *Rhizoctonia solani* AG 3 isolates. Isolates were grown on potato dextrose agar (PDA), and the young hyphal tips excised to fresh PDA for up to ten times before the dsRNA content was examined using CF11 cellulose chromatography.

5.2.2. Vegetative compatibility of hyphal tip subcultures

The vegetative compatibility of hyphal tip derivatives was examined to determine whether partially or fully cured isolates would be useful for dsRNA-transfer studies. Four hyphal tip subcultures from each generation were paired with their respective parental strains on cellophane-overlaid water agar, and examined microscopically for anastomosis reactions (Section 2.4.3). Each reaction was examined in triplicate. For isolates FT 10 and FT 204, a high degree of vegetative incompatibility of hyphal tip subcultures towards the parental isolates was observed (Figure 5. 1). Vegetative incompatibility reactions accounted for 73 % and 49 % of total observations for isolates FT 10 and FT 204, respectively. After the 7^{th} and 8^{th} generations, for isolates FT 204 and FT 10 respectively, all hyphal tip progeny showed triple incompatibility reactions with their parental isolates. Hyphal tip derivatives of isolate FT 201 showed much less incompatibility with their parental isolate, with only 7 % of total reactions being incompatible (Figure 5. 2). After the tenth generation, all four hyphal tip derivatives showed triple compatible reactions with the parental isolate.

5.2.3. Effect of cycloheximide on *R. solani*

Cycloheximide inhibits protein synthesis by inhibiting cytosolic peptidyl transferase activity of the 60S ribosomal subunit (Stryer, 1988). Organellar protein synthesis is not inhibited (Alberts *et al.*, 1989). A secondary effect of cycloheximide is inhibition of RNA synthesis, which has been reported to cure isolates of dsRNA in other species (Bottacin *et al.*, 1994; Elias and Cotty, 1996). Four isolates, PA1, Papa, FT 201 and FT 204, were therefore plated on potato dextrose agar (PDA) containing various concentrations of cycloheximide (Section 2.4.13). In each case ten replicates were incubated at 23°C for 2 weeks. For all isolates, a decrease in growth rate was observed with increasing cycloheximide concentration (Figure 5. 3). The morphology of colonies was also altered in response to cycloheximide (Figure 5. 4).

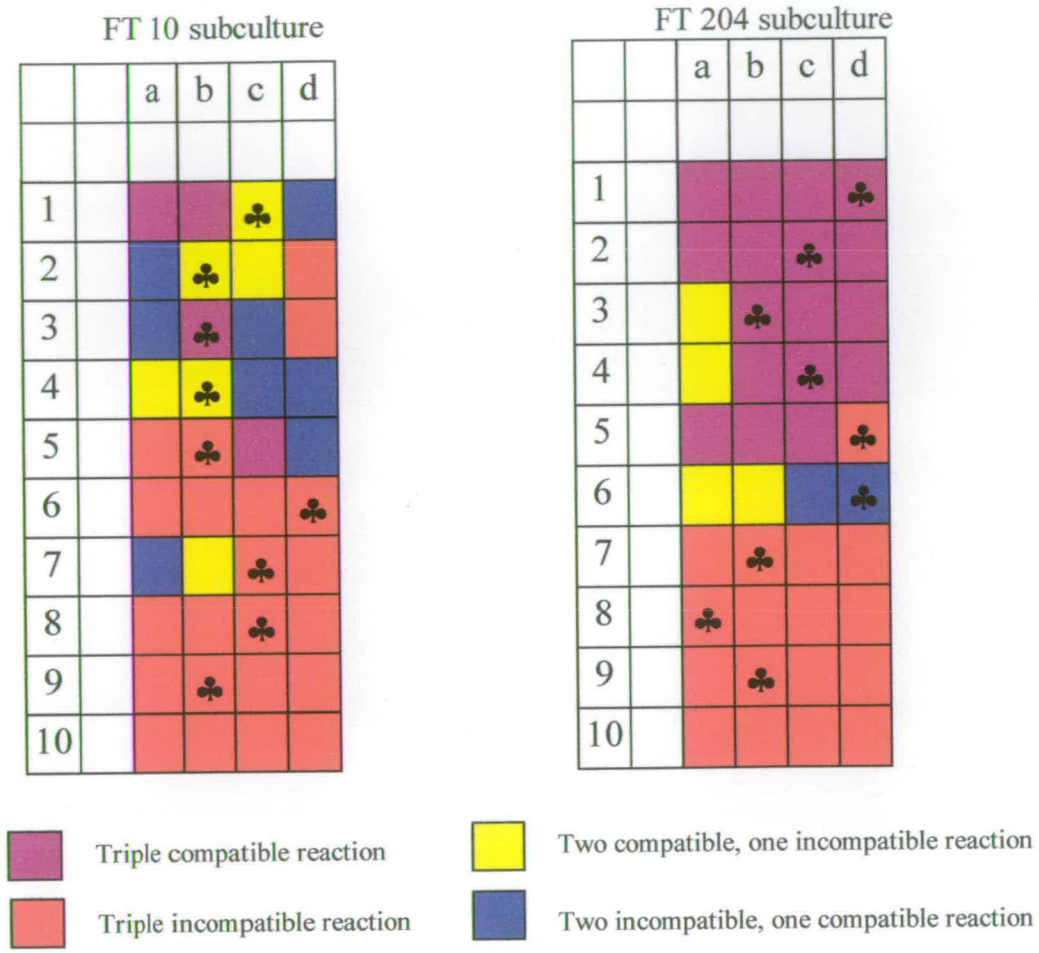


Figure 5. 1 Vegetative compatibility reactions of four hyphal tip subcultures (a-d), per generation, with their parental isolates, FT 10 and FT 204. Observations were made microscopically, in triplicate, on cellophane-overlaid water agar plates. ♣ denotes the isolates used to generate successive subcultures.

dsRNA content was examined for a selection of the above isolates from differing cycloheximide concentrations. Following two weeks incubation five cultures of PA1 and two cultures of Papa were unaltered in their dsRNA content compared with their parental isolates (Table 5. 2). Three cultures of cycloheximide-treated FT 201 were examined. One was partially cured, losing two of five dsRNA segments (Table 5. 2). Parental isolates were re-exposed for a four week period, to determine whether greater curing could be attained. Ten cultures were examined, of which six had undergone

partial curing (Table 5. 3). Cultures generally lost the smaller dsRNA segments, i.e. 1.6, 1.4 and 1.0 kb; however, one isolate of FT 201 lost a larger segment of size 6.5 kb. Due to the increased curative effect of a longer exposure time, parental isolates were incubated for an 8 week period on cycloheximide. Ten cultures were examined, with four showing partial curing (Table 5. 4). Three of these cultures lost 1.4 and 1.0 kb segments and one culture lost a 1.6 kb segment. One culture of strain Papa had gained two dsRNA segments of sizes 1.0 and 0.8 kb (Table 5. 4). Its corresponding parent contained only a single 12 kb dsRNA segment. This suggests that the smaller dsRNA elements may be formed from internal deletion events of larger dsRNA segments, in a similar manner to that occurring with dsRNA of *C. parasitica* (Shapira *et al.*, 1991a).

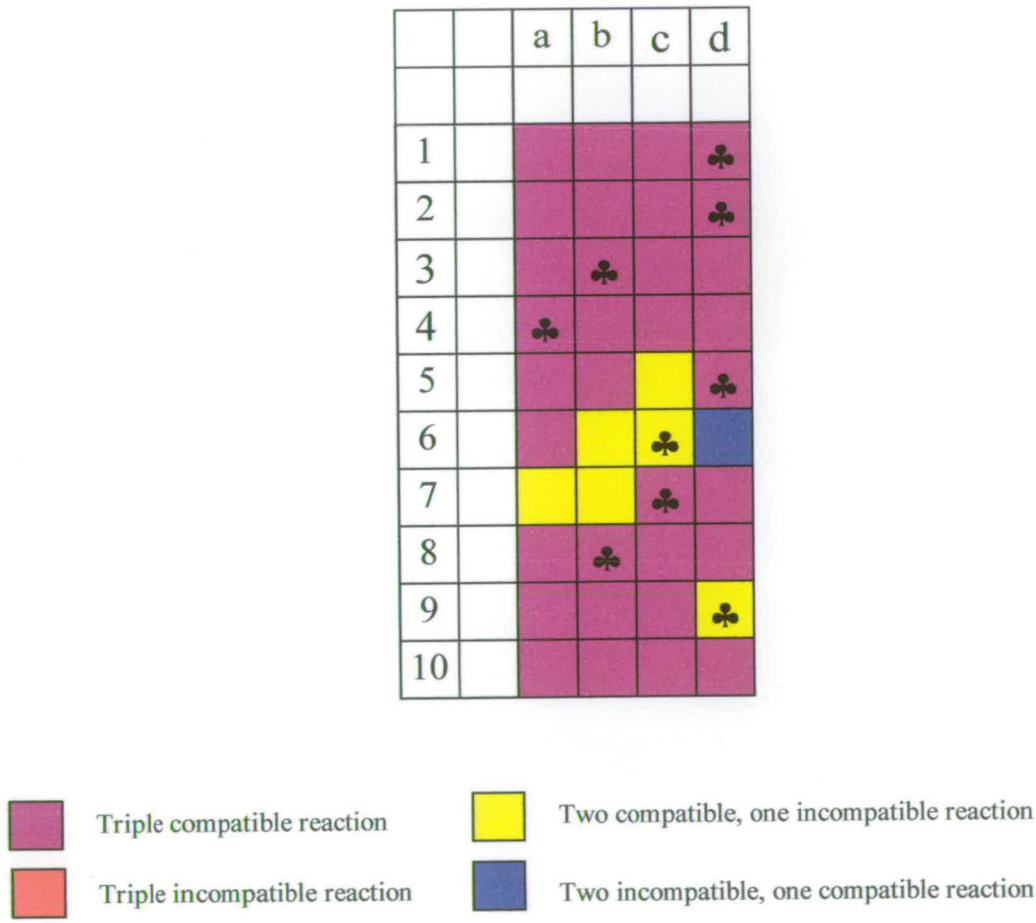
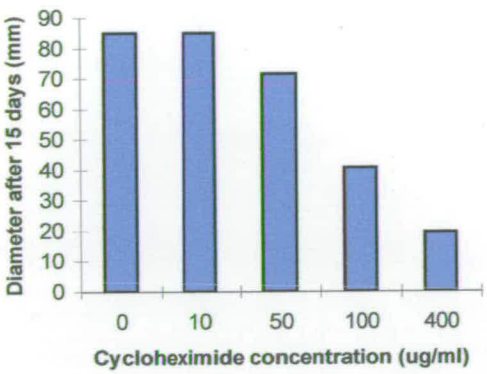
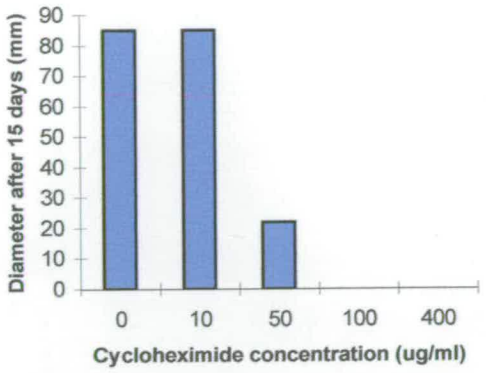


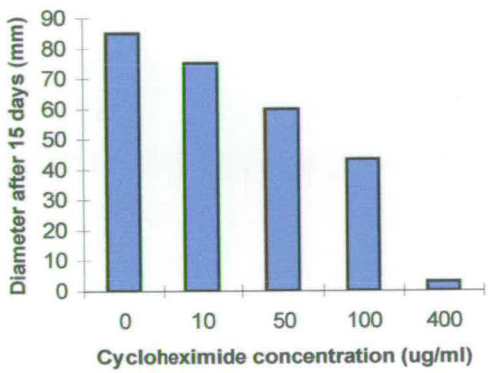
Figure 5. 2 Vegetative compatibility reactions of four hyphal tip subcultures (a-d), per generation, with their parental isolate FT 201. Observations were made microscopically, in triplicate, on cellophane-overlaid water agar plates. ♣ denotes the isolates used to generate successive subcultures.



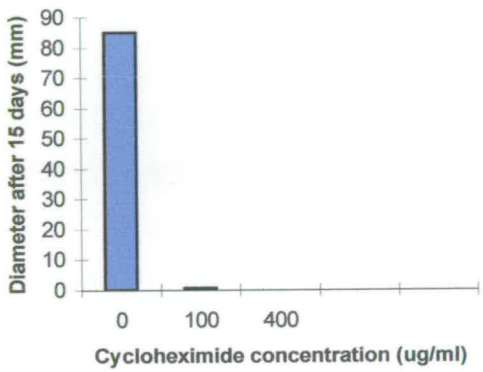
(a)



(b)



(c)



(d)

Figure 5. 3 Colony diameters of *R. solani* isolates when grown on PDA containing 0 to 400 μg ml^{-1} cycloheximide for 15 days at 23°C. Isolates are as follows: (a) PA1 (b) FT 201 (c) Papa and (d) FT 204.

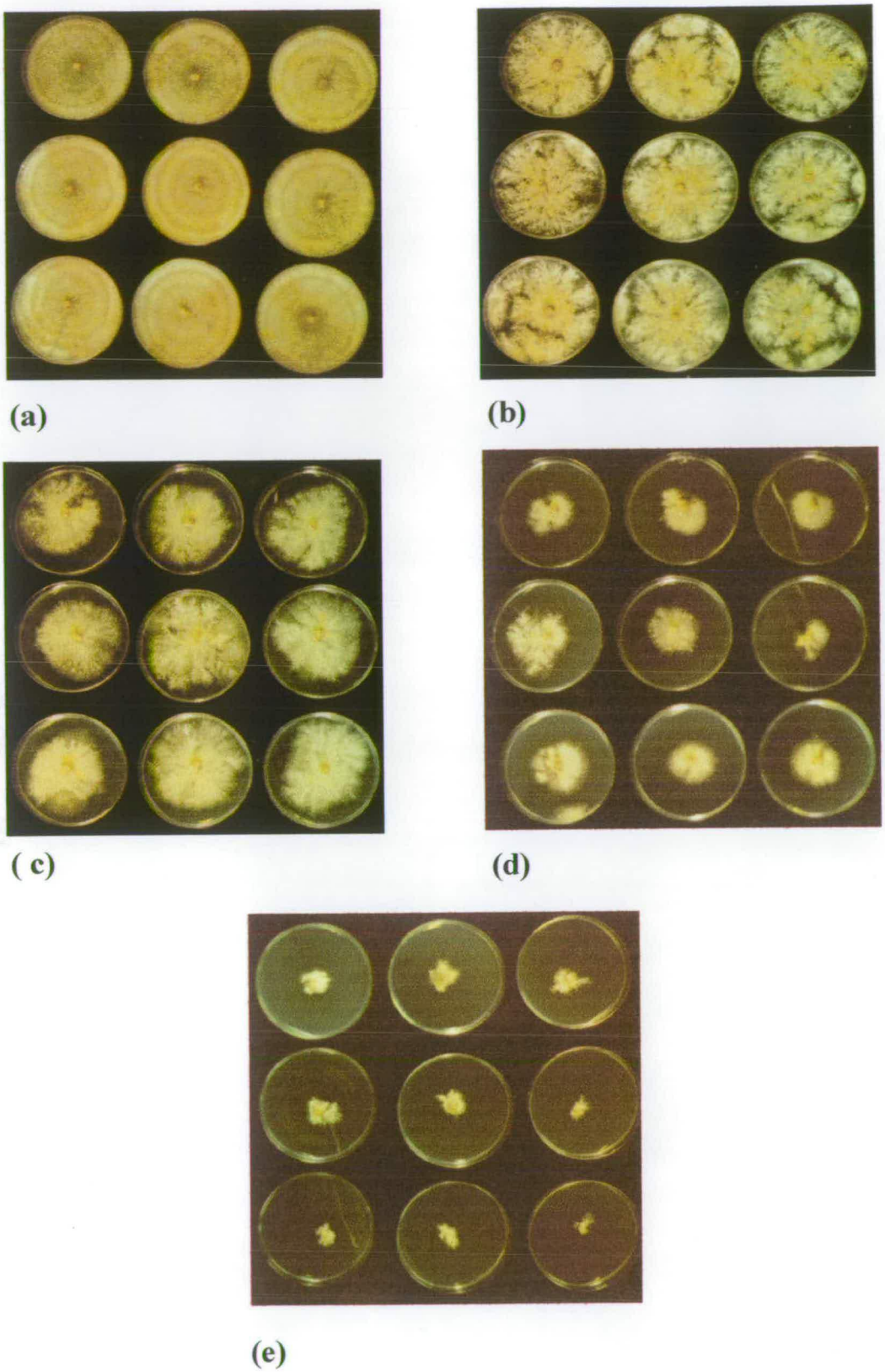


Figure 5. 4 Change in morphology of *Rhizoctonia solani* strain PAl, when exposed to cycloheximide at the following concentrations: (a) 0 μg ml^{-1} (b) 10 μg ml^{-1} (c) 50 μg ml^{-1} (d) 100 μg ml^{-1} and (e) 400 μg ml^{-1}. Plates photographed after 14 days incubation at 23°C.

Isolate	Cycloheximide concentration (μg ml^{-1})	dsRNA segments (kb)
PA1	**0**	**12, 1.4, 1.0**
PA1-CT2a	10	As parent
PA1-CT2b	10	As parent
PA1-CT2c	50	As parent
PA1-CT2d	400	As parent
PA1-CT2e	400	As parent
Papa	**0**	**12**
Papa-CT2a	100	As parent
Papa-CT2b	100	As parent
201	**0**	**9.5, 6.5, 1.6, 1.3, 1.0**
201-CT2a	50	Lost 6.5, 1.6 kb segments
201-CT2b	50	As parent
201-CT2c	50	As parent

Table 5. 2 Partial curing of dsRNA from *Rhizoctonia solani* isolates, following 2 weeks exposure to differing concentrations of cycloheximide. DsRNA content was determined using CF11 cellulose chromatography. CT2 denotes subcultures exposed to cycloheximide for 8 weeks, a to e indicates separate replicates of each parental isolate.

Isolate	Cycloheximide concentration (μg ml^{-1})	dsRNA segments (kb)
PA1 parent	**0**	**12, 1.4, 1.0**
PA1-CT4a	400	Lost 1.4, 1.0 kb segments
PA1-CT4b	400	As parent
PA1-CT4c	400	Lost 1.4, 1.0 kb segments
PA1-CT4d	400	Lost 1.4, 1.0 kb segments
Papa parent	**0**	**12**
Papa-CT4a	400	As parent
Papa-CT4b	400	As parent
201 parent	**0**	**9.5, 6.5, 1.6, 1.3, 1.0**
201-CT4a	50	Lost 6.5, 1.6 kb segments
201-CT4b	50	Lost 1.6 kb segment
201-CT4c	100	Lost 1.6 kb segment
201-CT4d	100	As parent

Table 5. 3 Partial curing of dsRNA from *Rhizoctonia solani* isolates, following 4 weeks exposure to differing concentrations of cycloheximide. DsRNA content was determined using CF11 cellulose chromatography. CT4 denotes subcultures exposed to cycloheximide for 4 weeks, a to d indicates separate replicates of each parental isolate.

Isolate	Cycloheximide concentration (μg ml^{-1})	dsRNA segments (kb)
PA1	**0**	**12, 1.4, 1.0**
PA1-CT8a	10	Lost 1.4, 1.0 kb segments
PA1-CT8b	10	As parent
PA1-CT8c	50	Lost 1.4, 1.0 kb segments
PA1-CT8d	50	As parent
PA1-CT8e	400	As parent
PA1-CT8f	400	Lost 1.4, 1.0 kb segments
Papa	**0**	**12**
Papa-CT8a	400	Gained 1.0, 0.8 kb segments
Papa-CT8b	400	As parent
201	**0**	**9.5, 6.5, 1.6, 1.3, 1.0**
201-CT8a	50	As parent
201-CT8b	50	Lost 1.6 kb segment

Table 5. 4 Partial curing of dsRNA from *Rhizoctonia solani* isolates, following 8 weeks exposure to differing concentrations of cycloheximide. DsRNA content was determined using CF11 cellulose chromatography. CT8 denotes subcultures exposed to cycloheximide for 8 weeks, a to f indicates separate replicates of each parental isolate.

5.2.4. Vegetative compatibility of isolates exposed to cycloheximide

Subcultures from isolates exposed to cycloheximide for 2, 4 and 8 weeks were paired with their parental isolates and their vegetative compatibility examined on cellophane overlaid water agar (Section 2.4.3). Pair-wise combinations were examined in triplicate. Following two weeks exposure to cycloheximide, 76.7 % of the total reactions were vegetatively compatible reactions (Table 5. 5). Subcultures of Papa and FT 201, when exposed to 100 μg ml^{-1} and 50 μg ml^{-1} cycloheximide respectively, showed triple compatible reactions. Subcultures of PA1 exposed to 10 μg ml^{-1} cycloheximide displayed triple compatible reactions, whereas subcultures exposed to 400 μg ml^{-1} showed triple incompatible reactions. Following four weeks exposure to

cycloheximide, the degree of vegetative compatibility had decreased sharply to 40 % of the total reactions (Table 5. 6). Subcultures of Papa exposed to 400 μg ml^{-1} showed triple incompatible reactions, as did three subcultures of PA1 exposed to 400 μg ml^{-1} cycloheximide. Isolates of FT 201 exposed to 50 μg ml^{-1} cycloheximide showed triple compatible reactions. Following eight weeks exposure to cycloheximide, only 10 % of total reactions were vegetatively compatible (Table 5. 7). No pair-wise combinations gave triple compatible reactions. Prolonged exposure to cycloheximide therefore decreases the probability of a vegetatively compatible reaction with the respective parental isolate.

		Compatibility with parental isolate		
Strain	**Cycloheximide concentration (μg ml^{-1})**	**1**	**2**	**3**
PA1	**0**	**C**	**C**	**C**
PA1-CT2a	10	C	C	C
PA1-CT2b	10	C	C	C
PA1-CT2c	50	C	C	I
PA1-CT2d	400	I	I	I
PA1-CT2e	400	I	I	I
Papa	**0**	**C**	**C**	**C**
Papa-CT2a	100	C	C	C
Papa-CT2b	100	C	C	C
201	**0**	**C**	**C**	**C**
201-CT2a	50	C	C	C
201-CT2b	50	C	C	C
201-CT2c	50	C	C	C

Table 5. 5 Vegetative compatibility reactions of subcultures exposed to cycloheximide for a two week period, when paired with their parental isolates. Vegetative compatibility reactions were observed microscopically, in triplicate, on cellophane overlaid water agar plates. C = compatible reaction and I = incompatible reaction. CT2 denotes subcultures exposed to cycloheximide for 8 weeks, a to e indicates separate replicates of each parental isolate.

Strain	Cycloheximide concentration (μg ml^{-1})	Compatibility with parental isolate		
		1	2	3
PA1	**0**	**C**	**C**	**C**
PA1-CT4a	400	C	C	C
PA1-CT4b	400	I	I	I
PA1-CT4c	400	I	I	I
PA1-CT4d	400	I	I	I
Papa	**0**	**C**	**C**	**C**
Papa-CT4a	400	I	I	I
Papa-CT4b	400	I	I	I
201	**0**	**C**	**C**	**C**
201-CT4a	50	C	C	C
201-CT4b	50	C	C	C
201-CT4c	100	C	C	C
201-CT4d	100	I	I	I

Table 5. 6 Vegetative compatibility reactions of subcultures exposed to cycloheximide for a four week period, when paired with their parental isolates. Vegetative compatibility reactions were observed microscopically, in triplicate, on cellophane overlaid water agar plates. C = compatible reaction and I = incompatible reaction. CT4 denotes subcultures exposed to cycloheximide for 4 weeks, a to d indicates separate replicates of each parental isolate.

Strain	Cycloheximide concentration (μg ml^{-1})	Compatibility with parental isolate		
		1	2	3
PA1	**0**	**C**	**C**	**C**
PA1-CT8a	10	C	I	I
PA1-CT8b	10	I	I	I
PA1-CT8c	50	I	I	I
PA1-CT8d	50	C	C	I
PA1-CT8e	400	I	I	I
PA1-CT8f	400	I	I	I
Papa	**0**	**C**	**C**	**C**
Papa-CT8a	100	I	I	I
Papa-CT8b	100	I	I	I
201	**0**	**C**	**C**	**C**
201-CT8a	50	I	I	I
201-CT8b	50	I	I	I

Table 5. 7 Vegetative compatibility reactions of subcultures exposed to cycloheximide for a eight week period, when paired with their parental isolates. Vegetative compatibility reactions were observed microscopically, in triplicate, on cellophane overlaid water agar plates. C = compatible reaction and I = incompatible reaction. CT8 denotes subcultures exposed to cycloheximide for 8 weeks, a to f indicates separate replicates of each parental isolate.

5.2.5. Effect of partial curing on pathogenicity

Previous reports have linked the presence of specific dsRNA-segments to increased or decreased virulence of the fungal host (Castanho *et al.*, 1978; Buck, 1986; Nuss and Koltin, 1990). In Section 5.2.3, isolates obtained following four weeks exposure to cycloheximide were partially cured of dsRNA. These isolates, plus isolates exposed to cycloheximide showing no change in dsRNA content, were examined for their pathogenicity and compared with parental isolates. Trials were completed as described in Section 2.4.6 using the following host plants: tomato (*Lycopersion esculentum*), radish (*Raphanus sativus*), carrot (*Daucus carota*), lettuce (*Lactuca sativa*), cress (*Barbarea praecox*) and onion (*Allium cepa*). Pathogenicity was recorded following 10 days incubation, on a disease index scale ranging from 0 to 5 based on the relative size of the necrotic area on the hypocotyl as follows: 0 = no disease; 1 = 1 - 10

%; 2 = 11 - 30 %; 3 = 31 - 50 %; 4 = 51 - 80 % and 5 = entire hypocotyl infected. Isolates with a mean infectivity between 0 and 1 were considered non-pathogenic.

Examination of roots indicated that for PA1, the disease index was fairly high, with the mean index for the six host crops ranging from 2.5 to 3.8, and an overall mean of 3.4 (Table 5. 8). Analysis of variance indicated no significant differences between the isolates regarding disease severity. Thus, for isolate PA1, loss of the 1.4 and 1.0 kb segments, plus exposure to cycloheximide with no change in dsRNA content, had no effect on overall fungal pathogenicity. For isolate FT 201, the disease index was low, with the mean index for the six host crops examined ranging from 1.2 to 1.5, and an overall mean of 1.3 (Table 5. 8). Again, no significant differences occurred between the isolates regarding disease severity. Thus the 6.5 and 1.6 kb segments have no significant effect on overall fungal pathogenicity.

Infection of shoots was less severe than root infection for both FT 201 and PA1 (Table 5. 8 and Table 5. 9). Analysis of variance indicated no significant difference between strains regarding disease severity for both PA1 and FT 201 (Table 5. 9).

	Disease index on host crops*						
Isolate	**Radish**	**Tomato**	**Carrot**	**Lettuce**	**Onion**	**Cress**	**Mean**
PA1	4.7 ± 0.1	0.5 ± 0.1	3.7 ± 0.3	3.6 ± 0.2	3.9 ± 0.4	4.6 ± 0.2	3.5
PA1-CT4a	4.7 ± 0.1	0.7 ± 0.2	4.5 ± 0.2	4.1 ± 0.2	4.1 ± 0.5	4.9 ± 0.1	3.8
PA1-CT4b	4.7 ± 0.1	0.2 ± 0.1	4.0 ± 0.3	3.5 ± 0.3	4.0 ± 0.6	5.0 ± 0	3.6
PA1-CT4c	4.7 ± 0.1	0.3 ± 0.1	4.1 ± 0.3	4.2 ± 0.2	4.3 ± 0.3	4.6 ± 0.1	3.7
PA1-CT4d	4.7 ± 0.1	0.4 ± 0.1	2.4 ± 0.3	3.6 ± 0.2	2.3 ± 0.7	1.4 ± 0.2	2.5
FT201	4.5 ± 0.1	1.0 ± 0.2	0.8 ± 0.2	1.0 ± 0.2	0.6 ± 0.2	0.4 ± 0.2	1.4
201-CT4a	4.5 ± 0.1	2.0 ± 0.3	1.3 ± 0.2	0.6 ± 0.2	0.6 ± 0.4	0.3 ± 0.2	1.5
201-CT4b	4.6 ± 0.1	1.2 ± 0.2	0.9 ± 0.2	0.2 ± 0.1	0.4 ± 0.3	0	1.2
201-CT4c	4.5 ± 0.1	0.8 ± 0.2	0.5 ± 0.1	1.2 ± 0.2	0.6 ± 0.4	0.4 ± 0.2	1.3
201-CT4d	4.4 ± 0.1	1.0 ± 0.2	0.8 ± 0.2	0.9 ± 0.2	0.2 ± 0.2	0.2 ± 0.1	1.2

* Means of 50 replicates

Table 5. 8 Disease index on roots over six host crops, of isolates PA1 and FT 201 following 4 weeks exposure to varying concentrations of cycloheximide. Disease index was recorded for 50 seedlings after 10 days incubation at 22°C. Disease index was recorded on a scale of 0 to 5; 0 = no disease; 1 = 1-10 %; 2 = 11-30 %; 3 = 31-50 %; 4 = 51- 80 % and 5 = entire root infected. Isolates with a mean infectivity between 0 and 1 were considered non-pathogenic. CT4 denotes subcultures exposed to cycloheximide for 4 weeks, a to d indicates separate replicates of each parental isolate.

	Disease index on host crops						
Isolate	**Radish**	**Tomato**	**Carrot**	**Lettuce**	**Onion**	**Cress**	**Mean**
PA1	2.6 ± 0.2	0.5 ± 0.2	1.7 ± 0.4	2.5 ± 0.4	0	4.0 ± 0.3	1.9
PA1-CT4a	2.5 ± 0.3	0.2 ± 0.1	2.2 ± 0.4	2.4 ± 0.4	0	4.9 ± 0.1	2.0
PA1-CT4b	2.1 ± 0.3	0.2 ± 0.1	1.7 ± 0.4	1.6 ± 0.4	0	4.4 ± 0.2	1.7
PA1-CT4c	3.2 ± 0.3	0.5 ± 0.2	2.4 ± 0.5	3.3 ± 0.4	0	4.1 ± 0.3	2.3
PA1-CT4d	1.1 ± 0.2	0.1 ± 0.1	0.6 ± 0.3	1.1 ± 0.3	0	0.8 ± 0.3	0.6
FT201	0.7 ± 0.2	0	0	0	0	0	0.12
201-CT4a	0.9 ± 0.3	0.1 ± 0.1	0	0	0	0.2 ± 0.1	0.2
201-CT4b	0.6 ± 0.2	0.1 ± 0.1	0	0	0	0.2 ± 0.1	0.1
201-CT4c	0.7 ± 0.2	0.2 ± 0.1	0	0	0	0.2 ± 0.1	0.2
201-CT4d	0.6 ± 0.2	0.1 ± 0.1	0	0	0	0	0.1

* Means of 50 replicates

Table 5. 9 Disease index on shoots over six host crops, of isolates PA1 and FT 201 following 4 weeks exposure to varying concentrations of cycloheximide. Disease index was recorded for 50 seedlings after 10 days incubation at 22°C. Disease index was recorded on a scale of 0 to 5; 0 = no disease; 1 = 1-10 %; 2 = 11-30 %; 3 = 31-50 %; 4 = 51- 80 % and 5 = entire shoot infected. Isolates with a mean infectivity between 0 and 1 were considered non-pathogenic. CT4 denotes subcultures exposed to cycloheximide for 4 weeks, a to d indicates separate replicates of each parental isolate.

5.2.6. Comparison of dsRNA from *R. solani* and *C. parasitica*

Partial curing of segments ranging in size from 1.0 to 6.5 kb from isolates FT 201 (AG 3) and PA1 (AG 4) resulted in no change in fungal virulence (Section 5.2.5). Further attempts to elucidate the role of dsRNA in *R. solani* used cDNA of CHV1-713 from *C. parasitica* in low stringency hybridization studies (Section 2.5.11). The plasmid pXH9, which contains the complete cDNA sequence of CHV1-713 (12 712 bp) (Figure 1.7c), was used as a radiolabelled probe (Section 2.5.12). Initial comparisons used dsRNA extracted from four isolates, FT 204 (AG 3), FT 201 (AG 3), PA1 (AG 4) and Papa (AG 4). No hybridization was observed between *R. solani* dsRNA and CHV1-713 from *C. parasitica* at low stringency (Figure 5. 5). Hybridization only occurred towards pAXHY2 and pXH9 (both constructed using sequence from CHV1-713) and dsRNA isolated from *C. parasitica*. Further isolates were examined from a broader range of

anastomosis groups encompassing AG 2-1, 3, 4 and 5. Again no hybridization was observed between CHV1-713 and *R. solani* at low stringency (Figure 5. 6).

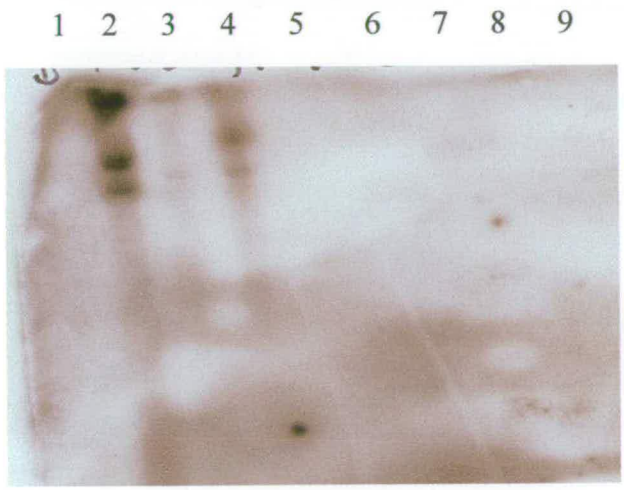


Figure 5. 5 Low stringency hybridization (42°C) of pXH9 (cDNA of CHV1-713 extracted from *C. parasitica*) to dsRNA from *Rhizoctonia solani* isolates from anastomosis groups 3 and 4. Lanes 1 to 10 contain: lane 1, λDNA digested by *Hin*dIII; lane 2, pXH9; lane 3, *C. parasitica* dsRNA; lane 4, pAXHY2; lane 5, FT 204; lane 6, FT 201; lane 7, PA1; lane 8, Papa; and lane 9 FT 201. The two plasmids are constructed from hypovirus CHV1-713 of *C. parasitica*.

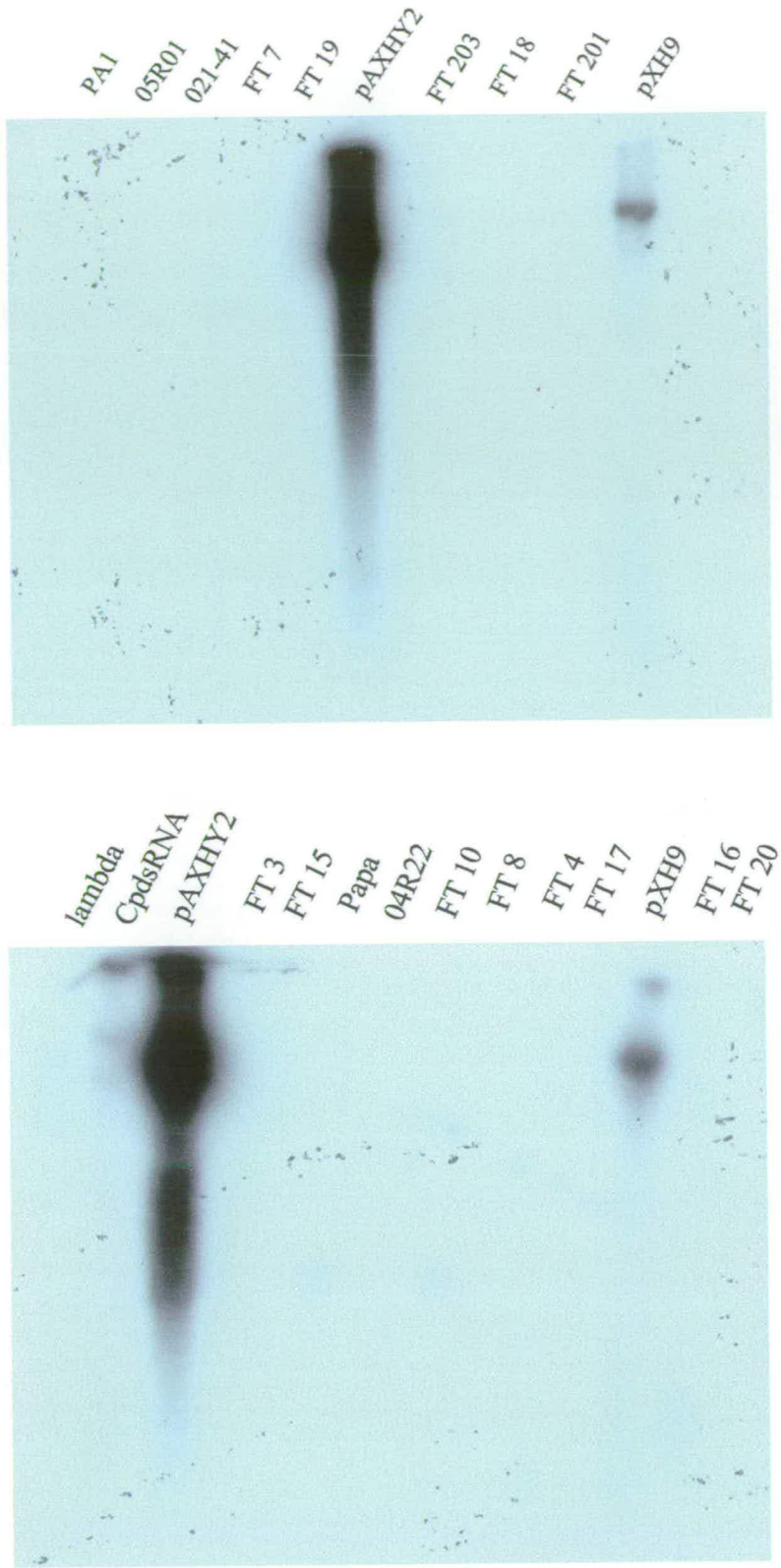


Figure 5. 6 Low stringency hybridization (42°C) of pXH9 (cDNA of CHV1-713 extracted from *C. parasitica*) to dsRNA from *Rhizoctonia solani* isolates from anastomosis groups 2-1, 3, 4 and 5.

A secondary approach in determining function of dsRNA would be to sequence the dsRNA. Initial attempts to construct cDNA, to facilitate sequencing, were unsuccessful. Reactions involved constructing ssDNA from dsRNA using moloney murine leukemia virus (MMLV) reverse transcriptase. Second strand synthesis was completed using polymerase I, with any cDNA inserts formed being ligated into blunt-ended pUC18 vectors. Plasmids formed were transformed into competent DL-5 *Escherichia coli* cells and plated onto LB agar supplemented with ampicillin, IPTG and X-Gal. X-Gal/ IPTG provides a blue/ white screen for transformed colonies resistant to ampicillin. Colonies containing re-ligated pUC18 were differentiated by their blue colour, from those containing the ligated cDNA insert which were white in colour. The transformation reaction was highly inefficient, with only five white colonies forming. These along with three of the blue colonies (re-ligated pUC18) were examined for plasmid content. Plasmids were extracted using mini-preps (Section 2.5.7) and the resulting DNA separated using agarose gel electrophoresis. Only re-ligated pUC18 could be detected (Figure 5. 7), indicating that cDNA construction had been unsuccessful.

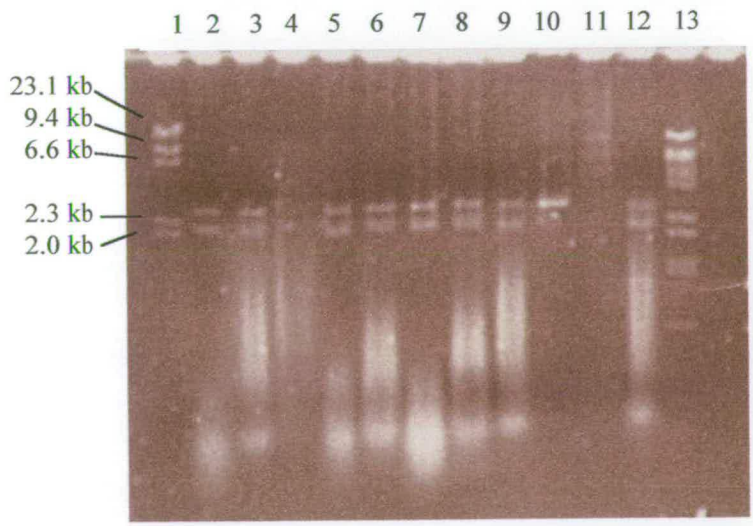


Figure 5. 7 DNA isolated from *Escherichia coli* DL-5 colonies. Colonies were previously transformed with plasmids constructed from pUC18 and cDNA inserts of dsRNA from *Rhizoctonia solani*. Lane 1 contained λ DNA digested with *Hind*III, lanes 2 to 10 contained DNA extracted from transformed colonies, lane 12 contained pUC18 DNA and lane 13 contained λ DNA digested with *Bst*I. All colonies contained pUC18 re-ligated to itself, with no cDNA insert.

5.3. Discussion

To compare phenotypic characteristics, and try and correlate them with the presence or absence of dsRNA, isolates cured of dsRNA are required. Initial attempts used hyphal-tip subculturing as a means of eliminating dsRNA. This technique relies on dsRNA not being located in the hyphal tip cells, which has been shown by electron microscopy to be the case in many fungal species, including *R. solani* (Buck, 1986; Varga *et al.*, 1994). However, for some species, such as *Cryphonectria parasitica*, this is not the case and dsRNA predominates in the tip cells (Newhouse *et al.*, 1983). Hyphal tip subculturing has been reported to give curing frequencies of 25 % in *R. solani* (McCabe, 1994), so this technique was employed initially. The present study examined dsRNA of three isolates following successive hyphal-tip subculturing. Isolate FT 10 contained four dsRNA segments which were unaltered following three successive subcultures and isolate FT 204 was unaltered in dsRNA content following ten successive subcultures. Isolate FT 201 lost a 1.3 kb segment following the second subculture, with no further alteration in dsRNA content following a further nine subcultures. Difficulty in curing isolates by this technique has been reported previously. Castanho and Butler (1978b) examined 150 hyphal tip cultures, of which only 6 grew to form healthy cultures, devoid of dsRNA, giving a curing frequency of 3 %. Finkler *et al.* (1985) obtained a curing frequency of 1 % for AG 4 isolates, although the isolates studied were not fully cured of dsRNA: isolate 53 lost all the major segments of dsRNA, whereas isolate 82 lost two dsRNA segments. These changes, however, were sufficient to alter the host phenotype from virulent to hypovirulent. Tavantzis (1994) also reported partial curing by hyphal tip subculture, eliminating three of four segments from the AG 3 isolate Rhs41.

The lack of curing by hyphal tip subculture in the present study led to incorporation of cycloheximide in the growth medium. Cycloheximide inhibits protein synthesis by inhibiting cytosolic peptidyl transferase activity of the 60S ribosomal subunit (Stryer, 1988); however, a secondary effect of cycloheximide is inhibition of RNA synthesis, which has been reported to cure isolates of dsRNA in other species (Bottacin *et al.*, 1994; Elias and Cotty, 1996). Following two weeks incubation on

cycloheximide, five isolates of PA1 (AG 4) and two isolates of Papa (AG 4) were unaltered in their dsRNA content compared with their parental isolates. One of two isolates of FT 201 (AG 3) was partially cured, losing two (6.5 and 1.6 kb) of five segments. Isolates were re-exposed for a four week period, to determine whether greater curing could be attained. Ten isolates were examined, with six being partially cured. The isolates had generally lost the smaller dsRNA segments, i.e. 1.6, 1.4 and 1.0 kb; however, one isolate of FT 201 lost a larger segment of size 6.5 kb. Consistent with this finding, Fulbright (1984) found that increasing the exposure times to four weeks increased the degree of curing of dsRNA in *Endothia (= Cryphonectria) parasitica.* Due to the increased curative effect of a longer exposure time, isolates were re-incubated for an eight week period on cycloheximide. Four of ten isolates showed partial curing, involving the 1.6, 1.4 and 1.0 kb segments. One isolate (Papa) had gained two dsRNA segments of sizes 1.0 and 0.8 kb, providing evidence that internal deletion events might have occurred. Internal deletions of L-dsRNA elements giving rise to smaller dsRNA segments has previously been reported in *C. parasitica* (Shapira *et al.*, 1991a). Additionally, Bharathan and Tavantzis (1990) provide evidence for internal deletions in *R. solani*, in that the 1.8 kb dsRNA element of Rhs11 cross-hybridized to three other segments from the same isolate.

Therefore, partial curing of *R. solani* seems to be readily attainable following cycloheximide exposure. Partial curing has been reported frequently following cycloheximide exposure in other species. For example, Castillo and Cifuentes (1994) obtained partial curing of dsRNA in the yeast *Phaffia rhodozyma.* The extent of curing is greatly affected by the concentration of cycloheximide used. Fulbright (1984) struggled to remove dsRNA segments from *C. parasitica* using 10 $\mu g\ ml^{-1}$ cycloheximide, whereas 20 $\mu g\ ml^{-1}$ was highly effective in eliminating dsRNA. This, however, was not the case in the present study, as a range of cycloheximide concentrations (0 to 400 $\mu g\ ml^{-1}$) were used, and 400 $\mu g\ ml^{-1}$ inhibited growth of all isolates. The curing achieved in the present study was restricted to the smaller sized segments e.g. 1.0, 1.4, 1.6 and 6.5; the larger elements, such as the 10 to 12 kb segments, were unaffected. Differential curing of specific segments has previously been reported by Bottacin *et al.* (1994). Upon cycloheximide exposure, isolates of *Chalara elegans* lost the large size bands, with a small 2.8 kb band always remaining. The

intensity of the 2.8 kb band increased following loss of larger segments, possibly indicating fragmentation of the larger segments.

Various other methods have been reported to eliminate dsRNA from fungi. Castanho and Butler (1978b) used a variety of techniques to cure *R. solani* of dsRNA, with varying degrees of success. Antibiotics such as streptomycin, tetracycline, chloramphenicol and gentamycin had no curative abilities. Elevated temperatures of 30°C led to a 3 % curing frequency, whereas temperatures of 52°C had no curative effect. Acridine dyes, which can cure fungi of plasmids (Fink and Styles, 1972) gave a 2.5 % recovery frequency (Castanho and Butler, 1978b). Similarly, use of acridine dyes in *Zygosaccharomyces bailli* gave unsuccessful curing (Radler *et al.*, 1993). These methods, generally, were not promising and were therefore not examined here.

It is possible that the difficulties in curing *R. solani* of dsRNA in the present study may have arisen from a resident, integrated DNA copy of the dsRNA elements. Finkler *et al.* (1985) found no evidence of similarity between the host DNA and the viral genome. However, subsequent studies by Tavantzis (1994) and Lakshman *et al.* (1998) indicated homology between dsRNA segments and the host genome of *R. solani.* In further support of this argument, McCabe (1994) reported isolate Papa to be cured of dsRNA segments. However, re-isolation of dsRNA from this isolate in the present study indicated the presence of a 12 kb segment, perhaps because a resident DNA copy was present, or alternatively poor experimental technique. The isolation procedure employed by McCabe (1994) frequently resulted in faint smearing at high molecular weight positions, which were resolved to form distinct bands when the isolation procedure indicated in Section 2.4.12 was employed. However, in further support of the possibility of a resident DNA copy, Koltin *et al.* (1987) re-examined the isolates of Castanho and Butler (1978b) which were reported dsRNA-free, and found them to contain dsRNA. An integrative state may have evolved, as the dsRNA may have a role to play in the host lifestyle. This is supported by A. Finkler (pers. comm.) who found that isolates cured of dsRNA were unstable upon subculture. McCabe (1994) also obtained similar results, with one of four cured derivatives not being maintained upon subculture.

As previously mentioned in chapter 4, the presence/ absence of dsRNA can affect fungal virulence. Since alteration of fungal virulence may require only single

segments or sequences of dsRNA, the virulence of isolates exposed to cycloheximide for four weeks was examined. For isolate PA1, loss of the 1.4 and 1.0 kb segments had no effect on overall fungal pathogenicity. Similarly for isolate FT 201, loss of the 6.5 and 1.6 kb segments had no significant effect on overall fungal pathogenicity. However, since these may be internal deletions of the larger dsRNA segments, the genetic information affecting virulence may still remain, in which case no change in virulence would be expected. Bharathan and Tavantzis (1990) have previously reported evidence for internal deletions, with low molecular weight segments cross-hybridizing to larger segments in the same isolate. In the present study, the loss of individual segments did not alter virulence; however, a recent report by Jian *et al.* (1997) indicates that individual dsRNA segments can affect virulence of *R. solani.* Removal of a 6.4 kb segment (M1) by hyphal tip subculture resulted in a loss of virulence, whereas acquisition of this segment led to increased virulence. In contrast acquisition of a 3.6 kb segment (M2) was associated with a decline in virulence. Direct evidence linking these two genes to virulence, though, is required via transformation studies. Chapter 6 outlines the development of a transformation protocol.

In an attempt to determine the potential coding capacity of the dsRNA elements used in the present study, and those reported in chapter 4, hybridization studies were completed using pXH9 as a radiolabelled probe. No hybridization was observed at low stringency between any dsRNA from *R. solani* and pXH9, indicating that no homology exists between *R. solani* dsRNA and hypovirus CHV1-713 from *C. parasitica.* In contrast, Lakshman *et al.* (1998) found M2 dsRNA to be phylogenetically related to the RNA-dependent RNA polymerase (RDRP) of a mitochondrial dsRNA associated with hypovirulence in *C. parasitica* strain NB631. This relation is likely to be segment specific with other unrelated dsRNA elements of *R. solani* occurring, some of which may indeed show sequence homology to CHV1-713. Earlier studies by Finkler *et al.* (1985) failed to show any homology between dsRNA from *Ustilago maydis* and *R. solani.*

Thus any attenuation of virulence in these *R. solani* isolates by dsRNA is mediated by a separate mechanism than that of CHV1-713 in *C. parasitica.* To elucidate the function of these dsRNA elements, sequencing would ideally be required. This initially involves the construction of cDNA, which is subsequently used for sequencing. This was attempted as described in Section 5.2.6; however, construction

was unsuccessful. Very recent reports by Jian *et al.* (1998) and Lakshman *et al.* (1998) were successful in constructing cDNA and report the sequences of M1 and M2 dsRNA, respectively.

The entire sequence of M1 consists of 6390 bp, with six proposed ORFs, as indicated in Figure 5. 8. M1 does not contain RDRP activity, and is consistently found associated with L2 dsRNA (22 kb). L2 dsRNA contains RDRP activity, so M1 is presumably dependent upon L2 for replicative functions (Jian, 1997). L2 shows sequence similarity to dsRNA from *C. parasitica* NB631, and to the two enzymatic domains of the penta functional AROM gene from yeast and filamentous fungi (Lakshman *et al.*, 1998). Of the six ORFs of M1, no significant homology was reported between ORF 1, 3, 4 or 6 and sequence databases (Jian *et al.*, 1998). Regarding the remaining ORFs, ORF 5 shows sequence homology to the cytochrome c oxidase assembly factor (CcOAF) and ORF 2 shows homology to the broad bean mottle virus and other plant Bromoviruses (Jian *et al.*, 1998). This includes six conserved helicase-related domains, which contain the NTP binding site motif. In addition, homology exists between ORF 2 and the conserved regions for a zinc finger. Jian *et al.* (1998) propose that ORF 5 leads to extra CcOAF being produced, which in turn increases the amount of cytochrome c oxidase present, which may enhance ATP synthesis in the cell. This would then partly explain why the M1-containing isolates show enhanced vigour. It is noteworthy that hypovirulent isolates of *O. novo-ulmi* contain low levels of cytochrome oxidase in the mitochondria (Rogers *et al.*, 1987). Potentially, dsRNA elements are similar between M1-containing *R. solani* isolates and *O. novo-ulmi* isolates, eliciting opposing effects within the mitochondria upon cytochrome c oxidase.

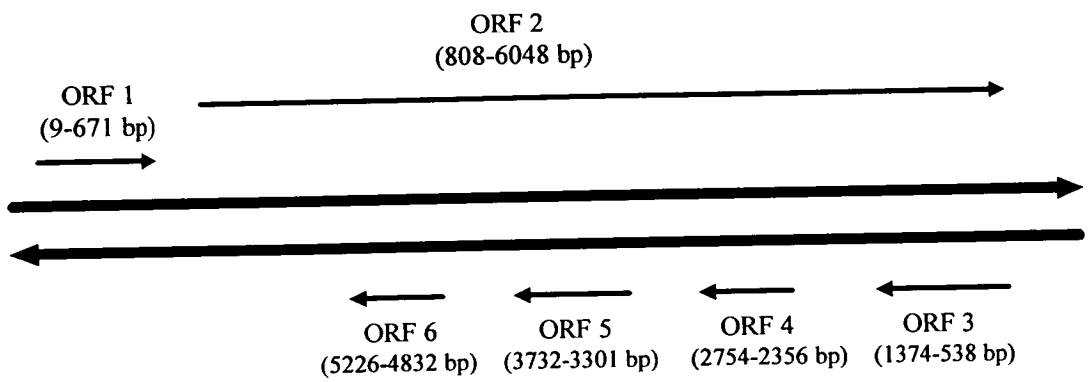


Figure 5. 8 Diagrammatic representation of the open reading frames (ORFs) encoded by the M1 double-stranded RNA of *R. solani* (Jian *et al.*, 1998).

The entire sequence of M2 consisted of 3570 bp, with one major proposed ORF (ORF A) and four minor ORFs (ORF 1 to 4), as indicated in Figure 5. 9. ORFA encodes 754 amino acids, with a specific stretch showing 72 % sequence identity with a region of hypovirulence-associated mitochondrial dsRNA from *C. parasitica* NB631 (Lakshman *et al.*, 1998). This is surprising, as little similarity has previously been observed between dsRNA viruses from either the same family or genera (Ghabrial, 1998). In addition, the segment from 190 to 517 amino acids is phylogenetically related to two domains (3-dehydroquinase, AroD, and shikamate dehydrogenase, AroE) of the pentafunctional polypeptide AROM from *Saccharomyces cerevisiae*. AROM forms a mosaic of five domains which complete steps two to six of the shikimate acid pathway (Duncan *et al.*, 1987). This pathway enables the formation of the aromatic amino acids (phenylalanine, tyrosine and tryptophan) from phosphoenol pyruvate and erythrose-4-phosphate via shikimic acid. Lakshman *et al.* (1998) propose that ORF A interferes with specific steps of the shikimate pathway resulting in reduced levels of phenylalanine. Phenylalanine is phytotoxic and responsible for some of the *Rhizoctonia* disease symptoms on potato, including root necrosis, leaf curling, stunting and leaf margin chlorosis. Therefore, reduced levels of phenylalanine may, in part, explain the reduction in virulence associated with isolates harbouring the M2 dsRNA element.

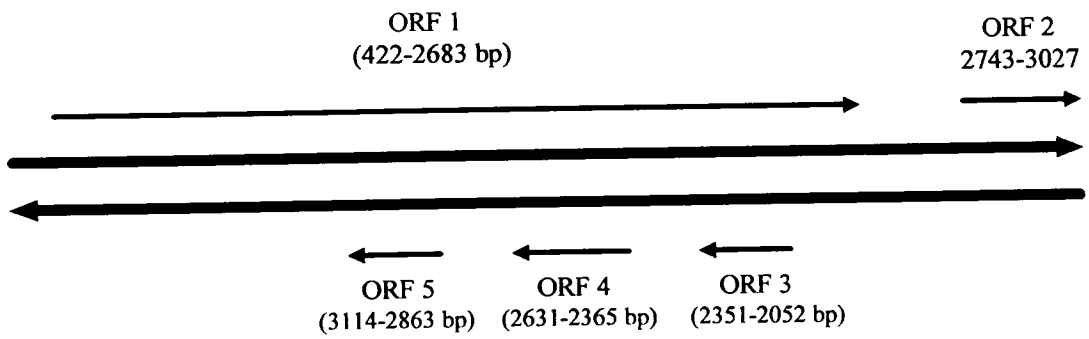


Figure 5. 9 Diagrammatic representation of the open reading frames (ORFs) encoded by the M2 double-stranded RNA of *R. solani*.

During attempted curing of isolates in the present study, the vegetative compatibility of derivatives was observed. Hyphal tip subculturing of isolates FT 10 and FT 204 resulted in a high degree of incompatibility in the subcultures, being 73 % and 49 %, respectively. Vegetative compatibility within hyphal subcultures of FT 201 was more stable, with 93 % of reactions being compatible. Hyphal tip subcultures of an AG 4 isolate yielded 8 of 78 colonies that were incompatible with the parental colony, whereas a second colony produced less than 1 % of incompatible daughter colonies (McCabe, 1994; Deacon, 1996). In the study of McCabe (1994) no daughter incompatible isolates were obtained for hyphal tip subcultures of AG 3 isolates.

When using cycloheximide in attempts to cure strains of dsRNA, the longer the exposure and the greater the concentration of cycloheximide, led to increased incompatibility of the subcultures. This, however, is not unexpected as cycloheximide is a protein synthesis inhibitor, causing altered phenotype of the isolates. With respect to vegetative compatibility reactions, it may prevent translation of proteins required for self-recognition, as uninhibited ribosomes will be involved in translating genes essential for cell survival. Alternatively, the ratio of nuclei may alter to favour those containing genes which provide a degree of resistance to cycloheximide. Ultimately, these alterations in vegetative compatibility are indicative of changes in the genotype of the host, which may indicate that direct comparisons of isolates lacking dsRNA may not be strictly valid. This also impedes dsRNA transfer, which requires compatible anastomosis.

CHAPTER 6

PROTOPLAST GENERATION AND TRANSFORMATION

6.1. Introduction

To study the structure and regulation of genes within *R. solani*, a suitable transformation system needs to be developed. In addition, this would enable further elucidation of the effects of dsRNA on the phenotype of *R. solani*, both by transforming dsRNA from *R. solani* and by examining whether cDNA constructed from dsRNA of *C. parasitica* is able to elicit hypovirulence in *R. solani*.

A wide variety of transformation protocols exist, which use differing types of cells; however, osmotically-sensitive protoplasts are most frequently used. These are generated by degradation of the fungal cell walls, which would otherwise form a barrier to the uptake of macromolecules such as DNA. Protoplasts can be generated from cells at different stages of development, with conidiospores being preferentially used when available, as they generate uninucleate protoplasts. Hyphal cells can also be used for species which lack a conidial phase, such as *Podospora anserina* (Brygoo and Debuchy, 1985), although the protoplasts generated tend to be multinucleate (Goosen *et al.*, 1992). In addition, basidiospores and oidia are sometimes used (Munoz-Rivas *et al.*, 1986; Binninger *et al.*, 1987). The choice of cell type is usually biased by the type which is most readily obtained. Protoplasts can be generated from most fungi, although different species, and even different isolates of the same species, vary greatly in the conditions required for protoplast generation. Therefore, finding the optimal conditions for protoplast production is empirical (Peberdy, 1976). The key factors which can be varied to attain optimal protoplast production include lytic enzymes, osmotic stabilizer systems, enzyme digestion time, temperature, pH, age and concentration of fungal material (Fincham, 1989; Hashiba, 1992). Unfortunately, development of protoplast production protocols usually relies on trial and error (Gadau, 1992; Hashiba, 1992). Once protoplasts are obtained, exogenous DNA may be added by transformation. Subsequently, suitable media must be selected to enable selection plus regeneration of protoplasts to the mycelial phase.

Various techniques for transformation of filamentous fungi exist. These include the following (Goosen *et al.*, 1992): (i) Fusionogenic methods whereby protoplasts are incubated with DNA and calcium chloride, prior to the addition of polyethylene glycol (PEG) as a fusionogenic medium. PEG causes the protoplasts to clump together, which

facilitates the trapping of DNA (Fincham, 1989). (ii) Electroporation, where strong electric fields are applied to protoplasts, resulting in structural rearrangements of the cell membrane, creating temporary pores for the uptake of DNA (Weaver, 1995). (iii) Physical damage, although this method is not particularly successful (Goosen *et al.*, 1992). A newer approach involves using biolistic transformation whereby cells are bombarded with microprojectiles coated with DNA (Klein *et al.*, 1987; Johnston *et al.*, 1988).

The work of this chapter outlines the development of a protoplast generation protocol, and subsequently describes a transformation procedure for *R. solani*.

6.2. Materials and Methods

A range of commercial lysing enzymes was used to determine which enzymes generated protoplasts from the mycelia of *Rhizoctonia solani* strain 3R8. Following successful protoplast production, various parameters of the protocol were altered to achieve optimum conditions for protoplast release. Section 6.2.1 describes the protocol developed for optimal production of *R. solani* protoplasts and Sections 6.2.1.1 to 6.2.1.5 describe the experiments used to determine optimal conditions.

6.2.1. Production of protoplasts from *R. solani*

Rhizoctonia broth (200 ml, consisting of 5 % glucose, 5 % malt extract, 5 % yeast extract) was inoculated with multiple plugs of 4 to 7 day old *R. solani*, and incubated aerobically for 3 days at 23°C. Mycelia were harvested by filtration through a double layer of sterile muslin and subsequently washed with 100 ml of cold (4°C) 0.6 M $MgSO_4$ solution. Mycelia were transferred to a 500 ml Erlenmeyer flask containing 40 ml osmotic medium (OM) (consisting of 980 ml 1.0 M $MgSO_4$, 16.8 ml 0.5 M Na_2HPO_4, 3.2 ml 0.5 M NaH_2PO_4) and 10 ml Novozyme enzyme solution (3 mg ml^{-1} OM). Flasks were incubated with shaking (80 rpm) for 2.75 hours at 30 to 33°C. Mycelia were removed by filtration through a double layer of sterile muslin and washed with 200 ml STC (1.0 M sorbitol, 10 mM tris-Cl pH 7.5, 50 mM $CaCl_2$), with the eluate forming a protoplast suspension. Protoplasts were sedimented by centrifugation at 4°C

for 10 minutes at 1 800 rpm in a MSE Mistral 4L centrifuge. Pellets were resuspended in 1 ml of STC and the number of protoplasts determined using a Neubaeur counting chamber. For protoplast regeneration, aliquots were added to 6 ml of cooled regeneration medium (*Rhizoctonia* broth containing 2 % Oxoid agar no. 3, plus 1.0 M mannitol as an osmoticum) containing 240 µl of antibiotics (50 mg streptomycin sulphate, 50 mg chloramphenicol, 50 mg penicillin, 50 mg bacitracin dissolved in 20 ml 48 % (v/v) ethanol). The cooled mixture was subsequently mixed and poured into 75 mm diameter Petri dishes and incubated at 27°C for 4 to 7 days to enable protoplast regeneration.

6.2.1.1. Lysing enzymes

Commercial lysing enzymes were used as indicated in Table 6. 1, to determine which, if any, led to the release of protoplasts from *R. solani* 3R8. Combinations of enzymes were also examined as indicated in Table 6. 2.

Mycelia were standardly produced (Section 6.2.1) following 45 hours aerobic incubation at 23°C, harvested by filtration and washed with 0.6 M $MgSO_4$ solution. Mycelia were added to flasks containing 40 ml OM, 10 ml enzyme solution (as indicated in Table 6. 1 and Table 6. 2), plus 1.2 mg ml^{-1} BSA (bovine serum albumin). Enzyme incubation was for 3 hours at 30 to 33°C, following which protoplasts were harvested and counted as indicated in Section 6.2.1.

The dry weight of mycelial inoculum was determined after oven-drying the inoculum in pre-weighed tins at 80°C for 2 days.

6.2.1.2. Optimal concentration of osmotic stabilizer

To determine the optimal concentration of osmotic stabilizers, the osmotic solutions OM and STC were adjusted using $MgSO_4$ and sorbitol, respectively, to concentrations of 0.4, 0.6, 0.8, 1.0 or 1.2 M. Protoplasts were prepared as described in section 6.2.1.1.

Enzyme	Concentration (mg ml^{-1})	Source Organism	Supplier
Cellulase	5	*Trichoderma viride*	Miles Kali-Chemie, Hannover, Germany
Cellulase C	5	?	Rohm enzyme, Philadelphia, USA
Cellulase "onozuka"	5	*Trichoderma viride*	Yakult Pharmaceutical Ind. Co. Ltd., Tokyo, Japan
Driselase	5	Basidiomycetes	Sigma Chemical Co., St. Louis, USA
Finizym 2004	5 µl ml^{-1}	?	Novo Industries AS, Copenhagen, Denmark
Glucanase GV-L	5 µl ml^{-1}	?	Grindsted Products AS, Denmark
β-Glucuronidase	5	*Helix pomatia*	Miles Laboratories (PTY) Ltd., South Africa
Hemicellulase	5	*Aspergillus niger*	Miles Kali-Chemie, Hannover, Germany
Lysing enzymes	5	*Rhizoctonia solani*	Sigma Chemical Co., St. Louis, USA
Lysing enzymes	5	*Trichoderma harzianum*	Sigma Chemical Co., St. Louis, USA
Maxazym CL	5	?	Gist-Brocades nv, Industrial Products, Delff, Holland
Novozyme 234	5	*Trichoderma harzianum*	Interspex Products Inc., California
Pectinase	0.056	*Aspergillus niger*	Sigma Chemical Co., St. Louis, USA
Rhozym HP150	5	?	Rohm & Haas Co., Philadelphia, USA

Table 6. 1 Commercial lysing enzymes tested for ability to produce protoplasts from *Rhizoctonia solani* strain 3R8.

Sample no.	Enzymes used (mg ml^{-1})					
	1		2		3	
1	Cellulase	1.67	Hemicellulase	1.67	Maxazym	1.67
2	Cellulase	1.67	Hemicellulase	1.67	Novozyme	1.67
3	Cellulase	2.50	Hemicellulase	2.50		
4	Novozyme	2.50	Maxazym	2.50		
5	Maxazym	2.50	Cellulase	2.50		
6	Maxazym	2.50	Hemicellulase	2.50		
7	Rhozym	2.50	Cellulase	2.50		
8	Pectin esterase	2.50	Hemicellulase	2.50		
9	Maxazym	2.50	Pectinase	2.50		
10	Novozyme	2.50	Cellulase	2.50		

Table 6. 2 Combinations of lysing enzymes tested to determine optimal release of protoplasts from *Rhizoctonia solani* strain 3R8.

6.2.1.3. Optimal period of enzyme digestion

To determine the time course of protoplast release, enzyme digestion was terminated following 0, 1, 1.5, 2, 2.5, 3, 3.5, 4 or 5 hours incubation at 30 to 33°C. Protoplasts were prepared as described in Section 6.2.1.1, either with or without the incorporation of BSA.

6.2.1.4. Optimal mycelial age

To determine the optimal mycelial age for protoplast release, mycelia was incubated at 23°C in *Rhizoctonia* broth for varying time periods between 0 and 86.5 hours. Mycelia was harvested and protoplasts were subsequently prepared as indicated in Section 6.2.1.

6.2.1.5. Regeneration of protoplasts

Protoplasts prepared as described in section 6.2.1 were placed in 6 ml of cooled *Rhizoctonia* broth either with or without 2 % Oxoid no. 3 agar. Liquid media contained either mannitol or sucrose as an osmotic stabilizer at concentrations of 0.6, 0.8, 1.0 or 1.2 M. Solid media contained sorbitol, mannitol, sucrose, or NaCl as an osmotic stabilizer at the concentrations noted above. Antibiotics were also incorporated as

described in Section 6.2.1. Aliquots were mixed and poured into 75 mm diameter Petri dishes and incubated at 27°C for 2 to 4 days to enable protoplast regeneration.

6.2.1.6. Protoplast sensitivity to fungicides

Protoplasts were prepared as described in section 6.2.1 and 100 000 protoplasts were plated onto selective regeneration media which consisted of an underlay agar (*Rhizoctonia* broth containing 2 % agar and 0 to 750 μg ml^{-1} hygromycin B) and an overlay agar (*Rhizoctonia* broth containing 2 % agar, antibiotics (described in section 6.2.1), and 1.0 M mannitol). Alternatively, protoplasts were plated onto a nitrogen-free underlay/ overlay agar system. Overlay agar (250 ml) consisted of 0.05 % glucose, 0.05 % fructose, 2 % sorbose, 5 ml 50 X Vogels salt solution, 0.5 % proline and 1.0 M mannitol. Underlay agar contained the above, with 1.0 M mannitol being substituted with 0 to 750 μg ml^{-1} ignite. In both cases, protoplasts were added to the cooled overlay agar and incubated for 4 days, before colonies were counted.

6.2.2. Transformation of protoplasts

Protoplasts (approximately 5 x 10^6 in 100 μl) were prepared as described in Section 6.2.1, mixed with 20 μl of plasmid DNA suspended in dH_20 and 1 μl of 1 M aurintricarboxylic acid (a DNase inhibitor), and incubated on ice for 25 minutes. Subsequently 250 μl of 60 % (w/v) polyethylene glycol (PEG) was added and the contents mixed gently. This step was repeated and followed by an addition of 850 μl of 60 % PEG which was again mixed gently. Following 20 minutes incubation at 10°C, 12 ml of STC was added. Transformed protoplasts were sedimented by centrifugation at 4°C for 10 minutes at 1 800 rpm in a MSE Mistral 4L centrifuge. Protoplasts were resuspended in 200 μl STC, and plated on selective regeneration agar consisting of an underlay agar (*Rhizoctonia* broth containing 2 % agar and 50 μg ml^{-1} hygromycin B) and an overlay agar (*Rhizoctonia* broth containing 2 % agar, 1.0 M mannitol plus antibiotics, as described in Section 6.2.1). Plates were incubated at 27°C for 3 days and regenerated transformed protoplasts transferred to potato dextrose agar (PDA) plates containing 75 μg ml^{-1} hygromycin B.

6.3. Results

6.3.1. Effect of different lysing enzymes

The ability of several lysing enzymes to release protoplasts from *Rhizoctonia solani* strain 3R8 was examined following a 3 hour enzyme incubation period (Section 6.2.1.1). Using single enzyme preparations, only Novozyme 234 and Lysing enzymes (from *Trichoderma harzianum*) released protoplasts, yielding 202 ± 10.6 and 15.6 ± 2.4 x 10^4 protoplasts per ml respectively (Table 6. 3). Rhozym HP150, Pectinase, Finizym 2004, Glucanase GV-L, β-glucuronidase, cellulase C, Maxazym CL, Cellulase (from *T. viride*), lysing enzymes (from *R. solani*), Driselase, Hemicellulase and cellulase "onozuka" were all inactive. Increasing enzyme concentration from 1 mg ml^{-1} to 4 mg ml^{-1} for the enzymes Maxazym CL, Cellulase (from *T. viride*) and cellulase "onozuka" yielded no protoplast release (Table 6. 4). Alteration of the enzyme incubation period from 3 hours to 2 or 4 hours incubation for both Maxazym and cellulase "onozuka" resulted in no protoplast release (Table 6. 5).

Enzyme preparation	**Concentration (mg ml^{-1})**	**Number of protoplasts (x 10^4 per ml.)**
Cellulase (*T. viride*)	1	0
Cellulase C	1	0
Cellulase "onozuka"	1	0
Driselase	1	0
Finizym 2004	1 µl ml^{-1}	0
Glucanase GV-L	1 µl ml^{-1}	0
β-Glucuronidase	1	0
Hemicellulase	1	0
Lysing enzymes (*R. solani*)	1	0
Lysing enzymes (*T. harzianum*)	1	15.6 ± 2.4
Maxazym CL	1	0
Novozyme 234	1	202 ± 10.6
Pectinase	1.1 x 10^{-2}	0
Rhozym HP150	1	0

Table 6. 3 Number of protoplasts produced (x 10^4 per ml) following 3 hours incubation of *Rhizoctonia solani* 3R8 mycelia with differing enzymes. Data presented as means of 4 replicates ± SEM.

Concentration (mg ml^{-1})	Enzyme preparation (No. of protoplasts produced per ml enzyme solution)		
	Cellulase "onozuka"	Cellulase (*T. viride*)	Maxazym CL
1	0	0	0
4	0	0	0

Table 6. 4 Number of protoplasts produced (x 10^4 per ml) following 3 hours incubation of *Rhizoctonia solani* 3R8 mycelia with differing enzymes at low (1 mg ml^{-1}) and high (4 mg ml^{-1}) enzyme concentrations. Data presented as means of 4 replicates ± SEM.

Enzyme incubation time (hours)	Enzyme preparation (No. of protoplasts produced per ml enzyme solution)	
	Cellulase "onozuka"	Maxazym CL
2	0	0
3	0	0
4	0	0

Table 6. 5 Number of protoplasts produced (x 10^4 per ml) following 2-4 hours incubation of *Rhizoctonia solani* 3R8 mycelia with cellulase "onozuka" and Maxazym CL at 1 mg ml^{-1}. Data presented as means of 4 replicates ± SEM.

6.3.2. Effect of combinations of lysing enzymes

The effectiveness of various combinations of lysing enzymes to release protoplasts was examined (Section 6.2.1.1). The enzyme mixture of Novozym 234 and Maxazym CL (Sample no.4) produced 4.7 ± 1.6 x 10^4 protoplasts per ml (Table 6. 6). This yield was lower than that for Novozyme 234 alone, which produced 202 ± 10.6 x 10^4 protoplasts per ml (Table 6. 3). Each of nine further enzyme combinations produced no protoplasts. Due to a high yield of protoplasts, Novozyme 234 alone was selected for further study.

Sample	Enzyme preparation*			Number of protoplasts (x 10^4 per ml.)
	1	2	3	
1	Cellulase	Hemicellulase	Maxazym CL	0
2	Cellulase	Hemicellulase	Novozyme 234	0
3	Cellulase	Hemicellulase		0
4	Novozyme 234	Maxazym CL		4.68 ± 1.6
5	Maxazym CL	Cellulase		0
6	Maxazym CL	Hemicellulase		0
7	Rhozym HP150	Cellulase		0
8	Pectin esterase	Hemicellulase		0
9	Maxazym CL	Pectinase		0
10	Novozyme 234	Cellulase		0

* At a total concentration of 1 mg ml^{-1} evenly divided between enzyme types.

Table 6. 6 Number of protoplasts produced (x 10^4 per ml) following 3 hours incubation of *Rhizoctonia solani* 3R8 mycelia with combinations of enzymes, at a total concentration of 1 mg ml^{-1}. Data presented as means of 4 replicates ± SEM.

6.3.3. Effect of different osmotic concentrations

The concentration of osmotic stabilizers was varied from 0.4 to 1.2 M to determine the concentration for optimal protoplast production using Novozyme 234 (Section 6.2.1.2). Concentrations of 0.4 to 0.6 M resulted in no protoplast production by Novozyme 234 (Table 6. 7), whereas concentrations of 0.8 to 1.2 M yielded protoplasts. 1.0 M solutions were the most effective yielding 54.7 ± 2.8 x 10^4 protoplasts per ml, compared with 2.0 ± 0.7 and 7.75 ± 1.1 x 10^4 protoplasts per ml for 0.8 and 1.2 M solutions respectively.

Osmotic stabilizer concentration (M)	Protoplast yield (x 10^4 per ml.)
0.4	0
0.6	0
0.8	2.00 ± 0.7
1.0	54.7 ± 2.8
1.2	7.75 ± 1.1

Table 6. 7 Protoplasts produced (x 10^4 per ml) following 3 hours incubation of *Rhizoctonia solani* 3R8 mycelia with Novozyme 234 using $MgSO_4$ as an osmotic stabilizer, at concentrations ranging from 0.4 to 1.2 M. Data presented as means of 4 replicates ± SEM.

6.3.4. Effect of enzyme incubation time

The number of protoplasts produced was examined during a 5 hour enzyme (Novozyme 234) incubation period, to determine when the maximum yield occurred (Section 6.2.1.3). Protoplast yield gradually increased during the first 2.5 hours of enzyme incubation, reaching a peak of approximately 40 x 10^4 protoplasts per g dry weight at 2.5 to 3 hours incubation (Figure 6. 1). Between 3 to 3.5 hours, protoplast numbers rapidly declined from 41.0 ± 1.7 to 2.4 ± 0.4 x 10^4 protoplasts per g dry weight. Between 3.5 and 5 hours, protoplast yield remained low, fluctuating between 2.4 ± 0.4 and 6.9 ± 0.6 x 10^4 protoplasts per g dry weight.

6.3.5. Effect of BSA on protoplast production

BSA (Bovine serum albumin) was incorporated during enzyme incubation at a concentration of 1 mg ml^{-1}, as it is reported to stabilize protoplasts upon formation (Solis *et al.*, 1996). Protoplast yield was examined over a 5 hour Novozyme 234 incubation period (Section 6.2.1.3). Protoplast production was similar both in the presence and absence of BSA, with peak yield occurring at 2.5 to 3 hours incubation (Figure 6. 1). Following maximal yield of protoplasts, the presence of BSA had no stabilizing effect, with protoplast numbers declining rapidly, between 2.5 and 3.5 hours, from 49.9 ± 2.8 to 1.8 ± 0.9 protoplasts per g dry weight. Between 3.5 and 5 hours, protoplast yield remained low, irrespective of whether BSA was incorporated. BSA was therefore not included in the final protocol.

6.3.6. Effect of mycelial age on protoplast yield

Mycelia were harvested at differing ages between 0 and 86.5 hours to determine the age for optimal protoplast production using Novozyme 234 (Section 6.2.1.4). Mycelial age greatly affected protoplast yield (Figure 6. 2). Mycelia less than 38 hours old produced negligible levels of protoplasts with the yield gradually rising from 0 to 6.1 ± 0.6 protoplasts per g dry weight. Protoplast yield rapidly increased with mycelia older than 38 hours, to reach a peak at 63.5 hours with 120.1 ± 8.2 protoplasts produced per

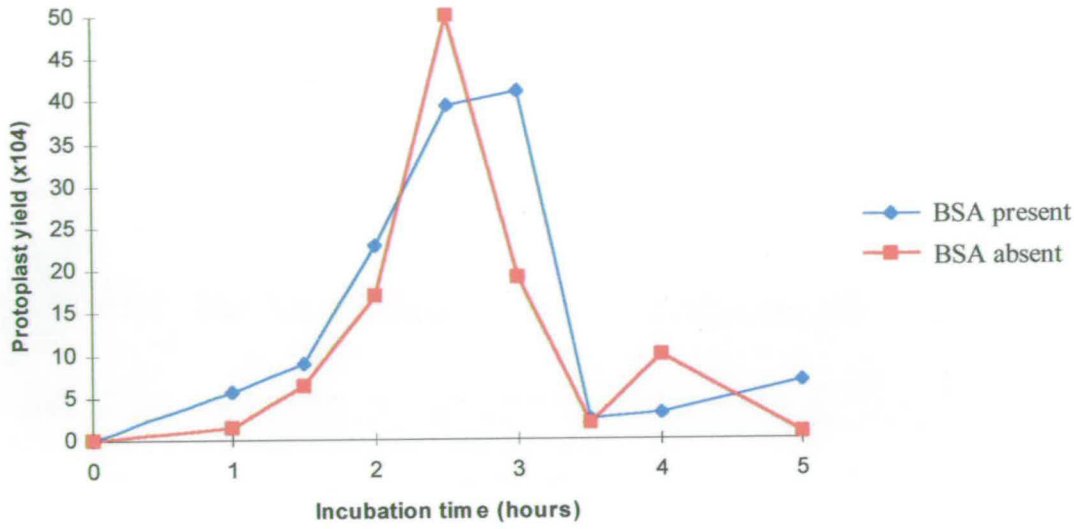


Figure 6. 1 Time course of protoplast release from *Rhizoctonia solani* strain 3R8 following incubation with Novozyme 234 (0.6 mg ml^{-1}), both in the presence and absence of BSA (bovine serum albumin).

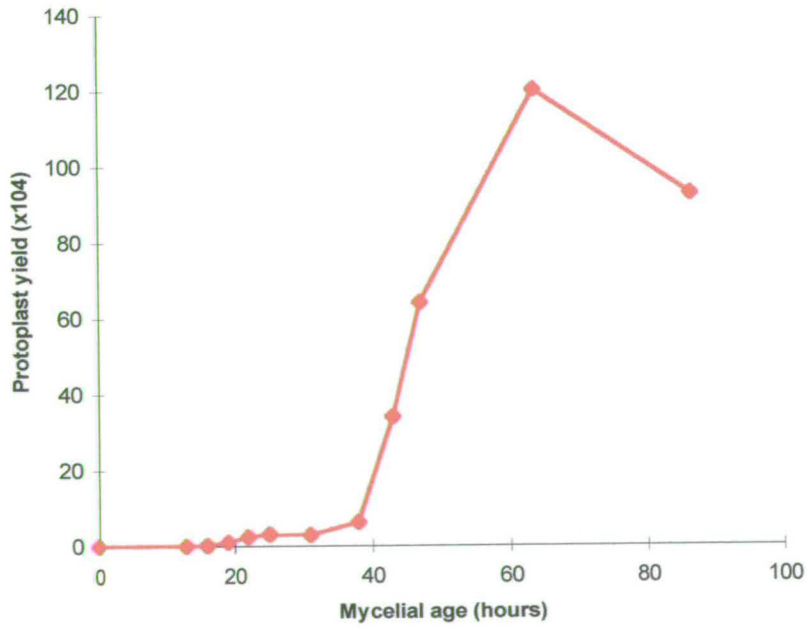


Figure 6. 2 The effect of mycelial age on protoplast yield (x10^4 per g dry weight) from *Rhizoctonia solani* strain 3R8, following 3 hours incubation with Novozyme 234 (0.6 mg ml^{-1}).

g dry weight. Following this, protoplast yield remained high, with only a slight decrease to 92.6 ± 7.8 protoplasts produced per g dry weight, with mycelia 86.5 hours old.

6.3.7. Regeneration of protoplasts

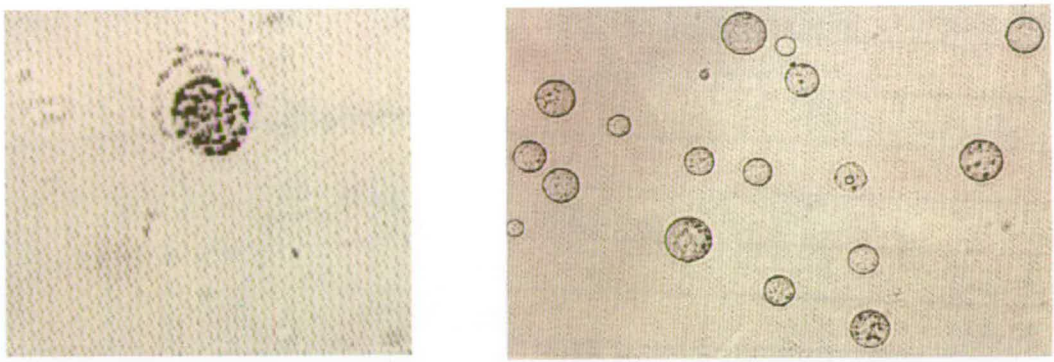
To determine the optimal conditions for protoplast regeneration, protoplasts were mixed with the following osmotic stabilizers mannitol, sorbitol, sucrose or sodium chloride at concentrations of 0.6 to 1.2 M. Osmotic stabilizers were incorporated in *Rhizoctonia* broth, either with or without 2 % Oxoid no. 3 agar (Section 6.2.1.5).

6.3.7.1. Protoplast regeneration in liquid media

In liquid media, sucrose and mannitol at concentrations of 0.6, 0.8 and 1.0 M were examined for protoplast regeneration. In both cases, at all concentrations, protoplast regeneration was highly inefficient following 4 days incubation at 27°C. Protoplasts incubated in sucrose at 0.6, 0.8 and 1.0 M showed 0 % regeneration. Upon microscopic examination very few protoplasts remained, with those present displaying a "sickly" phenotype characterized by cell contents which had shrunk away from the cell membrane (Figure 6. 3). Protoplasts incubated in 0.6 and 0.8 M mannitol did not survive the first 24 hours incubation. Incubation in 1.0 M mannitol resulted in 5 % regeneration following 4 days incubation at 27°C. Microscopic examination indicated that protoplasts unable to regenerate were highly vacuolated (Figure 6. 4a), whereas regenerated protoplasts had a healthy phenotype (Figure 6. 4b).

6.3.7.2. Protoplast regeneration on solid media

On agar, the osmotic stabilizers sodium chloride, mannitol and sorbitol at concentrations of 0.6, 0.8, 1.0 and 1.2 M were used for protoplast regeneration. Protoplast regeneration frequencies were highly variable depending on the osmotic stabilizer and concentration used (Figure 6. 5). Using sodium chloride, protoplast regeneration was not observed at any of the four concentrations examined after 4 days



(a) (b)

Figure 6. 3 Microscopical (x 250) appearance of *Rhizoctonia solani* 3R8 protoplasts. (a) A "sickly" protoplast following incubation for 4 days in 1.0 M sucrose regeneration broth and (b) healthy protoplasts suspended in 1.0 M osmotic stabilizer (STC).

(a) (b)

Figure 6. 4 Microscopical (x 250) appearance of *Rhizoctonia solani* 3R8 protoplasts, following 4 days incubation in 1.0 M mannitol regeneration broth. (a) Highly vacuolated protoplasts unable to undergo regeneration and (b) re-generated protoplast with no vacuoles visible.

incubation (Figure 6. 5a). On sucrose agar, protoplast regeneration was poor (Figure 6. 5b), with a maximum regeneration frequency of 12.7 ± 0.7 % after 4 days incubation in 1.2 M sucrose regeneration agar. For sorbitol regeneration agar, maximum regeneration frequency of 75.3 ± 2.4 % was observed using 0.6 M sorbitol after 4 days incubation (Figure 6. 5c). This frequency gradually decreased to 15.3 ± 4.4 % regeneration as the concentration of sorbitol was increased to 1.2 M. Using mannitol as an osmotic stabilizer, maximum regeneration frequencies occurred at 0.8 and 1.0 M showing 87.3 ± 3.9 % and 83.3 ± 4.8 % regeneration respectively (Figure 6. 5d). At 0.6 and 1.2 M,

regeneration was less efficient showing 38.3 ± 1.7 and 46.7 ± 3.0 % regeneration respectively.

Mannitol regeneration agar at a concentration of 1.0 M was selected for further studies.

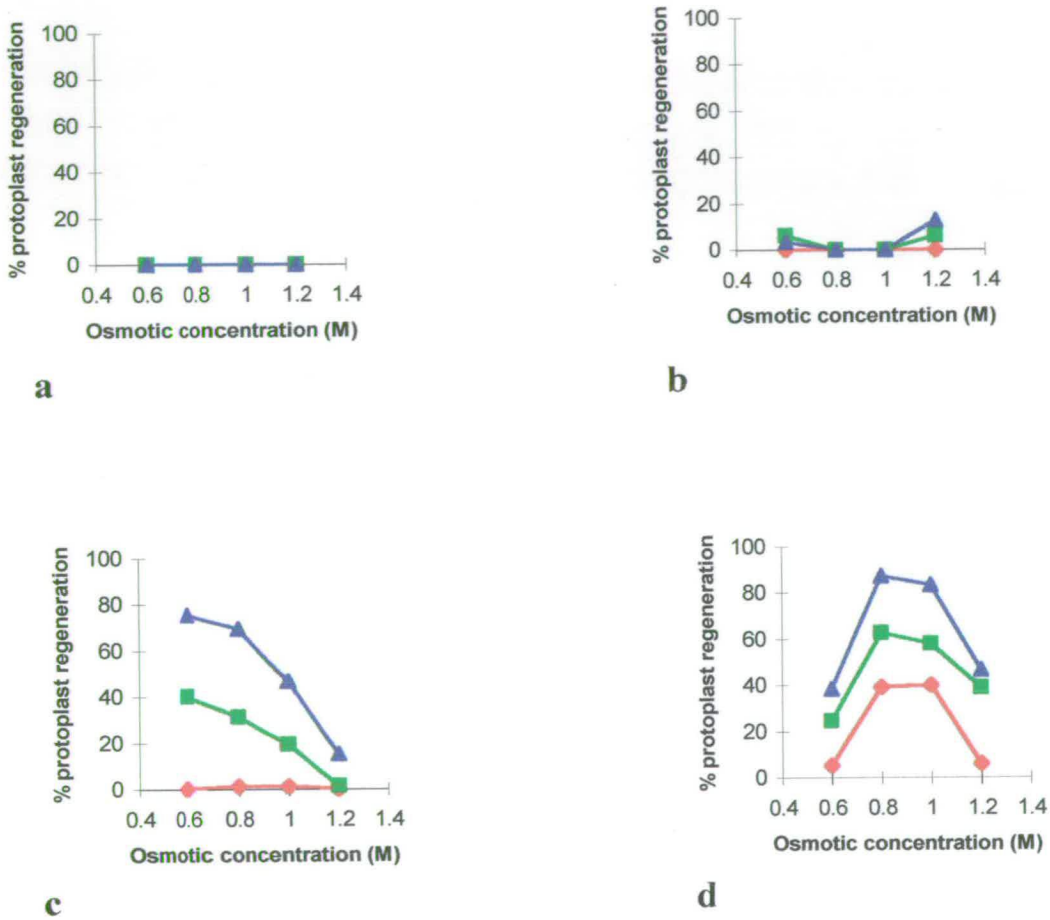


Figure 6. 5 Protoplast regeneration frequencies, following 2-4 days incubation at 27°C on *Rhizoctonia* broth agar containing 0.6, 0.8, 1.0 or 1.2 M concentrations of (a) sodium chloride (b) sucrose (c) sorbitol or (d) mannitol. Red, green and blue lines indicate % regeneration after 48, 72, 96 hours respectively.

6.3.8. Protoplast sensitivity to fungicides

To select for protoplast transformants, a suitable selective medium is required, upon which untransformed protoplasts do not survive. Transformed protoplasts survive due to a selectable marker, often drug resistance, encoded by the transformed plasmid.

R. solani 3R8 protoplasts were therefore screened for their sensitivity towards Ignite (Hoechst-Roussel Agri-Vet Company, Somerville, New Jersey, USA) and Hygromycin B at varying concentrations (Section 6.2.1.6). Ignite acts as an inhibitor of glutamine synthetase (Pall and Brunelli, 1993), whereas hygromycin B inhibits protein synthesis by causing mistranslation (Gonzalez, 1978) and by interfering with protein translocation (Singh, 1979). Resistance to hygromycin B is encoded by the bacterial hygromycin B phosphotransferase gene *hyg*B^R (Punt *et al.*, 1987) whereas the *bar* gene of *Streptomyces hygroscopicus* encodes resistance to Ignite (Avalos *et al.*, 1989).

R. solani 3R8 protoplasts showed a high level of natural resistance to Ignite, requiring a concentration greater than 750 μg ml^{-1} for 100 % death (Table 6. 8). At lower concentrations, i.e. 50 to 250 μg ml^{-1}, the number of colonies formed ranged from 234 to 419 per plate. Ignite would therefore not form a suitable selection strategy for *R. solani* 3R8 protoplasts.

Incorporation of hygromycin B into protoplast regeneration media (Section 6.2.1.6) was highly effective in preventing protoplast regeneration (Table 6. 9). At low concentration (50 μg ml^{-1}), only 4 protoplasts successfully formed colonies following 6 days incubation. Increasing the concentration to 100 μg ml^{-1} or greater resulted in 100 % suppression of protoplast regeneration Thus hygromycin B forms a highly effective selection strategy for *R. solani* 3R8 transformants.

Ignite concentration (μg ml^{-1})	**Colonies formed following 6 days incubation at 27°C**
0	> 1 000
50	419
200	234
250	293
500	29
750	10

Table 6. 8 Effect of Ignite, at varying concentrations, on the ability of protoplasts to regenerate into colonies. 100 000 protoplasts were inoculated per plate and incubated at 27°C for 6 days.

Hygromycin B concentration ($\mu g\ ml^{-1}$)	Colonies formed following 6 days incubation
0	> 1 000
50	4
100	0
150	0
250	0

Table 6. 9 Effect of Hygromycin B, at varying concentrations, on the ability of protoplasts to reform colonies. 100 000 protoplasts were inoculated per plate and incubated at 27°C for 6 days.

6.3.9. Transformation of *R. solani* protoplasts with pES200

Protoplasts were produced from *R. solani* isolate 3R8 as described in Section 6.2.1. These with plasmid pES200 (Figure 6. 6) were used to develop the PEG-based transformation procedure described in Section 6.2.2. This plasmid contains the bacterial hygromycin B phosphotransferase gene driven by a *trp*C promoter from *Aspergillus nidulans* (Staben *et al.*, 1993). This enables positive selection of transformants, as *R. solani* 3R8 is sensitive to low concentrations of hygromycin B (Section 6.2.1.6). In initial studies the DNase inhibitor, aurintricarboxylic acid (ATA), was not incorporated in the first 25 minutes incubation period and transformants were not obtained. Examination of plasmid concentration after 0, 10 and 20 minutes incubation at 4°C indicated an approximate 55 % reduction in plasmid concentration every 10 minutes (Table 6. 10). This led to incorporation of ATA, in addition to a ten fold increase in the quantity of plasmid used. Transformed protoplasts were subsequently obtained. Successful transformation occurred when the second incubation period was 10 to 12 °C, whereas temperatures in excess of 15°C prevented successful transformation.

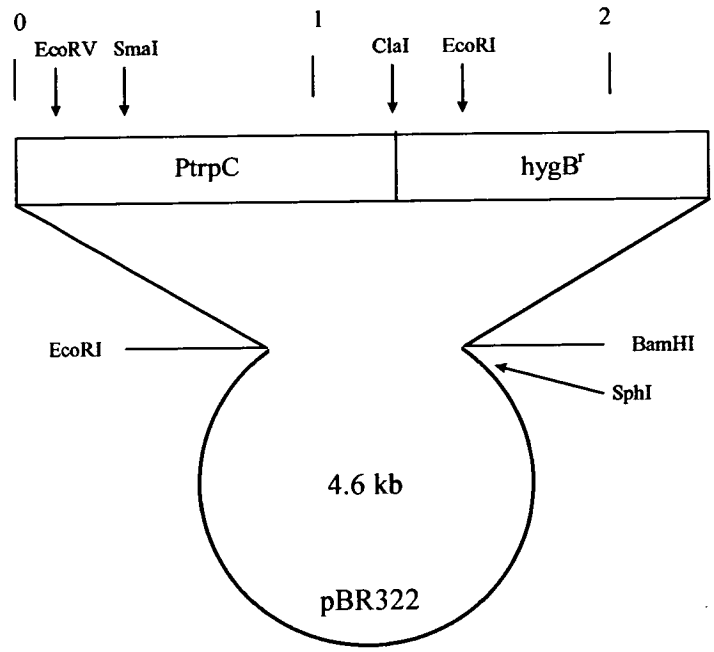


Figure 6. 6 Structure of pES200, containing the bacterial hygromycin resistance gene (*hygB*^{r}) downstream from the *Aspergillus nidulans trp*C start codon, denoted P*trp*C. pBR322 formed the vector sequence, which is indicated by the circle. Unique cloning sites are indicated in blue.

Time of incubation (min at 4°C)	Quantity of pES200 present (ng)
0	723
10	419
20	238

Table 6. 10 Reduction in quantity of vector (pES200) during transformation of *R. solani* 3R8. Reduction occurred during the initial 25 minute incubation period at 4°C, prior to uptake of pES200.

Transformed protoplasts were initially plated onto selective regeneration agar containing 100 μg ml^{-1} hygromycin. This enabled germination of transformants, but death of the cells rapidly ensued. Colonies grew, branched several times, but never exceeded overall diameters of 1 to 2 mm. Microscopic examination indicated that tip cells of the hyphae primarily died and this was followed by death of the whole colony (Figure 6. 7). Subsequent experiments decreased the hygromycin B concentration on

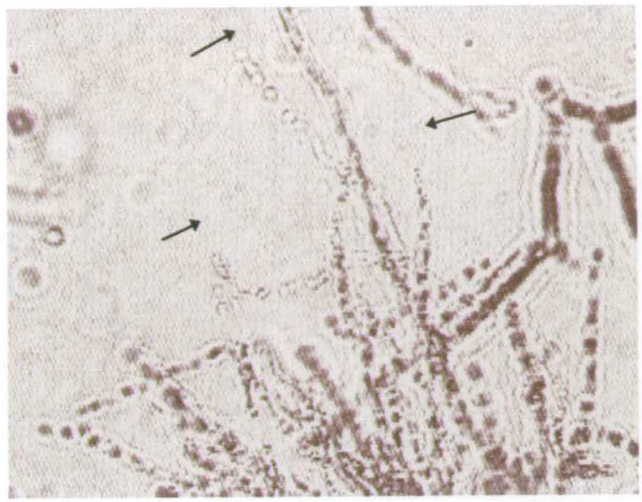
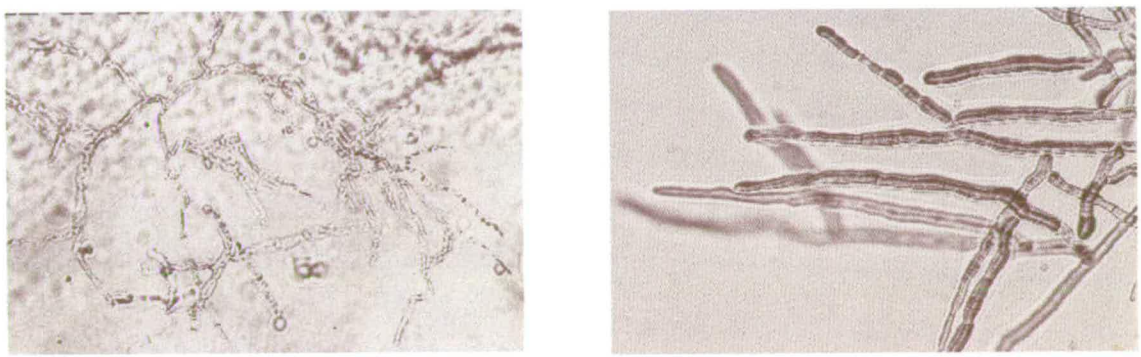


Figure 6. 7 Germinated protoplast colony on agar containing 100 μg ml^{-1} hygromycin B. Growth was slow reaching about 1 to 2 mm diameter after 14 days incubation, following which tip cells died first, as indicated by the arrows, rapidly followed by the rest of the colony.



(a) (b)

Figure 6. 8 Appearance of hyphae after 14 days incubation at 27°C on agar containing (a) 100 μg ml^{-1} and (b) 50 μg ml^{-1} hygromycin B. Colonies on 100 μg ml^{-1} reached a maximum growth of 1 to 2 mm diameter, following which death occurred. Colonies on 50 μg ml^{-1} grew to form colonies reaching approximately 20 mm diameter over 14 days incubation.

selection plates from 100 to 50 μg ml^{-1}. Protoplasts germinated readily, and hyphal structure was healthy in comparison to that on 100 μg ml^{-1} (Figure 6. 8). No germination of untransformed protoplasts was observed on 50 μg hygromycin ml^{-1}. Colonies were fairly slow growing reaching a diameter of approximately 20 mm following 14 days incubation at 27°C. Later growth was even slower, with the colonies formed having a stressed phenotype, with an abundance of vertical aerial mycelium.

6.3.10. Confirmation of pES200 transformation

To confirm that protoplast-derived colonies had been successfully transformed, southern blots were performed (Section 2.5.11). Total DNA was harvested from mycelia by grinding mycelia-coated agar with liquid nitrogen, followed by homogenization as described in Section 2.4.12. Cell debris was removed by centrifugation, and the resulting supernatant was phenol-extracted three times and ethanol precipitated overnight at 4°C. Centrifuged nucleic acid pellets were resuspended in small volumes of dH_20, and separated by agarose gel electrophoresis (Section 2.5.3). Untransformed 3R8 nucleic acid was included as a control.

At high stringency hybridization (60°C), using pES200 as a radiolabelled probe (Section 2.5.12), hybridization occurred to nucleic acid from transformed 3R8 colonies, but not from untransformed colonies (Figure 6. 9). Nucleic acid hybridization occurred towards non-integrated plasmid corresponding in molecular migration to circular pES200 (approximately 12 kb).

6.3.11. Transformation of *R. solani* protoplasts with pAXHY2

Transformation was completed as described in Section 6.2.2. This yielded transformed protoplasts, which grew more rapidly than pES200 transformants. Again growth ceased following approximately 21 days incubation at 27°C. Overall diameter obtained was 55 to 65 mm, compared with 25 to 35 mm diameter for pES200 transformants. Southern blots confirmed the presence of non-integrated pAXHY2. Following prolonged incubation for 8 to 10 weeks no sectoring of the colonies occurred.

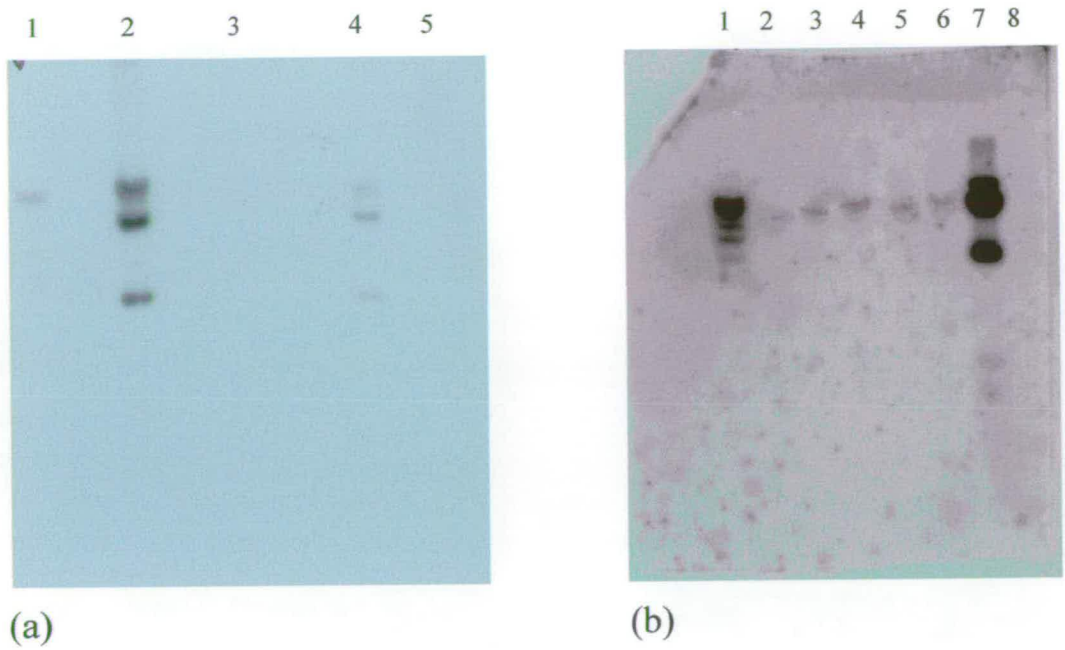


Figure 6. 9 Hybridization at 60°C of pES200 to total nucleic acid of (a) untransformed 3R8 and (b) 3R8 transformed with pES200. In (a) lane 1 contains λ DNA digested with *Hind*III, lanes 2 and 4 contain pES200, lanes 3 and 5 contain total nucleic acid extracted from untransformed 3R8. In (b) lane 1 contains λ DNA digested with *Hind*III, lanes 2 to 6 contain total DNA extracted from transformed 3R8, lane 7 contains pES200 and lane 8 contains untransformed 3R8.

6.4. Discussion

Protoplasts have been obtained from all the major taxonomic groups of fungi, with their production being highly variable depending on the species, and even the isolate, from which they are generated. The optimum conditions require to be determined empirically for each fungus, with several variable parameters being optimized prior to protoplast formation. These include: temperature, enzyme(s), osmotic stabilizer and concentration, pH, age of mycelia and enzyme incubation period. The range of commercial enzymes currently available for protoplast generation includes: helicase, β-glucuronidase, glusulase, cellulases, streptozyme, driselase, chitinase, chitosanase, zymolase, Novozyme 234, snail (*Helix pomatia*) digestive juices and Rhozym HP150 (Goosen *et al.*, 1992; Hashiba, 1992). Rhozym HP150 is more commonly used for plant, rather than fungal, protoplast production (Davey, 1983).

However in fungi with a mucilage layer such as *Pseudocercosporella herpotrichoides* (Fron) Deighton, Rhozym HP150 enhances protoplast production by degrading the mucilage, allowing greater access to the cell wall (Riess, 1971; Hocart *et al.*, 1987). Various preparations of cellulases exist, such as cellulase "Onozuka" R-10 which effectively releases protoplasts from *Coprinus cinereus* (Akamatsu *et al.*, 1983), and cellulase CP, which, in combination with Novozym 234 releases protoplasts from *Gaeumannomyces graminis* (Stanway and Buck, 1984) and *Fusarium oxysporum* (Marriott *et al.*, 1984). *H. pomatia* digestive juices were commonly used in the past (Gull *et al.*, 1972; Fawcett *et al.*, 1973; Ferenczy *et al.*, 1974), whereas Novozyme 234 is currently the most widely used preparation (Goosen *et al.*, 1992). Novozyme 234 contains a mixture of hydrolytic enzymes from *Trichoderma harzianum*, which includes endo β-1,4-glucanase, cellobiose, β-1,3-glucanase, protease, α-mannanase, α-mannosidase and chitinase (Kolar *et al.*, 1985).

The lytic enzymes cellulase (from *Trichoderma viride*), cellulase C, cellulase "Onozuka", driselase, Finizym 2004, glucanase GV-L, β-glucuronidase, hemicellulase, lysing enzymes (from *R. solani*), Sigma lysing enzymes (from *T. harzianum*), Maxazym CL, Novozyme 234, pectinase (from *Aspergillus niger*) and Rhozym HP150 were examined in the present study to determine which could release protoplasts from *R. solani* isolate 3R8. Only Novozyme 234 and Sigma lytic enzymes (from *T. harzianum*) produced protoplasts, following a 3 hour enzyme incubation period, with yields of 2.0 x 10^6 and 1.6 x 10^5 per ml, respectively. Since Novozyme 234 is a preparation based on lytic enzymes from *T. harzianum* (Kolar *et al.*, 1985), it is likely to contain similar components to the Sigma lytic enzymes. Protoplast production by Novozyme 234 from *R. solani* has previously been reported by Yang *et al.* (1993b) in 1.0 M NaCl buffered at pH 5.7 with 0.1 M $KHC_8H_4O_4$-NaOH. Thus, Novozyme 234 is effective at generating protoplasts from *R. solani* in a variety of osmotic stabilizers and buffers. The lack of protoplast production by *T. viride* enzyme preparations has been observed previously by de Vries and Wessels (1973). de Vries and Wessels (1973) used a *T. viride* enzyme preparation which released protoplasts from all species of basidiomycetes examined, with the exception of *R. solani*. Hashiba and Yamada (1982) reported protoplast production in *R. solani* isolate 0-0 using cellulase "Onozuka" R-10, Driselase or β-glucuronidase. No protoplasts were obtained in the present study with any of these

three enzyme preparations. Hashiba and Yamada (1982), however, used an osmotic stabilizer of 0.6 M mannitol at pH 5.2, which may be a more optimal system for these three enzymes. In addition, enzyme batches have highly variable effects and their ability to produce protoplasts varies between isolates (Wang *et al.*, 1988; Fincham, 1989). For example, protoplast yield was good using Driselase for three isolates of *Trichoderma reesei* but for a fourth isolate a low protoplast yield was obtained (Kumari and Panda, 1992). Additionally, variations in protoplast yields of 100 fold have been reported for differing batches of glusalase (Kinnaird *et al.*, 1982; Kinsey and Rambosek, 1984).

Combinations of enzymes were also examined, as they may act synergistically to enhance cell wall degradation, and thus increase protoplast yield. This has been reported in a wide range of species (e.g. Tilburn *et al.*, 1983; Stasz *et al.*, 1988; Solis *et al.*, 1996). In this study, ten combinations were examined, with the only combination yielding protoplasts being Novozyme 234 with Maxazym CL. This yield was significantly lower than that of Novozyme 234 alone, being 4.7×10^4 protoplasts per ml, compared with 2.0×10^6 protoplasts per ml for Novozyme 234 alone. Additionally, Novozyme 234 combined with cellulase and Novozyme 234 combined with cellulase and hemicellulase yielded no protoplasts. Thus the cellulase used in this study appears to be inhibiting the action of Novozyme 234. These results indicate that *R. solani* is highly resistant to cell wall degradation, despite attempted digestion by a diverse array of enzymes. This is unusual, with several fungi releasing protoplasts much more readily. For example, *Fusarium graminearum* releases protoplasts in the presence of Driselase (from Basidiomycetes), Glucanex (from *Trichoderma* sp.), Sigma lytic enzymes (from *R. solani*), Novozyme 234, β-glucuronidase (from *H. pomatia*) or ICN yeast lytic enzymes (from *Arthrobacter luteus*) (Wiebe *et al.*, 1997).

As previously mentioned, Novozyme 234 contains a mixture of hydrolytic enzymes from *T. harzianum*, including endo β-1,4-glucanase, cellobiose, β-1,3-glucanase, protease, α-mannanase, α-mannosidase and chitinases (Kolar *et al.*, 1985). *T. harzianum* is a mycoparasite of *R. solani* and is an effective biocontrol agent. Mycoparasitism is achieved by coiling round the hyphae of *R. solani*, followed by penetration of the hyphal cell wall (Ridout *et al.*, 1988). Of the lytic enzymes produced by *T. harzianum*, only β-1,3 glucanase, protease and chitinases are thought to be important in mycoparasitic degradation of the cell wall of *R. solani*. The chitinolytic

system present in *T. harzianum* is a complex composed of six distinct enzymes (Haran *et al.*, 1995): two β-1,4-N-acetyl glucosamidases and four endochitinases. Therefore, chitinases, proteases and β-1,3-glucanases are presumably the major factors in determining protoplast release from *R. solani*. This is further supported by studies examining the interaction between *Gliocladium virens* and *R. solani*. *G. virens* produced β-1,3-glucanase, N-acetyl glucosamidases and proteinases during attack of *R. solani* (Vantilburg and Thomas, 1993)

The ability of the enzymes to attack hyphal cells and release protoplasts depends upon the wall structure of the fungus. The walls are generally composed of 80 to 90 % carbohydrates, plus 10 to 20 % protein and lipids (Bartnicki-Garcia, 1968). These are organized into an inner fibrillar layer (composed mainly of chitin), and an outer amorphous layer (composed predominantly of glucans, proteins, and mannans). However, the composition varies greatly between species. For example, the Oomycetes, such as *Phytophthora infestans*, contain 20 % cellulose, 68 % other glucans, 1 % mannans and a trace of chitin, whereas the Chytridiomycetes, such as *Allomyces*, contain 58 % chitin and 16 % glucans, with no cellulose (Bartnicki-Garcia, 1968). In addition, the wall composition varies depending on the life cycle and stage of growth of the species examined (Hunsley and Burnett, 1970; Goosen *et al.*, 1992). The variation in enzymes required to release protoplasts, therefore, reflects the diversity of fungal cell wall structures. *R. solani* is a Basidiomycete, of which the typical wall composition consists of chitin and glucans (Bartnicki-Garcia, 1968). Indeed in mycoparasitic interactions, β-1,3-glucanase is thought to primarily degrade the amorphous layer of the cell wall, subsequently exposing the chitin microfibrills (Elad, 1996). However, since de Vries and Wessels (1973) obtained protoplasts from a range of Basidiomycetes, excluding *R. solani*, with a *T. viride* enzyme preparation, this may indicate differences in composition of the cell wall of *R. solani* compared with other Basidiomycetes.

Although the wall of *R. solani* is highly resistant to degradation, addition of compounds which alter the composition of the cell wall may increase enzyme susceptibility and hence enhance protoplast yield. For example, addition of thiol compounds reduces disulphide bonds in the cell wall, opening up the molecules, enabling penetration of the lytic enzymes (Anderson and Millbank, 1966). This has been used successfully with *Cephalosporium acremonium* (Fawcett *et al.*, 1973), *Histoplasma*

capsulatum (Berliner and Reca, 1969) and *Geotrichum candidum* (Dooijewaard-Kloosterziel *et al.*, 1973). In *Pythium* species, the addition of Triton X-100 removes a lipid layer, and subsequently improves protoplast yield (Sietsma and de Boer, 1973).

From a wide range of osmotic stabilizers, the inorganic salts such as potassium, sodium and magnesium tend to be used for filamentous fungi, whereas sugars and sugar alcohols are used for yeasts (Peberdy, 1979). However, exceptions do exist. For example, 1.0 M sorbitol was used as the osmotic stabilizer for protoplast production from *Fusarium culmorum* 15902 (Curragh *et al.*, 1992). pH in the range of 5.8 to 6.5 is reported to be suitable for a wide range of fungi (Peberdy *et al.*, 1976; Curragh *et al.*, 1992), thus the present study used magnesium sulphate buffered to pH 5.9 as an osmotic stabilizer. Protoplast yield was determined at varying concentrations (0.6, 0.8, 1.0 and 1.2 M), with 1.0 M $MgSO_4$ producing the greatest yield. Yang *et al.* (1993b) obtained protoplasts from *R. solani* AG 8 using buffered 1.0 M NaCl, whereas Hashiba and Yamada (1982) produced protoplasts from *R. solani* isolate 0-0 using 0.6 M sucrose.

Using the digestive enzyme Novozyme 234, the effect of enzyme incubation period was examined. The length of enzyme incubation period was found to be critical, with optimal protoplast yield occurring at 2.5 hours. Subsequently, protoplast yield rapidly declined to negligible levels. This is presumably due to proteolytic activity present in the Novozyme 234 lytic system (Mann and Jeffery, 1986; Kitamoto *et al.*, 1988) attacking the recently-formed protoplasts. This led to incorporation of BSA in the incubation period, in an attempt to stabilize protoplast yields. However, no stabilization occurred and protoplast yield declined just as dramatically. Similar declines in protoplast yields were obtained by Solis *et al.* (1996) using lytic enzymes from *Arthrobacter luteus* and *T. harzianum*. The decline in protoplast yield over time was less gradual using enzymes from *Aspergillus* species, which presumably have a lower proteolytic content. Previous results of Hashiba and Yamada (1982) found that maximal protoplast yield in *R. solani* occurred at approximately 2 hours incubation, with no decrease observed following 4 hours incubation. They used a combined enzyme system containing cellulase "Onozuka" R-10, macerozyme R-10 and β-glucuronidase from *H.*

pomatia. Presumably, the proteolytic content of this enzyme system was negligible, and thus protoplasts remained stable.

Enzyme incubation was completed at 30 to 33°C in the present study. Temperatures of 37°C, which have been previously reported for Novozyme 234 (Cullen *et al.*, 1987), gave decreased protoplast yields from *R. solani* 3R8. Curragh *et al.* (1992) reported temperatures of 15 to 20°C to be inefficient compared with 30°C, whereas Kumari and Panda (1992) reported insufficient protoplast production below 30°C, with optimal yield at 30 to 31°C, and increasing the temperature beyond 31°C resulted in a rapid decline in protoplast yield.

A further critical factor determining protoplast yield is mycelial age. Many species require young cultures 20 to 30 hours old to release high yields of protoplasts. For example, 21 to 24 hour old cultures produced high protoplast yields in *F. culmorum* (Curragh *et al.*, 1992), *Fulvia fulva* gave optimal yields with cultures 24 to 48 hours old (Harling *et al.*, 1988), and *Phytophthora parasitica* required 48 hours incubation to yield high levels of protoplasts (Gu and Ko, 1998). However, in the present study, low yields of protoplasts were obtained with young mycelial cultures less than 40 hours old. The yield increased one hundred fold between 40 and 60 hours, reaching a peak of 1.2 x 10^6 protoplasts per g dry weight. Between 60 and 85 hours, a slight decrease in numbers was observed. This differs from the work of Hashiba and Yamada (1982) where 20 hour old *R. solani* cultures gave optimal protoplast yields, and yields from 3 to 4 day old cultures were very low. Yang *et al.* (1993b) found *R. solani* cultures of 48 hours to be satisfactory. The differences in optimal ages of mycelia between species, and even between isolates, of *R. solani*, are readily explained. Several reports indicate that maximum yields of protoplasts are obtained from cultures nearing the end of exponential growth (Peberdy *et al.*, 1976). During exponential growth phase, the mycelia show maximum branching and new cell wall formation, thus the greatest number of young hyphal tips are present (Peberdy, 1979). Protoplasts are released from small pores formed at the growing tip of the hyphae, where the walls are thin and actively growing (Hocart and Peberdy, 1989). In *R. solani* the tip walls are approximately 80 nm thick, whereas the older walls are more than 1 μm thick (Butler and Bracker, 1970).

Older walls are thicker and more resistant to digestion due to alterations in their composition. For example, deposition of α-1,3-glucans occurs in *A. nidulans*, which subsequently decreases protoplast yield (Zonneveld, 1973). The resistance of the older wall of *R. solani* to digestion is supported by the absence of "ghost-like" ungranular protoplasts formed from the empty, older hyphae. Thus, rapidly growing isolates may undergo exponential phase at around 20 hours, given a suitable growth media, whereas other slow-growing isolates may not reach exponential phase until 3 to 5 days. Indeed, in the present *R. solani* culture collection, isolate 3R8 grew slowly in comparison to the majority of isolates. This may partly explain the differences in optimal mycelial age observed between the present study and those of Hashiba and Yamada (1982) and Yang *et al.* (1993b).

For protoplasts to be useful in transformation studies, successful regeneration must occur. Regeneration frequencies vary between species and strains, with the majority of filamentous fungal protoplasts showing reversion frequencies of 0.1 to 50 % (Stasz *et al.*, 1988; Peberdy, 1991). Hashiba and Yamada (1982) obtained regeneration frequencies of 10 to 20 % from *R. solani* on water agar adjusted to 0.6 M with respect to mannitol. The present work initially examined regeneration in *Rhizoctonia* broth containing either sucrose or mannitol as osmotic stabilizers. Regeneration frequencies were negligible (0 to 5 %) following four days incubation, with the protoplasts remaining displaying "sickly" phenotypes. In sucrose broth, cell contents had shrunk away from the cell membrane, whereas in mannitol, protoplasts became highly vacuolated. A similar degree of vacuolation has been reported for several species in the presence of magnesium sulphate (Gascon and Villanueva, 1965; Sietsma and Wouters, 1971). This enables a convenient means of purification of protoplasts from hyphal fragments, as the protoplasts become buoyant.

In yeasts, such as *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*, high reversion frequencies of 50 to 90 % were obtained in agar-solidified media (Svoboda, 1966). Therefore, due to the poor regeneration obtained in liquid media, subsequent attempts used agar medium containing NaCl, sucrose, mannitol or sorbitol as osmotic stabilizers. The protoplasts were added to cooled agar and poured immediately. It was vitally important that this stage was done quickly using agar cooled just prior to

the point of solidification. Incorporation of agar seemed to protect the protoplasts such that regeneration frequencies of 90 % could be obtained. No regeneration was observed in agar containing NaCl. This was anticipated, as studies by Carrera (1951) indicated that *R. solani* was sensitive to high concentrations of sodium. In addition, regeneration was poor on sucrose. High levels, however, were obtained on sorbitol and mannitol. Deed and Seviour (1990) reported protoplast regeneration to be higher in organic than inorganic osmoticum although the reason for the differential response was not understood. The higher regeneration frequencies obtained in the present study, compared with those of Hashiba and Yamada (1982) on agar, may be due to protoplasts being incorporated in the agar. Hashiba and Yamada (1982) spread the protoplasts on the agar surface, which might have resulted in physical damage to the fragile protoplasts.

Prior to transformation, a suitable selection strategy for transformants must be developed. Selection usually involves one of two methods. (i) Auxotrophic complementation systems, which require isolation of auxotrophic mutants and then transformation with the wild type gene. Successful systems include *leu* in *Mucor circinelloides* (van Heeswijk and Roncero, 1984) and *pyr* in *A. niger* (Goosen *et al.*, 1987; van Hartingsveldt *et al.*, 1987). (ii) Dominant selectable markers, which usually involves transforming genes which give resistance to toxins. This procedure is generally more acceptable, as it does not require the time-consuming isolation of auxotrophic mutants.

The present study examined the susceptibility of protoplasts to Ignite and Hygromycin B. Ignite is a tripeptide compound composed of two L-alanine residues and an analogue of glutamic acid known as phosphonothricin (PPT) (Thompson *et al.*, 1987). The two alanines are cleaved from PPT by endogenous cellular peptidases, producing a substance which is a potent inhibitor of glutamine synthetase (Straubinger *et al.*, 1992). This leads to an accumulation of ammonia and cell death. Hygromycin B is an aminoglycoside that inhibits protein synthesis by causing mistranslation (Gonzalez, 1978) and by interfering with protein translocation (Singh, 1979). *R. solani* isolate 3R8 was resistant to high concentrations of Ignite (> 500 $\mu g\ ml^{-1}$) and sensitive to low concentrations (< 100 $\mu g\ ml^{-1}$) of Hygromycin B. Thus, Hygromycin B was used as a selection system to develop a transformation system for *R. solani*.

It should be possible to develop transformation systems for each and every species of fungus, although the major stumbling block is achieving the first successful experiment (Fincham, 1989; Upshall, 1992)! The present study developed a PEG/ Ca^{2+} based transformation technique for *R. solani* 3R8. Transformation is reported to occur due to PEG acting as a polycation, resulting in clumping of the protoplasts (Stewart, 1981), with subsequent molecular bridges forming between adjacent membranes, trapping DNA intracellularly (Constabel and Kao, 1974). The transformation can then be either integrative or non-integrative, depending upon whether the plasmid is incorporated into the host chromosome. Three types of integrative transformation are recognized (Hinnen *et al.*, 1978). Type 1 (addition) involves a single crossover event between homologous host and non-host DNA, resulting in linked duplications of the non-host DNA. Type 2 (ectopic) is where the non-host DNA integrates at non-homologous sites by single crossover events. Type 3 (replacement) involves a double crossover where the host DNA is replaced by non-host DNA.

Initial transformation was achieved with pES200 (Figure 6. 6), which contains the bacterial hygromycin resistance gene (*hyg*B^R) downstream from *Aspergillus nidulans trp*C start codon. The *hyg*B^R gene has been used successfully in transformation of several species, including *Fusarium culmorum* (Curragh *et al.*, 1992), *Trichoderma harzianum* (Thrane *et al.*, 1995) and *Fusarium graminearum* (Wiebe *et al.*, 1997). The initial attempts to transform *R. solani* failed due to highly active DNases secreted by *R. solani*. Washing the protoplasts prior to transformation is usually sufficient to remove contaminating nucleases. However, since DNase activity remained high, the nuclease inhibitor aurintricarboxylic acid was incorporated into the protocol, in addition to a ten fold increase in plasmid concentration. Successful transformation then occurred. When hygromycin B was incorporated at 100 $\mu g\ ml^{-1}$, protoplasts germinated and grew 1 to 2 mm, then hyphal tip death occurred, which was closely followed by death of the entire colony. The hygromycin B concentration was subsequently reduced to 50 $\mu g\ ml^{-1}$, and transformants were readily obtained. Initially these displayed a "normal" phenotype, growing at a similar rate to protoplasts on non-selective agar. Following 14 to 18 days, growth rate declined dramatically, and colonies essentially stopped growth at 25 to 35 mm diameter. Transformation with pAXHY2 was also

obtained, but again growth stopped following 14 to 18 days incubation, with a final diameter of approximately 55 to 65 mm.

Non-integrative transformation is characterized by a slow growth rate of transformants, with 99 % of these isolates failing to grow upon subculture (Monke and Schafer, 1993). This is due to the DNA being able to replicate independently from the chromosomes; however, they are subject to frequent losses during growth. Some species, however, can maintain non-integrated plasmids indefinitely, for example *Mucor circinelloides* (van Heeswijk, 1986). Thus the phenotype of the transformants indicates that non-integrative (transient) transformation has occurred. This is supported by Southern blot analysis. Transient transformation has previously been reported for several species including *F. oxysporum* (Kistler and Benny, 1988), *Penicillium roquefortii* (Durand *et al.*, 1991), *Glomerella cingulata* (Rikkerink *et al.*, 1994) and *Pyricularia oryzae* (Kimura *et al.*, 1995). Thus, it appears that the limiting step in transformation, for *R. solani* 3R8 and other species, is not the uptake of foreign DNA, but its incorporation into the chromosome.

The length of time the plasmid remains in a non-integrated state appears to be highly variable, as determined by the duration of "normal" hyphal growth. *R. solani* 3R8 grew for 14 days before growth became limiting, whereas *Aspergillus nidulans* grew for 5 to 6 days (Tilburn *et al.*, 1983). Potentially, the prolonged growth observed in *R. solani* may be due to highly efficient hyphal translocation, which *R. solani* possesses for its saprotrophic lifestyle. This may then increase movement of either the hygromycin B phosphotransferase molecules, or the plasmid itself, towards the hyphal tip enabling growth to continue for 14 days, as opposed to the 3 to 5 days observed for many other species. This possibility is supported by the death of hyphal tips prior to the older mycelia, when plated onto media containing 100 $\mu g\ ml^{-1}$ hygromycin B. *Aspergillus nidulans* transformants, when grown in submerged culture, are sensitive to 10 $\mu g\ ml^{-1}$ hygromycin, whereas when grown on the surface of media growth could be tolerated at > 1000 $\mu g\ ml^{-1}$ hygromycin (Cullen *et al.*, 1987). The continuing slow growth of 3 to 4 mm per week observed in *R. solani* after the initial 14 days may well be explained by a similar decrease in sensitivity towards hygromycin B when grown upon agar, as opposed to within agar. This is confirmed, as untransformed mycelial blocks

occasionally showed slight (< 5 mm) growth upon agar containing 100 μg hygromycin B ml^{-1}, whereas submerged, untransformed protoplasts never showed growth.

Transformants of *R. solani* containing pAXHY2 showed greater growth than transformants containing pES200. pAXHY2 contains *Hyg*B^R flanked by the *A. nidulans* *trp*C promoter and terminator sequences (Choi and Nuss, 1992b), whereas pES200 contains *hyg*B^R preceded by the *trp*C promoter, with the *trp*C terminator deleted (Staben *et al.*, 1993). In *Neurospora crassa*, Staben *et al.* (1993) found the addition of the *trp*C terminator to pES200 (forming pDH25) did not affect the resistance to hygromycin B. However, in other species, such as *A. niger*, the lack of a terminator sequence greatly reduces expression (Punt *et al.*, 1987). In *R. solani* 3R8, during non-integrated transformation, the lack of terminator sequences appears to reduce the expression of *Hyg*B^R, and thus decreases the resistance of transformants to hygromycin B.

In the majority of transformation experiments, 99 % of transformants contain non-integrative DNA, and fail to grow on subculture (Liljeroth *et al.*, 1993; Monke and Schafer, 1993). In the present study, 100 % of transformants failed to grow on subculture. However, transient transformants may produce sectors of vigorous growth. These sectors frequently arise from the centre of the inoculum, and contain integrated plasmid DNA (Tilburn *et al.*, 1983). Following 8 to 10 weeks incubation, sectors were never observed in *R. solani* 3R8, transformed with either pES200 or pAXHY2. Since the possibility of integrative events is 1 %, increasing the number of transformants should increase the probability of obtaining stable transformants. One way could involve increasing the starting concentration of protoplasts (Fincham, 1989). However, this effect is not universal, as exemplified by transformation in *Cochliobolus heterostrophus*, where the number of transformants declined greatly when 10^7, as opposed to 10^6, protoplasts were used. Additionally, plasmid DNA can be constructed to include homologous DNA or ribosomal repeats. Ribosomal repeats have been reported to enhance transformation frequency in *Aspergillus niger* (O'Connell, 1998). Alternatively, transformation could be attempted using other methods such as electroporation or biolistics.

Once stable transformants are readily obtained, it will be interesting to transform *R. solani* with cDNA of dsRNA from *C. parasitica*, to determine the effect it may have

on the phenotype. Since fungi are in many ways promiscuous in their ability to express foreign DNA, *R. solani* may well express *C. parasitica* cDNA and generate dsRNA. This has been shown to occur in several species phylogenetically related to *C. parasitica*, including *C. radicalis*, *C. havanensis*, *C. cubensis* and *Endothia gyrosa* (Chen *et al.*, 1994; Chen *et al.*, 1996). However, transformation with CHV1-713 cDNA in *Leucostoma* sp., was unstable, indicating the range may be limited (D. Nuss, pers. comm.). Thus, if *C. parasitica* cDNA can generate dsRNA in *R. solani*, and the transformants are subsequently hypovirulent, then an effective biological control strategy may potentially be developed.

CHAPTER 7

CONCLUDING DISCUSSION

7.1. Concluding Discussion

Currently, the majority of phytopathogens are controlled by chemical means, with few biological control strategies being employed. The main drawbacks with biological control are high developmental costs of the biocontrol agents in relation to their potential "market share", because many of the agents are either disease or environment specific. Ideally biocontrol agents should require a one-time inoculation to a site to maintain control; however, many need to be applied repeatedly, when and where they are needed. One example of a successful biocontrol strategy requiring one-time inoculative release involves prevention of black crust on the foliage of rubber, caused by *Phyllachora huberi*. This is maintained in a cyclical state of suppression following application of the mycoparasites *Cylindosporum concentricum* and *Dicyma pulvinata* (reviewed by Sutton and Peng, 1993). A second example of a successful, self-sustaining biocontrol strategy is that against chestnut blight in Europe, caused by *C. parasitica*, using hypovirulent strains of the pathogen containing dsRNA genetic elements.

Chestnut blight was first reported on the European chestnut (*Castanea sativa*) in 1938 and by 1950 it was widespread throughout Southern Europe (Anagnostakis, 1992). *C. parasitica* isolates which contained dsRNA were observed to have an altered phenotype which includes hypovirulence, reduction of pigmentation, reduced conidiation and down-regulation of virulence-associated proteins such as laccase, cellulase, cutinase, protease and polygalacturonase. When co-inoculated with virulent *C. parasitica* isolates, the dsRNA elements were cytoplasmically transmissible, converting the recipient to a hypovirulent phenotype (Day *et al.*, 1977). Subsequently, a biological control program of chestnut blight was initiated in France. This involved successive treatments of ten cankers for a three year period, followed by treatment of five cankers per hectare for two to three years. The plantations were cured of chestnut blight within a ten year period (Grente and Berthelay-Sauret, 1978). To date, chestnut blight has spread throughout most of central Europe, including Portugal, Switzerland, Germany and Greece (Heiniger and Rigling, 1994). However, in many of these plantations, the frequency of hypovirulent isolates is high. For example, in chestnut coppices in

Switzerland, 59 % of isolates recovered were hypovirulent (Bissegger *et al.*, 1997); therefore chestnut blight is no longer a major problem in Europe.

In the USA, chestnut blight rapidly destroyed native chestnut (*Castanea dentata*) stands, following the first appearance of the disease in New York in 1904. Following the success in controlling chestnut blight in Europe, it was hoped that the same could be achieved in the USA. Anagnostakis and Jaynes (1973) found that European hypovirulent strains could diminish the growth of cankers in greenhouse seedlings. This led to field trials involving 300 trees being inoculated with an American hypovirulent strain derived from the original French strain (Jaynes and Elliston, 1980). This proved successful in that 86 % of the inoculated cankers were controlled after one year. Further field trials, however, were unsuccessful, with virulent *C. parasitica* infection levels remaining high and natural dissemination of dsRNA not being achieved. Upon examination of the population structure, it was found that American isolates of *C. parasitica* showed a high degree of natural incompatibility, with more than 100 vegetative compatibility groups present, compared to only a handful of such groups in Europe (Newhouse, 1990). This complex population structure is thought to have limited the dissemination of dsRNA in field trials, under natural conditions, in the eastern United States, thus preventing dsRNA being used as a successful biological control agent, as occurred in Europe.

The work in this thesis examined the potential for developing biological control for *Rhizoctonia*-incited diseases, modelled upon the biological control attained against chestnut blight in Europe. The work first examined the natural distribution of dsRNA in *R. solani* AG 3 field isolates harvested from a single site in Scotland, in addition to isolates from differing anastomosis groups. The study found all isolates to contain at least one and up to eight dsRNA segments, ranging in size from 0.3 to 15 kb. Zanzinger *et al.* (1984) also found dsRNA to be present in 98 % of isolates examined in the USA. However, several other reports indicate dsRNA distribution to be more variable, with one study reporting 54 % of isolates to contain dsRNA (Kousik *et al.*, 1994), whereas another failed to extract dsRNA from 77 % of isolates (Castanho *et al.*, 1978). The banding patterns of extracted dsRNA in the present study were highly variable, with some isolates containing the same segmentation. Since the isolates were harvested from a small locality, and contained similar banding patterns, it is likely that some of the segments may be the same, possibly the result of horizontal transmission by vegetative anastomosis. To determine whether the segments are related, or are just similar sizes,

northern hybridization studies are required, which were not completed in the present study. Previous work indicates sequence homology between dsRNA segments extracted from *R. solani* isolates of the same anastomosis group, but not from different anastomosis groups (Bharathan and Tavantzis, 1987). This, though, is not unexpected since the differing anastomosis groups are genetically isolated, due to their lack of attempted hyphal fusion (Vilgalys and Cubeta, 1994; Adams, 1996). Bharathan and Tavantzis (1991) subsequently examined homology between isolates of the same anastomosis group that were geographically distant. No hybridization was observed between Japanese and American isolates from AG 1, 2, 4 and 5. However, isolates harvested from within Maine, USA, showed a certain degree of cross-hybridization of dsRNA, both with each other and also towards isolates from Colorado. The isolates from the present study were all from AG 3 and isolated from close proximity, so it would be interesting to determine the degree of cross-hybridization between them, which may be quite high.

The mere presence of dsRNA in *R. solani* does not indicate it to be a useful biocontrol agent, as many dsRNA elements are non-symptomatic in their host fungi. For example, *Puccinia sorghii* contains at least twenty dsRNA segments, none of which alters the fungal phenotype (Zhang *et al.*, 1994). Initial reports by Castanho *et al.* (1978) indicated that dsRNA was correlated with a hypovirulent phenotype in *R. solani*. However, a subsequent report by Finkler *et al.* (1985) contradicted this by reporting dsRNA to be associated with increased virulence. Several subsequent reports indicate dsRNA to be ubiquitous within *R. solani*, with no apparent correlation between the presence of dsRNA and the degree of pathogenicity (Zanzinger *et al.*, 1984; Hyakumachi *et al.*, 1985; Bharathan and Tavantzis, 1990; Bharathan and Tavantzis, 1991; Washington and Martin, 1991; Kousik *et al.*, 1994). The present work shows that for fifteen dsRNA-containing, AG 3 isolates the virulence is low (mean 2.0), but not so low as to be regarded as hypovirulent. The virulence of a dsRNA-containing AG 4 isolate was also examined and was found to be significantly higher than that of the AG 3 isolates (mean 3.5). It is likely that specific dsRNA elements give rise to certain phenotypes. A similar situation occurs in *Ophiostoma novo-ulmi* where many isolates contain multiple dsRNA segments, but three specifically sized segments (2.43, 0.95 and 0.33 kb) are required to invoke the hypovirulent phenotype (Rogers *et al.*, 1986; Rogers *et al.*, 1988). To determine this, isolates both fully and partially cured of dsRNA are

required. The present study attempted curing of *R. solani* isolates initially by hyphal-tip subculturing and secondly by exposure to cycloheximide, a known RNA synthesis inhibitor (Bottacin *et al.*, 1994; Elias and Cotty, 1996). Hyphal tip subculturing has been reported to give curing frequencies of 25 % in *R. solani* (McCabe, 1994); however, total curing of dsRNA was not observed in the present study. Two isolates remained unaltered in dsRNA content following three and ten successive subcultures of excised hyphal tips. A third isolate lost one (1.3 kb) of five segments upon the second subculture, with no further alterations in dsRNA content following a further nine subcultures. Difficulty in curing isolates by this technique has been reported previously by Castanho and Butler (1978a), who obtained a 3 % curing frequency. Attempted curing by cycloheximide exposure was more successful, with several isolates being partially cured. The degree of curing increased with cycloheximide exposure time. The isolates generally lost smaller dsRNA segments, i.e. 1.6, 1.4 and 1.0 kb; however, one subculture of FT 201 lost a larger segment of 6.5 kb. The virulence of partially cured isolates was subsequently examined. For isolate PA1, loss of the 1.4 and 1.0 kb segments had no effect on overall fungal virulence. Similarly for isolate FT 201, loss of the 6.5 and 1.6 kb segments had no significant effect on overall fungal virulence. However, since these may be internal deletions of the larger dsRNA segments, the genetic information affecting virulence may still remain. To determine this, northern hybridization studies are required.

To elucidate precisely the effects that these dsRNA elements have on fungal phenotype, isolates fully cured of dsRNA are required, such that segments can be individually transferred into a dsRNA-free background. It may be that the segments encode hypovirulence, as evidenced by the relatively low virulence of the fifteen dsRNA-containing isolates, or some segments may encode virulence, with others encoding hypovirulence. The two opposing effects may be maintained in a delicate balance following interaction with the host phenotype.

The difficulties in curing *R. solani* of dsRNA in the present study may arise from a resident, integrated DNA copy of the dsRNA elements. Hybridization studies would be required to determine whether this is the case. Finkler *et al.* (1985) found no evidence of similarity between the host DNA and the viral genome. However, subsequent studies by Tavantzis (1994) and Lakshman *et al.* (1998) indicated homology between dsRNA segments and the host genome. In further support of this argument,

McCabe (1994) reported isolates to be cured of dsRNA, but re-isolation of the same isolates during the present study indicated dsRNA to be present. Additionally, Koltin *et al.* (1987) re-examined the isolates of Castanho and Butler (1978a) which were reported to be dsRNA-free, and found them to contain dsRNA. An integrative state may have arisen, as the dsRNA may have a role in the fungal host lifestyle. This is supported by A. Finkler (pers. comm.) who found that isolates cured of dsRNA were unstable upon subculture.

Although removal of the dsRNA segments from isolates in the present study resulted in no alteration of the host phenotype, it is possible that the dsRNA present shows homology to hypovirus CHV1-713 of *C. parasitica*. Hybridization studies were completed to determine this. No hybridization was observed at low stringency between any dsRNA from *R. solani* and pXH9 (cDNA of CHV1-713), indicating that that no homology exists between *R. solani* dsRNA and hypovirus CHV1-713 from *C. parasitica*. In contrast, a recent study found homology between M2 dsRNA from *R. solani* isolate Rhs1A1 and the RNA-dependent RNA polymerase (RDRP) of dsRNA associated with hypovirulence in *C. parasitica* strain NB631. It is likely that this relation is segment specific with several other unrelated dsRNA elements occurring within *R. solani*. Some of these may show sequence homology to CHV1-713.

Thus the precise function of the dsRNA elements in the present study has not been elucidated. However, Jian *et al.* (1998) and Lakshman *et al.* (1998) have recently reported the sequence of two segments of dsRNA (M1 and M2) found in other *R. solani* isolates. M1 consists of 6390 bp and encodes six proposed ORFs, four of which show no significant homology with databases (Jian *et al.*, 1998). ORF 5 shows sequence homology to the cytochrome c oxidase assembly factor (CcOAF) and ORF 2 shows homology to the broad bean mottle virus and other plant Bromoviruses (Jian *et al.*, 1998). M2 consists of 3570 bp, with one major proposed ORF and four minor ORFs (Lakshman *et al.*, 1998). The major ORF shows significant homology to a region of hypovirulence-associated mitochondrial dsRNA from *C. parasitica* NB631, as well as to the pentafunctional polypeptide AROM from *Saccharomyces cerevisiae* (Lakshman *et al.*, 1998).

Direct evidence for the involvement of M1, M2 and other dsRNA segments in virulence regulation can only be obtained by transformation of *R. solani* with cDNA copies of the respective dsRNA elements. The group of Victor Rubio (CNB, Madrid,

Spain) has been developing a transformation protocol for the past 7 to 8 years. They are currently trying transformation using boilistic transformation methods. The present study examined transformation of *R. solani* using a PEG/ Ca^{2+}-mediated transformation protocol. Once transformation is attained, it can be either integrative or non-integrative, depending upon whether the plasmid is incorporated into the host chromosome. Initial transformation was achieved with pES200 (Figure 6.6), which contains the bacterial hygromycin resistance gene (*hyg*B^R) downstream from *Aspergillus nidulans trp*C start codon. Transformation, however, was non-integrative, which is characterized by a slow growth rate of the transformants (Monke and Schafer, 1993). Subsequent transformation using pAXHY2 (cDNA of hypovirus CHV1-713) was slightly more successful in that greater growth occurred than with pES200 transformants. However, these cultures also failed to grow on subculture, indicating non-integrative transformation, with the increased growth displayed by pAXHY2 transformants potentially due to the presence of a terminator sequence following *hyg*B^R in pAXHY2, and not in pES200. In both cases, non-integrative transformation was confirmed by hybridization analysis. For the transformation protocol to be a viable tool for genetical manipulation, integrative transformation would be desirable. Various means exist to increase the possibility of integrative transformation. The possibility of integrative events is 1 % (Monke and Schafer, 1993), so increasing the number of transformants should increase the probability of obtaining stable transformants. The plasmids used in this study contained no DNA with known homology to the genome of *R. solani*. Therefore construction of plasmids containing homologous DNA should enhance the probability of obtaining integrative transformants. In addition, ribosomal repeats have been reported to enhance transformation frequency in *Aspergillus niger* (O' Connell, 1998 http), so a similar situation may occur in *R. solani*. In addition, some isolates of *R. solani* harbour plasmid sequences (Miyasaka *et al.*, 1990; Miyashita *et al.*, 1990; Hongo *et al.*, 1994). It may be possible to construct plasmids with homology to the resident plasmids, and in this manner obtain stable transformantion.

Once stable transformants are readily obtained, it will be interesting to transform *R. solani* with cDNA of dsRNA from *C. parasitica*, to determine the effect it may have on the phenotype. Since fungi are in many ways promiscuous in their ability to express foreign DNA, *R. solani* may well express CHV1-713 cDNA and generate dsRNA. This has been shown to occur in several species phylogenetically related to *C. parasitica*

including *C. radicalis*, *C. havanensis*, *C. cubensis* and *Endothia gyrosa* (Chen *et al.*, 1994, 1996). Thus, if CHV1-713 can generate dsRNA in *R. solani*, and the transformants are subsequently hypovirulent, then a highly effective biological control strategy may potentially be developed. However, this becomes a transgenic organism, which leads to additional problems with licensing for environmental release.

It appears that dsRNA-mediated biocontrol may be possible in *R. solani*, due to the presence of segments, such as M2 from isolate Rhs1A1, encoding a hypovirulent response; however, for it to be successful, dsRNA transfer between isolates must occur. Transformation is generally limited to horizontal modes via hyphal anastomosis. Several reports indicate that dsRNA transfer occurs in *R. solani* (Castanho and Butler, 1978; Finkler *et al.*, 1985; Jian *et al.*, 1997); however, no direct microscopical evidence has been presented. This would require the elements to be labelled, possibly with radio-isotopes, or alternatively with fluorescing dyes. In addition, the elements may be tagged with reporter genes, such as the *Vibrio* luciferase genes or green fluorescent protein (GFP) which emit light upon expression. A similar tagging system with GFP was applied to Potato Virus X, to study movement of this virus between cells (Santa Cruz *et al.*, 1998).

The development of a dsRNA-mediated biocontrol strategy for *C. parasitica* was successful in Europe, but largely unsuccessful in the USA (reviewed by Nuss, 1992). This was due to a low number of VCGs in Europe, such that dsRNA dissemination via hyphal anastomosis was successful, whereas in the USA a greater number of VCGs were present, such that the majority of hyphal anastomosis events were followed by incompatible reactions. At the tuber level, isolates of *R. solani* were fully compatible with one another, indicating that dsRNA dissemination should not be impeded. However, the degree of incompatibility increased greatly when isolates from differing tubers from within a single field site were examined, with 44 % of the total reactions being incompatible. This increased to 100 % of pair-wise combinations being incompatible when isolates were harvested from differing areas of Scotland, and indeed from differing countries. It seems possible that dissemination of dsRNA throughout natural field populations of *R. solani* will be limited to small areas, with no widespread transmission beyond adjoining colonies. Potentially, this could be overcome by releasing isolates from multiple VCGs. Alternatively, it may be possible to suppress the

vegetative incompatible reactions using anti-sense mRNA to block *vic* gene expression (Benedetti *et al.*, 1987).

A further factor to be examined before a reliable biocontrol strategy is developed, is whether the hypovirulent isolates can persist in the environment. Ideally, they should succeed following a one-time inoculative release, as opposed to repeated releases each time they are required. Release of hypovirulent isolates of *C. parasitica* in Europe was self-sustaining following the initial release programme. In contrast, in the USA, following the release of hypovirulent *C. parasitica* containing CHV1-713 throughout eastern North America, later studies failed to re-detect its presence (Peever *et al.*, 1997). Since hypovirulent isolates tend to show reduced vigour, in addition to hypovirulence, problems with persistence are likely to arise. This can potentially be circumvented by altering the transformed DNA, such that hypovirulence is expressed without the reduction in vigour. For example, Craven *et al.* (1993) have constructed strains of *C. parasitica* possessing increased conidiation levels, which remain hypovirulent. In addition, Chen and Nuss (1998) constructed a cDNA clone from *C. parasitica* Euro7 (CHV1-Euro7). Isolates subsequently infected with CHV1-Euro7 were hypovirulent, as well as being more aggressive in colonizing chestnut tissue, and less reduced in asexual sporulation than strains infected with CHV1-713.

CHAPTER 8

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8.1. References

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