CHARACTERISATION OF EXTRACHROMOSOMAL ELEMENTS 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

A	adenine
ADP	adenosine diphosphate
AG	anastomosis group
amp	ampicillin
ATP	adenosine triphosphate
dATP	deoxyadenosine triphosphate
ddATP	dideoxyadenosine triphosphate
bp	base pairs
^{o}C	degrees Celsius
cDNA	complementary deoxyribonucleic acid
cm	centimetre
dCTP	deoxycytidine triphosphate
ddCTP	dideoxycytidine triphosphate
Ci	Curies
Da	Daltons
DMF	dimethyl formamide
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetate
g	grams
g	standard acceleration of gravity
GCG	genetics computer group
dGTP	deoxyguanosine triphosphate
dH_2O	distilled water
IPTG	isopropyl-β-D-thiogalactoside
Kb	kilobases
kg	kilogramms
LB	Luria broth
M	molar
μ	micro
min	minutes
mm	millimeters
mM	millimolar
mg	milligrams
ml	millilitres
MW	molecular weight
μl	microlitres
nm	nanometers
dNTP	deoxynucleotide triphosphate
OD	optical density
ORF	open reading frame

PCR	polymerase chain reaction
PDA	potato dextrose agar
psi	pounds per square inch
pM	picomoles
RACE	rapid amplification of cDNA ends
RAPD	randomly amplified polymorphic DNA
RNA	ribonucleic acid
RNase	ribonuclease
sdH_2O	sterile distilled water
SDS	sodium dodecyl sulphate
sp	species
T	thiamine
TCA	trichloroacetic acid
TEMED	N,N,N'-trtramethylethylenediamine
Tris	tris (hydroxymethyl) aminomethane
UV	ultra-violet
v/v	volume to volume ratio
w/v	weight to volume ratio
X-gal	5-bromo-4-chloro-3-indoyl-β-D-galactoside

ABSTRACT

The plant pathogenic basidiomycete, *Rhizoctonia solani* contains extrachromosomal double-stranded RNA and DNA elements, but the role of these elements in the biology and pathology of the fungus is uncertain. Aspects of these elements in *R. solani* and the role of anastomosis (hyphal fusions) in their transmission is examined here.

Anastomoses between hyphae, leading to successful cell fusions and death of fused cells (vegetative incompatibility) were observed by video microscopy and by fluorescence microscopy when hyphae were loaded with fluorochromes. However, attempts to monitor organelle transfer were unsuccessful and ultra-violet irradiation of hyphae containing fluorochromes led rapidly to hyphal death.

Two strains of anastomosis group (AG) 4 could readily be 'cured' of dsRNA by subculture of hyphal tips, although one strain which contained a 2.5Kb DNA element could not be freed in this way, nor by ultra-violet irradiation or heating to 30^{o}C. Several of the resulting hyphal tip subcultures showed an incompatibility reaction when paired with the respective parent strain. These parent-incompatible strains (6 from parent strain PA1 and 6 from parent strain I13) fell into two groups - mutually compatible within each group, but incompatible with the other group and the parent. Anastomosis during pairings of strains within any one group never led to a parent-compatible strain when subcultures were taken from the zone of hyphal fusion. There was no evidence that dsRNA influenced compatibility; instead it is suggested that hyphal tip subculturing led to segregation (or expression) of nuclear compatibility genes. Counts of nuclei in tip cells, by DAPI staining and fluorescence microscopy, showed variation in different parts of the fungal colonies, and significant tendency for some juxtaposed branch tips (arising as clusters from a single hypha) to have similar nuclear numbers to one another. The parent strains (AG4 strains PA1 and I13) that produced incompatible daughters after hyphal tip subculture had relatively low nuclear numbers per tip cell (2-9) whereas strains which did not produce such incompatible daughters (eg AG3 strain R41) had higher nuclear numbers per tip cells (14-16).

Only 3 of 15 strains of *R. solani* assayed for presence of dsRNA, using a cellulose CF-11 column, were found to contain it. There was no evident relationship between presence of dsRNA and phenotype of strains, but cured derivatives of both PA1 and I13 were found to have enhanced celluloytic activity and cured derivatives of strain PA1 showed increased pigmentation on PDA, increased mycelial mass when grown in liquid culture and increased production of two pectic enzymes, polygalacturonase and pectin lyase. However they were as pathogenic as the parent strain in seedling assays on carrot, cress, radish and cabbage. It is suspected that these effects were due to segregation or expression of nuclear genes in the cured strains rather than to curing of dsRNA, because other cured strains did not show phenotypic changes.

Isolation and partial characterisation of the extrachromosomal 2.5Kb DNA element from *R. solani* strain AG4 I13 showed it to be linear with blocked 5' ends and free 3' ends. A base sequence was obtained for 637bp (approximately 1/3) of this element but it showed no similarity to other detected DNA sequences and no continuous open reading frame. Southern blotting showed the element to be specific to AG4.

CHAPTER 1

INTRODUCTION

1.1. GENERAL INTRODUCTION

Agricultural crops are under constant attack from noxious organisms. It is estimated that 25-45% of the yearly harvest of the world is lost as a result of parasite damage and adverse environmental conditions. Of this, approximately 12% results from damage caused by fungal and bacterial pathogens (Jutsum, 1988). Economically this loss is vast, even when considered solely in terms of lost revenue from farm crops in the United Kingdom, £759 million in 1991. Although crop losses due to harsh environmental conditions cannot be controlled, losses which result from attack by parasites and pathogens can be reduced with the aid of chemical or biological control agents.

Chemical pesticides and fungicides are the most frequently employed method for controlling plant pests and pathogens and have been used extensively and successfully in controlling many plant diseases (Becker & Schwinn, 1993). However, the effectiveness of plant disease control by chemicals is diminishing due to the emergence of fungicide resistance in the pathogens. Indeed, development of resistance appears to have been actively promoted by the widespread and indiscriminate use of biocidal agents (Becker & Schwinn, 1993). Thus, there is now a pressing need for new 'improved' chemicals to combat such pests. However, as development of pesticides and fungicides is a difficult, costly and long term process, biological approaches have assumed greater importance as alternative control mechanisms (Whipps *et al.*, 1993).

Biological control is defined as 'the action of parasites, predators and pathogens in maintaining another organism's density at a lower average than would occur in their absence' (DeBach, 1964). Thus, biological control of pathogens exploits natural processes as a means of reducing damage to plants and, although at present less than 1% of the market for crop control is held by such agents (Powell & Jutsum, 1993), the potential for expansion of this control is great. However, to be effective, biological control agents must become established in their natural habitat, so it is essential that they are as fit environmentally as the target organism. The fates of released organisms are important, especially if they are genetically modified, as

there are several possible outcomes upon the release of a genetically engineered organism into the environment. (1). The organism may be unable to survive and is therefore of no use as a biological control agent. (2). The organism may establish itself and perform its desired function. (3). The organism may become a rogue and affect the environment detrimentally by phenotypic means, such as expression of unwanted growth or competition characteristics, or by genetic means, such as the transfer of some or all of the genetic material of the engineered organism to indigenous organisms, changing their characteristics in an environmentally negative way. Therefore before biological control mechanisms can be implemented an assessment of their influence on the environment must be carried out by closely monitored field studies.

One of the most successful approaches to biological control was observed with the fungus *Cryphonectria parasitica* (Murrill) Barr, virulent forms of which cause chestnut blight *C. parasitica* devastated both the European and American chestnut tree population. Perhaps surprisingly, successful biological control of chestnut blight emerged naturally upon the emergence of protective (hypovirulent) strains of *C. parasitica,* to the extent that after only 24 years from their discovery, Mittempherger (1978) reported that, in Italy, chestnut blight had lost its epidemic character and had found a more even host-pathogen balance. Despite these early observations, it was not until 20 years after discovery of the hypovirulent isolates that the latter were shown to differ from the virulent strains in that they contained double-stranded ribonucleic acid (dsRNA) (Day *et al.*, 1977). The dsRNA was thought to belong to a fungal virus, or mycovirus. *C. parasitica* will be discussed in more detail later in this chapter.

Mycoviruses are intracellular viruses found in both unicellular and filamentous fungi. The typical mycovirus is usually present in the cytoplasm as RNA (often double-stranded and multi-segmented) encapsidated in a protein coat (Buck, 1986a). Mycoviruses have been identified from a variety of plant pathogenic fungi such as *Ustilago maydis* (DC.) Corda which causes corn smut (Koltin & Day, 1976), *Ophiostoma ulmi* (Buisman) L. Moreau, the causative agent of Dutch elm disease (Brasier, 1983) and *Gaeumannomyces graminis* (Sacc.) v. Arx & Olivier which

causes take-all of wheat (Rawlinson *et al.*, 1973). Furthermore, the finding that chestnut blight could be suppressed by strains of *C. parasitica* which carried dsRNA resulted in numerous attempts to correlate the presence of a mycovirus or dsRNA in other fungal pathogens with changes in virulence (reviewed by Nuss & Koltin, 1990). In most species, however, the cause and effect relationship between the dsRNA and pathogenicity was not as clear as with *C. parasitica.*

A particular example of conflicting views on the effect of dsRNA on virulence and pathogenicity occurs with *Rhizoctonia solani* Kuhn. In this fungus the presence of dsRNA elements has been correlated with both reduced virulence (Castaño *et al.*, 1978) and increased virulence (Finkler *et al.,* 1985). In addition to this, yet other reports claim that dsRNA plays no major role in *R. solani* virulence (Zanzinger *et al.,* 1984; Kousik *et al.,* 1994). As *R. solani* is a pathogen of over 130 plant species (Baker, 1970), it is of significant economic importance. Further investigation is needed into the role of extrachromosomal elements in this organism, with regards to their potential for biological control. The prospect of their successful inter-strain transfer in natural environments also requires further investigation.

1.2. TRANSMISSION OF EXTRACHROMOSOMAL GENETIC ELEMENTS IN FILAMENTOUS FUNGI

Extrachromosomal genetic elements are ubiquitous in fungi. In order for them to persist a mechanism of transmission of the elements between strains, followed by their maintenance and stable inheritance, must exist. The most common extrachromosomal genetic elements in fungi are dsRNA, present either in mycovirus particles or unencapsidated, so the transmission and stability of these elements will be discussed.

Double-stranded RNA mycoviruses are distinct from other viruses in that they appear to be transmitted solely by intracellular routes, have no extracellular phase in their lifecycle and do not lyse their hosts (Buck, 1986a). Nonetheless the term mycovirus is applicable to most of these elements due to structural similarities with

known viruses (Stanway & Buck, 1984; Ghabrial, 1988). These viruses are often cryptic so their presence and possibly roles in the host can only be determined by thorough analysis.

When present in filamentous fungi, the viruses are carried by the flow of protoplasm towards the hyphal tip. The viruses often are not present in the tip itself and replication of the particles appears to take place at the peripheral growth zone of the hypha whereupon the virus is moved through the cells by cytoplasmic streaming towards the tip (Hollings, 1978; McFadden *et al.*, 1983). Movement of virus within a mycelial network is limited by the rate and extensiveness of septal pore blocking. Septal pores allow free movement of both viruses and cytoplasm between cells, enabling their spread throughout a colony. Blockage of these pores restricts the movement of protoplasm and so prevents spread of extrachromosomal elements. The rate of blocking after the formation of septal pores varies between species. In some fungi such as *Geotrichum* spp the pore is blocked almost immediately after septum formation (Buck, 1986a) whereas in *Neurospora crassa* Shear & Dodge, the septal pores remain unplugged for some time after septum formation so a number of cellular compartments are unplugged at any given time, allowing a free spread of the elements throughout the network (Bull & Trinci, 1977). If the septal pore in the apical compartment becomes blocked before virus transmission, then subsequent growth could be virus-free. This may explain 'self-curing' in some fungi (Buck, 1986a).

Transmission of viruses throughout the hyphal network is generally a very efficient process such that if one part of the hypha is infected the infection usually spreads throughout the whole mycelium. Efficient transmission of the virus through both sexual spores (Koltin & Day, 1976), in some cases, and vegetative spores (Bozarth *et al.,* 1971) allows stable inheritance of the virus within a species. The number of viral particles are similar in both vegetative spores and mycelium in some fungi. In sexual spores of *S. cerevisiae* the amount of dsRNA is higher than would be expected from the volume of cytoplasm (Brewer & Fangman, 1980). However, in some ascomycetes such as *Gaeumannomyces graminis* there appears to be exclusion

of mycoviruses from sexual spores so that many sexually reproducing ascomycetes are virus-free (Buck, 1986b).

It appears that transfer of the virus throughout the network and into spores is very efficient so once the virus is present in the mycelia its position is very secure. One of the notable features of mycoviruses is their apparent lack of ability to infect by other means. Numerous attempts have been made to infect fungal mycelium with purified virus preparations, all of which have been unsuccessful (Bozarth, 1975; Boissonnet-Menes & Lecoq, 1976; Mitchell *et al.,* 1976) and it is generally thought that the cell wall acts as a barrier to penetration (Buck, 1986a). This lack of infection limits transmission of the viruses to intracellular routes such as through fusion between hyphal cells (anastomosis). Once the new hypha has been infected by the virus, via cell fusion with a virus-containing cell, the virus can spread throughout it by anastomosis of hyphae within the newly infected colony. Transmission of dsRNA between cells via anastomosis has been shown using auxotrophic, coloured or fungicide-tolerant mutants as recipients in anastomoses of *Ustilago maydis* (Wood & Bozarth, 1973) and *R. solani* (Finkler *et al.,* 1988) so transmission via anastomosis is probably common in fungi. However, successful anastomosis is limited to a specific subset of the population-those individuals which are vegetatively compatible.

Vegetative incompatibility occurs either as fusion incompatibility, such as between different anastomosis groups of *R. solani* (described in section 1.3), or post fusion incompatibility, in which transmission is restricted, such as in *C. parasitica.* Anagnostakis (1983) showed that the efficiency of transmission of dsRNA in *C. parasitica* was inversely related to both the number of gene differences between the strains involved and the strength of the individual vegetative compatibility genes. The same conclusions were reached by Brasier (1984) for *Ophiostoma ulmi.*

As transmission in nature only occurs via anastomosis it is expected that the natural host range of any one type of dsRNA is limited to closely related individuals. Although extensive transmission studies have not been carried out hybridisation studies have been used to ascertain the degree of similarity of dsRNA between fungi from different species (Bharathan & Tavantzis, 1990,1991). Although in general only closely related species contain similar dsRNA, species of diverse genera such as

Penicillium stoloniferum (ATCC 14586) and *Diplocarpon rosea* Wolf have similar viruses (Bozarth *et al.,* 1972; Kim & Bozarth, 1985).

1.3. ANASTOMOSIS

Rhizoctonia solani is a basidiomycete and has a sexual stage in its lifecycle. But this sexual stage is inconspicuous, occurring as a crust of basidia on the surface of soil (Papavizas, 1970), and its significance in nature is unknown. A significant level of gene transfer might therefore occur by vegetative fusions of hyphae, because the fungus can persist in soil as aggregates of mycelia known as sclerotia. Anastomosis occurs between two growing hyphal tip cells. It may be a common method for gene exchange in fungi and in *R. solani* it is categorised in the following way. When isolates are placed 2-3 cm apart on water agar their mycelia grow towards each other (Parmeter *et al.*, 1969). Microscopic examination then reveals three types of event (Figure 1.1).

1. The hyphae grow over and under each other but never make tip to tip contact or physically interact with each other in any way. When two colonies behave in this way they are of different anastomosis groups which, in effect, may represent different species because of the breeding barrier.
2. The hyphae undergo an interaction between mycelial tip cells and fuse but upon fusion 5-6 cells on either side of the fused cells become vacuolated and die. Flentje *et al.* (1967) named this 'the killing reaction'. Hyphal fusion and the killing reaction only occur between isolates of the same anastomosis group.
3. Hyphal fusion is complete and cytoplasmic mixing occurs. The cells survive. These hyphae are usually genetically identical or at least very similar to one another.

Perfect hyphal anastomosis occurs as follows. As the hyphae grow they release one or more attracting substances which cause a chemotropic response in the other hypha. On contact of the two cells there is cessation of growth and the formation of a branch-like projection. The cell walls then dissolve locally at the contact point and the two protoplasts are joined. Only isolates of the same anastomosis group can recognise each other and fuse, suggesting there may be a

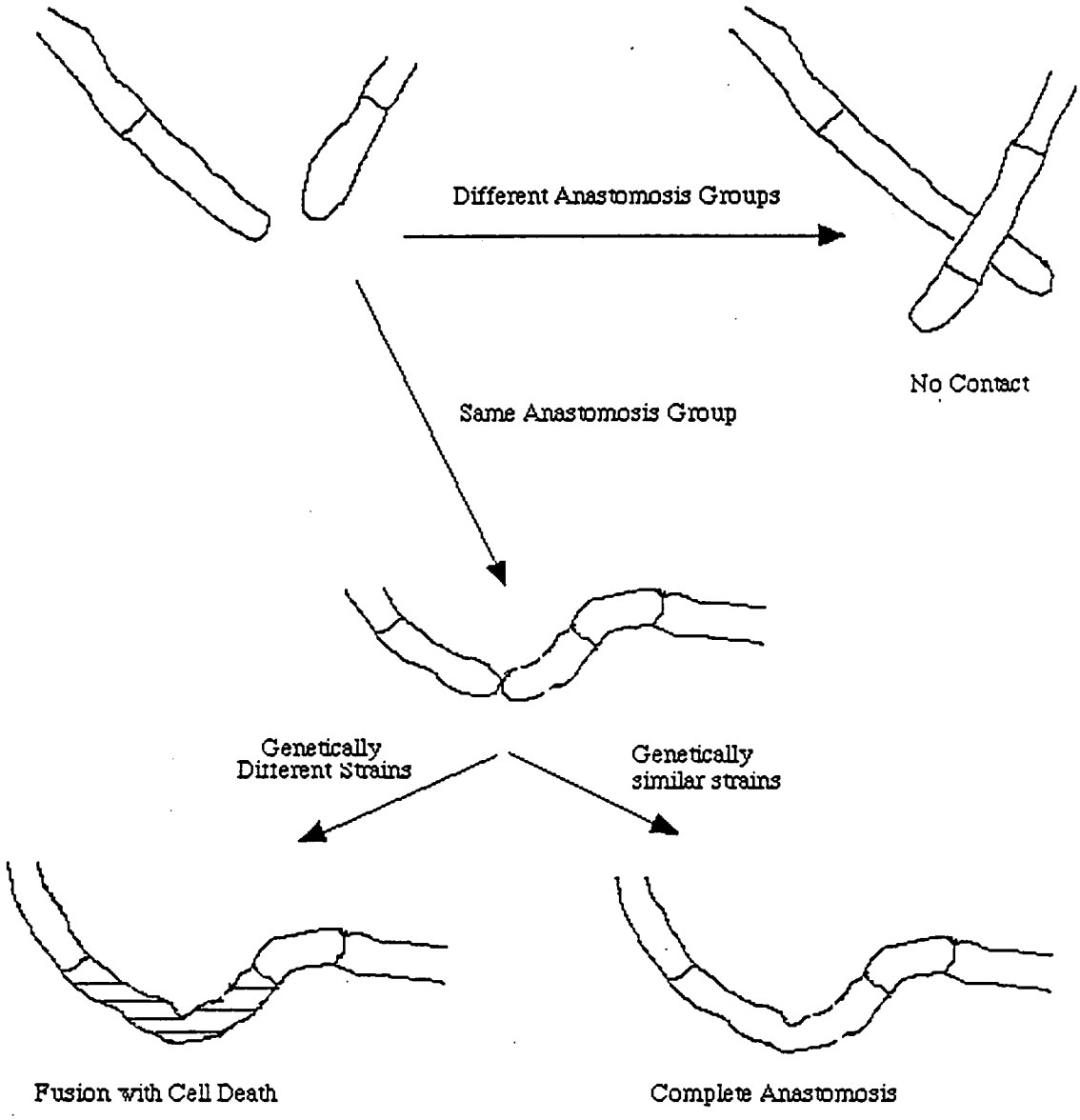


Figure 1.1. Types of hyphal interaction in filamentous fungi.

unique signal recognised by each anastomosis group. Hyphae may be attracted from distances of 100μm, causing the responsive tip to change its direction of growth.

One of the major mechanisms of classification of *Rhizoctonia solani* is via hyphal anastomosis on culture media. Each anastomosis group can be considered as a genetically isolated population as no genetic interaction is thought to occur between anastomosis groups since cellular fusion is observed solely between strains belonging to a single group (Anderson, 1982). Anastomosis was first described by Schultz (1937) and since then a variety of studies on it have been made on Japanese, American and British strains, among others. At present 12 anastomosis groups (AG) are recognised and subdivided by a combination of hyphal fusion, pathology and morphology (Ogoshi & Ui, 1985). Included in these were bridging isolates, BI, which can fuse with more than one group. Inevitably, there is also a mixing of nuclei of two strains following anastomosis, and this presence of two genetically distinct nuclei in a common cytoplasm is termed heterokaryosis. Thus, two compatible homokaryons can fuse to form a heterokaryon. A field isolate of *R. solani* can be paired in culture with a strain from a known specific anastomosis group, called a tester strain, and its anastomosis group can be clearly identified from this.

1.3.1. Vegetative compatibility

From the killing reaction (Flentje *et al.,* 1967), it was noted that isolates from within the same anastomosis group may not always be compatible. As a result of this incompatibility, *R. solani* is separated into vegetative compatibility groups which are subdivisions of anastomosis groups (Ogoshi, 1987). The genetic control of vegetative compatibility is unknown in Basidiomycetes but it is thought to be very specific (Anderson, 1982). In the chestnut blight fungus, *C. parasitica,* an ascomycete, there are no obvious anastomosis groups but there are over 70 vegetative compatibility groups, serving to limit cytoplasmic transfer (Anagnostakis, 1977). Such groups may also reduce the spread of cytoplasmic genetic elements between isolates in nature (Caten, 1972) and may contribute to the stability of the fungus. (But *C. parasitica* is still a simple species because sexual reproduction can occur between all members of the population.)

There are two types of cytoplasmic inclusions which may have detrimental effects on the fungus: viruses and defective mutant derivatives of cytoplasmic genetic elements which are normally present. Double-stranded RNA viruses occur commonly in fungi and infectivity of these has been correlated with hyphal fusions following hyphal anastomosis in *P. stoloniferum* (Lhoas, 1971), *C. parasitica* (Anagnostakis & Day, 1979) and *H. victoriae* (Ghabrial, 1988). Defective mutant derivatives of existing genes have also been identified in fungi, giving rise to phenomenon such as senescence in *Podospora anserina* (CES.) Rehm (Esser, 1990), stopper phenotypes in *Neurospora crassa* (Bertrand *et al.*, 1985) and vegetative death in *Aspergillus amstelodami* Thom & Church (Caten, 1972). With *A. amstelodami* cytoplasmic transfer of the vegetative death phenotype was reduced to 15% by vegetative incompatibility compared to 100% transfer with compatible reactions.

Genetic control of vegetative incompatibility

Cryphonectria parasitica contains at least 77 vegetative incompatibility groups in the USA alone and it is estimated that at least 7 nuclear genetic loci determine vegetative compatibility in this species (Anagnostakis, 1982). Parental lines with the same alleles at all vegetative compatibility loci yield progeny of the same vegetative compatibility group. If progeny fall into two vegetative compatibility groups, then the parents are assumed to have different alleles at a single locus. If progeny fall into more than two compatibility groups then more than one locus is assumed to be involved. The ability to form viable anastomoses decreases as the number of dissimilar alleles increases. In *C. parasitica* the large number of vegetative compatibility groups has prevented successful biological control of chestnut blight in the USA (see later). In addition, it is also thought to have markedly reduced the spread of cytoplasmic dsRNA between strains. Other ascomycetes such as *Fusarium* spp and *Neurospora* spp also have similar vegetative incompatibility systems (Leslie, 1993).

Although, as stated, the genetic control of vegetative incompatibility is not fully understood, there is not always a complete block to nuclear and cytoplasmic exchange as hemi-incompatibility has been reported in certain gene combinations

(Esser, 1964). It appears that even a short fusion time followed by cell death may be enough for transfer of certain elements (B. Hillman, pers. comm.)

1.4. EXTRACHROMOSOMAL ELEMENTS OF FILAMENTOUS FUNGI

Although ubiquitous in fungi, extrachromosomal double-stranded RNA elements have only been examined in detail in a few species. This is usually due to an obvious phenotype which can be associated with the element. The effect of the presence of dsRNA is variable and often there is no noticeable phenotypic change. When there is a phenotypic effect, it is due to the expression of specific viral coding sequences, as in *Ustilago maydis* where dsRNA-encoded killer systems produce a toxin which is active against other *U. maydis* strains (Koltin, 1988). Debilitation of the fungus due to dsRNA elements can lead to phenotypic effects such as abnormal morphology, slow growth, reduced sexual and asexual sporulation and a marked decrease in pathogenicity.

Like dsRNA viruses, linear DNA plasmids are common in plant and fungal species, but as most of the elements are cryptic they appear to be of little importance. In only a few cases, such as in the yeasts *Kluyveromyces lactis* (Gunge *et al.*, 1981) and *Saccharomyces cerevisiae* (Sinclair *et al.*, 1967) killer strains, were phenotypes associated with the plasmids.

Linear plasmids of eucaryotes were first isolated from *S. cerevisiae* (Sinclair *et al.*, 1967) and since then have been isolated from a variety of plants such as Brassica spp. (Palmer *et al.*, 1983) and fungal species such as *Morchella conica* CBS. (Baarn) (Meinhardt & Esser, 1984), *Ascobolus immersus* Rizet (Francou, 1981) and *Claviceps purpuria* (Fr.) Tul. (Tudzynski & Esser, 1983). The plasmids are normally located in either the cytoplasm or the mitochondria of the cells and in general, they have terminal inverted repeats (Meinhardt *et al.*, 1990) or proteins covalently bound to the 5' end. These proteins are thought to serve as primers for DNA replication.

Analysis of a group of linear DNA plasmids from a variety of species showed them all to have an open reading frame which has sequence homology to the

nucleotide binding site of viral DNA polymerase (Oeser & Tudzynski, 1989) and therefore they appear to encode their own polymerase. The overall structure of the plasmids are very similar to that of DNA viral genomes (Carusi, 1977) so the plasmids may share a common ancestral form.

1.5. VIRUSES OF PHYTOPATHOGENIC FUNGI

1.5.1. *Cryphonectria parasitica*

Pathology

The use of an extrachromosomal dsRNA element to control a fungal plant pathogen is best illustrated with chestnut blight caused by *Cryphonectria parasitica* which invades wounds of chestnut trees and destroys the surrounding healthy tissues. Necrosis and collapse of bark tissue produces a visible, sunken, bright orange canker which spreads until it has completely encircled the trunk or branch. Subsequent occlusion or destruction of the nutrient-conducting tissue results in wilting or death of the trees. The root systems often survive to produce new shoots, but these are susceptible to the fungus and so rarely reach maturity; as a result the trees are stunted (Herbard *et al.,* 1984).

History

Chestnut blight was first recorded in the Bronx Zoo in New York City in 1904 and by the early 1930's had spread throughout most of the United States. In 1938 the disease was also identified in Northern Italy and it continued to spread throughout Europe for the next 15 years. In 1951, Biraghi (1953) reported a coppice near Genoa with spontaneous healing of cankers of diseased trees: although 85% of the trees were infected with *C. parasitica,* very few exhibited extensive disease symptoms. Further investigations by Grente in the early 1960's revealed two types of *C. parasitica* in asymptomatic infected trees: those strains which appeared normal in pure culture and those with reduced sporulation and pigmentation (Grente, 1965). When applied to trees, the form of *C. parasitica* with reduced virulence (that with

reduced pigmentation and sporulation) was restricted to outer layers of the bark and could prevent the further spread of a wound caused by a virulent strain. The strains with reduced virulence were termed hypovirulent and this phenotype spread so successfully and naturally via anastomosis that Mittempergher (1978) reported that cultivation of chestnut was then no longer a problem in Italy. In France, chestnut blight was controlled by artificial application of hypovirulent strains directly to trees and within 10 years the disease was no longer a problem in that country (Grente & Betherlay-Sauret, 1978). Attempts to control chestnut blight in the USA by topical application of hypovirulent strains proved unsuccessful due to the fact that there is a much wider variety of anastomosis groups of *C. parasitica* in America than Europe, thereby preventing cytoplasmic exchange (Anagnostakis, 1982). This problem resulted in further studies into the hypovirulence agent.

Phenotypic characterisation of hypovirulence

By pairing the virulent and hypovirulent strains of *C. parasitica* on plates, and allowing fusion of cells, it was shown that the hypovirulence phenotype could be transferred to virulent strains, so the hypovirulence phenotype appeared to be cytoplasmically determined (Van Alfen *et al.*, 1975). Day and co-workers (1977) isolated double-stranded RNA (dsRNA) from hypovirulent strains but not virulent strains. Transfer of the hypovirulence phenotype correlated with transfer of dsRNA (Anagnostakis & Day, 1979) and equally, elimination of dsRNA restored the virulence phenotype (Fulbright, 1984). Decreased virulence is the only consistent phenotype associated with the dsRNA of *C. parasitica.* Saprophytic growth on agar is not affected and, although pigmentation is reduced in hypovirulent European strains, there is no clear reduction with American strains. However, in addition to decreased virulence, cultures which harbour dsRNA may exhibit one or more other phenotypic changes. These differences include altered colony morphology (Anagnostakis, 1982) and asexual spores which are produced as normal but in lower numbers (Anagnostakis, 1984a). Production of at least eight specific fungal proteins has been reported to be reduced in hypovirulent strains (Powell & Van Alfen, 1987). Laccase, a phenol oxidase which is secreted at the advancing edge of the colony, is

reduced 6-fold in hypovirulent strains. This enzyme is thought to retard the defensive process of lignification in the host tree (Rigling *et al.*, 1989). Reduction is thought to be due to a shortening of the half-life of the mRNA and repression of transcription of the laccase gene late in the fungal life cycle. Reduced levels of oxalate have also been recorded. Oxalate is a calcium chelator and lowers the pH of the tree's tissue, allowing pectic enzymes to act more effectively on the tree. Reduction of oxalate production may account for the inability of hypovirulent strains to colonise deep into the bark (Havir & Anagnostakis, 1983). Cryparin, an 18,000Da protein thought to be involved in formation of the cell wall in fruiting bodies, is abundant in virulent strains but not in hypovirulent strains (Powell & Van Alfen, 1987). Reduction of this protein may explain the decrease in conidiation by the hypovirulent strains. In addition to these, the expression of two early sporulation genes *vir1* and *vir2* is reduced. The exact function of *vir1* is unknown but failure to transcribe *vir2* is known to reduce the level of sporulation (Pfeiffer and Van Alfen, 1992).

Down-regulation of all of these genes could be explained by non-specific effects of the dsRNAs on protein kinases as this would debilitate the translational apparatus of the fungus. So molecular characterisation of dsRNA was carried out to determine if specific fungal genes are debilitated by the elements.

Molecular characterisation of hypovirulence

Reduction in the pathogenicity of *C. parasitica* is transferable to virulent strains of the same vegetative compatibility group, suggesting that the hypovirulence phenotype is encoded by a mobile genetic element (Van Alfen *et al.*, 1975). Double-stranded RNA was later positively correlated with hypovirulence (Day *et al.*, 1977). Electron microscopy has shown that *C. parasitica* dsRNA is unencapsidated but associated with membranous vesicles clustered in the hyphal apex. Transmission of the particles appears to be solely by anastomosis as cell free preparations are uninfectious (Havir & Anagnostakis, 1983).

Most hypovirulent strains contain a variety of dsRNA elements with considerable sequence similarity (L'Hostis *et al.*, 1985), a polyA tail at the 3' termini and a 28 nucleotide conserved sequence at the 5' end. On the basis of their size the

dsRNA's have been termed large (L-dsRNA), medium (M-dsRNA) and small (S-dsRNA). S-dsRNA and M-dsRNA's are derived by internal deletions from the 12,712bp L-dsRNA (Shapira *et al.*, 1991a). As there is variation in the S-dsRNA and M-dsRNA elements during culturing, and only the L-dsRNA is stable throughout the life-cycle of the fungus, characterisation was carried out on the L-dsRNA only. L-dsRNA contains 2 viral coding domains, ORF (open reading frame) A which is 622 codons long and ORF B which is 3165 codons in length; ORF A is preceded by a leader sequence of 495bp. The termination of the first ORF serves also as the initiation codon of the second ORF. Proteolytic processing of the proteins occurs such that ORF A encodes two polypeptides of 29,000Da and 40,000Da instead of the expected protein of 70,000Da (Choi *et al.*, 1991). All of the dsRNAs are transmissible, in concert, and cause conversion of the recipient strain to hypovirulence (Powell & Van Alfen, 1987), suggesting that the hypovirulence phenotype may result from an effect of the double-stranded RNAs on gene expression.

Cells transformed with a plasmid containing the viral coding domain of ORF A of the L-dsRNA produced the physiological traits of the hypovirulent strain - reduction of orange pigmentation, fewer conidia and less laccase, but there was no reduction of virulence (Choi & Nuss, 1992a). Reduction in virulence was achieved in addition to hypovirulence-associated traits upon transformation of a cDNA copy of the full length L-dsRNA (Choi & Nuss, 1992b). Therefore, the individual viral coding domains appeared to be directly responsible for the phenotypic changes in the fungus as opposed to a general debilitation of the strain. This provided the first direct evidence for the causal role of dsRNA in hypovirulence of a fungus.

Transmission

As the dsRNA is responsible for reduced virulence, its transfer into new strains is essential for conversion of the strains to hypovirulence and, therefore, control of the fungus. Vegetative incompatibility is a barrier to cytoplasmic transmission as there is no anastomosis between incompatible strains. As there are over 70 compatibility groups in the USA this poses a problem in using natural

transmission mechanisms. For transmission of this dsRNA there need only be partial compatibility then cell death, as the virus is present at the hyphal tip and is thus transferred very quickly (Havir & Anagnostakis, 1983). As the successful establishment of hypovirulence requires transfer of the dsRNA, the mobility of genetic information within the fungus must be explored. Gobbi *et al.* (1990) demonstrated that upon anastomosis, nuclei and mitochondria fail to migrate at detectable levels but dsRNA moved rapidly between the strains.

As the viruses will not infect fungi with intact cell walls in the absence of anastomosis a direct transformation system has been devised for *C. parasitica* (Churchill *et al.*, 1990). A full length cDNA clone of L-dsRNA has been constructed in which the viral open reading frames are under the control of constitutive fungal promoters (Choi & Nuss, 1992a). Upon transformation, the cDNA integrated into the fungal genome and was transcribed subsequently being present in the cell as both cDNA and dsRNA. The advantage of integration is that, in the chromosome, the cDNA will also be transmitted through sexual crosses. As there is only a single mating type locus for sexual reproduction (Anagnostakis, 1984b), involving two alleles, this greatly reduces the barrier to transmission erected by vegetative incompatibility (Figure 1.2).

Foreign DNA introduced into filamentous fungi is subject to frequent point mutations, rearrangements, or silencing by methylation. These post-integrative events result in mitotic (Pandit & Russo, 1992) and meiotic instability (Selker *et al.*, 1987) so the stability of the integrated cDNA was examined. There was no unwanted alteration of phenotypic traits or physical integrity of the strains even after growth on dormant chestnut tree stem tissue. Engineered strains were shown to be able to serve as male donors in a sexual cross with vegetatively incompatible virulent strains to give stable transmission of functional cDNA, so providing an efficient means of spread of hypovirulence.

At present, there has been no release of the engineered strains but field trials should show their environmental fitness and stability. Other fungi have not been as well studied as *C. parasitica* and there is limited information on the potential effects of dsRNA in other species.

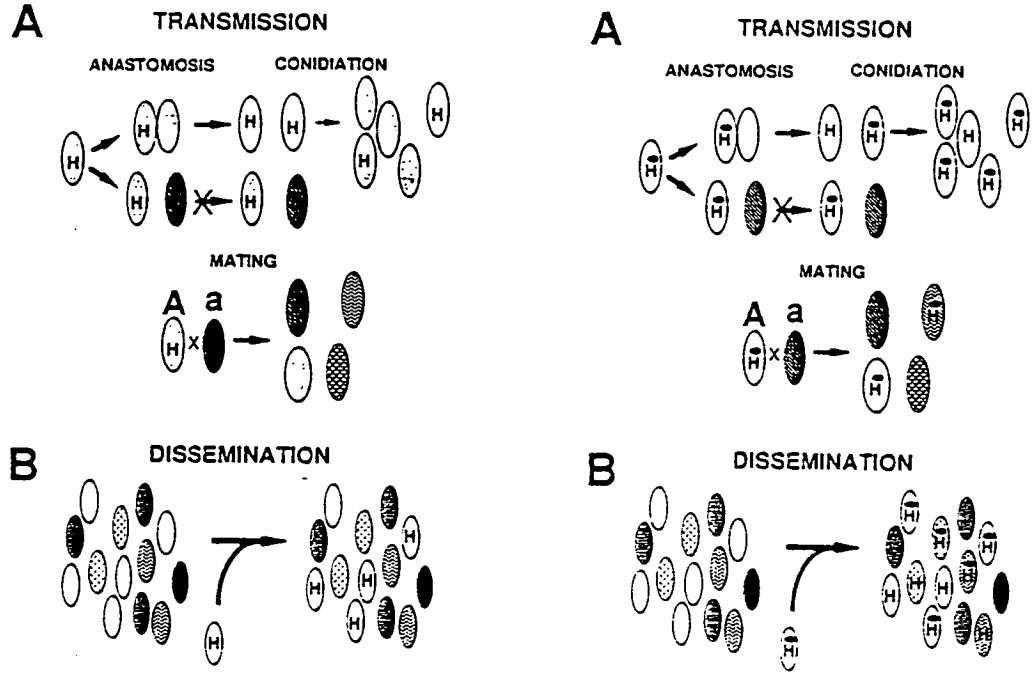


Figure 1.2. Transmission of double-stranded RNA in *Cryphonectria parasitica.*

1. Transmission of natural hypovirulence-associated viral dsRNAs and consequences for dissemination. Ellipses represent colonies of *C. parasitica.* The letter H denotes cytoplasmically replicating hypovirulence-associated dsRNAs. Different shading patterns indicate different vegetative compatibility groups. **A.** Three potential modes of transmission of the viral dsRNA. Efficient transmission to a virulent strain occurs only after anastomosis. However anastomosis is limited to strains of the same vegetative compatibility groups. Consequently viral dsRNAs are not transmitted to incompatible strains (indicated by X through arrow). A second potential mode of transmission is through asexual spores (conidia), which occurs at variable frequency. A third potential mode is through sexual spores (ascospores) resulting from mating. Mating compatibility is controlled by a single mating-type locus involving 2 alleles designated (A) and (a). Due to allelic rearrangement the progeny can be of different vegetative compatibility groups to the parent strain. However evidence is available which indicated that hypovirulence associated dsRNAs are not transmitted through ascospores at readily detectable levels. **B.**Predicted consequences of this natural transmission pattern on the dissemination of introduced natural hypovirulence. Introduction of a natural hypovirulent strain results in the efficient conversion of virulent strains of the same, or closely related, vegetative compatibility group.

2. Predicted transmission and dissemination of genetically engineered hypovirulent *C. parasitica* strains. Designations as in 1 except the integrated cDNA copy is indicated by a solid black oval within colonies. **A.** The predicted transmission of a genetically engineered strain differs as; nearly all of the asexual spores would carry the L-dsRNA as either integrated cDNA or resurrected dsRNA. Secondly, the integrated cDNA form has the potential for transmission into the progeny of sexual crosses. **B.** The predicted availability of the cDNA form of L-dsRNA to be integrated into new vegetative compatibility groups as a result of mating coupled with higher transmission frequency through sexual spores is likely to result in significantly increased dissemination and sustainability. (Reproduced from Nuss, 1992).

1.5.2. *Ophiostoma ulmi*

The causative agent of Dutch elm disease, *Ophiostoma ulmi*, is an ascomycete which occurs naturally as two distinct populations. The aggressive population is highly virulent and has been responsible for recent epidemics of Dutch elm disease; the non-aggressive strain is less pathogenic. Both populations contain 'diseased' isolates which show abnormal morphology, slow growth, reduced conidial viability and a deficiency in sexual reproduction when compared with healthy strains (Brasier, 1983). The disease has been shown to be caused by dsRNA elements, named d-factors. Ten of these dsRNA elements are needed for complete 'disease' symptoms. Removal of three specific segments results in the loss of the symptoms, suggesting that these are important in the diseased phenotype (Rogers *et al.*, 1988). 'Disease' symptoms are also lost during sexual reproduction, coinciding with the loss of most of the dsRNA elements. Both healthy and diseased strains are known to contain dsRNA and it is a specific combination of the ten dsRNA elements, ranging in size from 0.34 to 3.5 kb, which is needed for the full disease phenotype.

The dsRNA elements are associated with the mitochondria, and are thought to affect cytochrome aa_3; therefore repression of aerobic respiration may reduce the disease symptoms (Rogers *et al.*, 1987). The d-factors are easily transmitted via hyphal anastomosis, resulting in transfer of disease symptoms. These, d-factors therefore, have the potential for control of virulent strains as the diseased isolates have reduced pathogenicity due to reduced fitness. Unfortunately, the level of reduced fitness of the hypovirulent strains limits their use as biological control agents; also the vegetative incompatibility system of *O. ulmi* limits transmission of the disease. In some countries, like Portugal, over 90% of strains are of the same vegetative compatibility group (Buck, 1988) so there is the potential for the successful spread of low virulence in these countries, resulting in a possible form of control for Dutch Elm disease, if fitter cultures containing dsRNA could be engineered.

1.5.3. *Helminthosporum victoriae*

H. victoriae is a highly specialised pathogen of oats, affecting only the Victoria variety which is susceptible to crown rust. The 'diseased' form of the fungus carries two specific dsRNA viruses (Sanderlin & Ghabrial, 1978). Disease symptoms in the fungus include the development of small colonies which lyse their aerial mycelium, poor sporulation and generalised cell lysis. These colonies exhibit reduced levels of expression of the toxin, victorin and reduced pathogenicity. Diseased cultures of *H. victoriae* produce two serologically and electrophoretically distinct viral particles which have been designated 142s and 190s due to their sedimentation values. Healthy isolates are either virus free or contain a low concentration of the 190s virus (Ghabrial, 1988). Disease severity appears to correlate with the 145s titre in colonies, so it has been suggested that either the 145s alone or a mixed infection with the two viral dsRNA elements is the cause of the disease phenotype (Ghabrial *et al.*, 1979). However no isolate has been observed to contain the 145s virus alone, so the possibility that the 145s virus is a satellite of the 190s virus cannot be ruled out. The viruses are transmitted by hyphal anastomosis. Due to the reduction in toxin production the diseased strains have reduced pathogenicity on plants and so have the potential for use in control of plant disease.

1.5.4. *Gaeumannomyces graminis* var *triciti*

G. graminis is the causative agent of take-all disease of wheat, and natural isolates range from avirulent to highly pathogenic. Storage results in rapid loss of pathogenicity (Naiki & Cook, 1983). Attempts to correlate dsRNA with pathogenicity were unsuccessful, as viruses were detected in both virulent and non-virulent strains (Rawlinson *et al.*, 1973). However, a greater proportion of isolates with dsRNA had reduced pathogenicity when compared to those without dsRNA (Stanway, 1985) and further analysis of a hypovirulent strain containing 9 segments of dsRNA was used to correlate the dsRNA with hypovirulence. After sexual reproduction conidial isolates which had lost the dsRNA were virulent whereas those which had maintained it were hypovirulent, suggesting a correlation between dsRNA

and reduced virulence (Buck, 1986a) although it is unknown if specific dsRNA segments are important in decreased virulence.

1.5.5. *Ustilago maydis*

The basidiomycete, *Ustilago maydis*, is the causative agent of corn smut. Double-stranded RNA elements cause interstrain inhibition of other *Ustilago maydis* strains due to the production of specific toxins which inhibit growth (Koltin, 1988). These toxins are found in less than 1% of the natural population (Koltin *et al.*, 1986) and are encoded by up to 8 segments of dsRNA. The toxin consists of two polypeptides which are not covalently associated (Peery *et al.*, 1987) and are similar to the *Saccharomyces cerevisiae* killer toxins (Bostain *et al.*, 1984).

The toxins do not affect fungal virulence as the toxin has never been detected in infected plant tissue and appears to have no known adverse effects on plants. Thus, there is no difference in plant pathogenicity of strains with or without virus, but the toxin-containing strains could be used to control the levels of *U. maydis*.

1.5.6. *Rhizoctonia solani*

The soil fungus, *Rhizoctonia solani* is also known to contain dsRNA elements, but these have not been correlated conclusively with pathogenicity. In order to ascertain their role, an understanding of the biology of the fungus is needed, so the biology of *R. solani* will be described before discussing the present knowledge of the extrachromosomal elements.

1.6. *RHIZOCTONIA SOLANI*

Rhizoctonia solani was described in 1850 by Julius Kuhn, associated with potato tubers (Menzies, 1970). The fungus is now known to have a world wide distribution, and is capable of attacking such a large range of plants that it is more not enough if a plant cannot be parasitised by this organism. Although the name *Rhizoctonia* comes from the Greek meaning "death of roots" the organism attacks a

variety of plant structures, causing diseases such as seed decay, damping off, black scurf, root rots and basal stem rots (Bateman, 1970).

As well as being a very successful pathogen *Rhizoctonia* can grow saprophytically on roots and in soil (Menzies, 1970). It is the combination of competitive saprophytic ability, lethal pathogenic potential and an almost unlimited host range that makes *R. solani* such an economically important pathogen.

1.6.1. Morphology of *R. solani*

The name *Rhizoctonia solani* represents the imperfect (asexual) state of *Thanatephorus cucumeris*. In the absence of a sexual stage the fungus is described on the basis of its multinucleate cells and aerial hyphae. Vegetative hyphae consist of cells 4-12μm in diameter (Palo, 1926) and 50-250μm in length depending on the age of the cells. All cells are multinucleate and are separated by dolipore septa (Figure 1.3). These septa have a small pore in them allowing partial cytoplasmic streaming but preventing the transfer of organelles (Butler & Bracker, 1970).

Hyphal branching occurs either at an angle of approximately 45^{o} to the direction of growth, or at right angles to the main hyphae. Young hyphae bend towards the direction of growth of the main filaments. There is a constriction of the cell at both sides. A septum is always present near the origin of the new branch. In some cases a septum subsequently develops in the main hypha close to the branch point. As the hyphae mature the cell walls thicken and constrictions at the branch points may not be so marked. Branches from mature hyphae arise infrequently and give rise to sclerotia or infection cushions (Flentje *et al.*, 1970). Mature hyphae may produce a pigment on the surface of a substrate whereas young hyphae, or those within a substrate or host, are hyaline. Strains have varying degrees of pigmentation and aerial or surface hyphae can range in colour from hyaline, through yellow, to brown (Butler & Bracker, 1970).

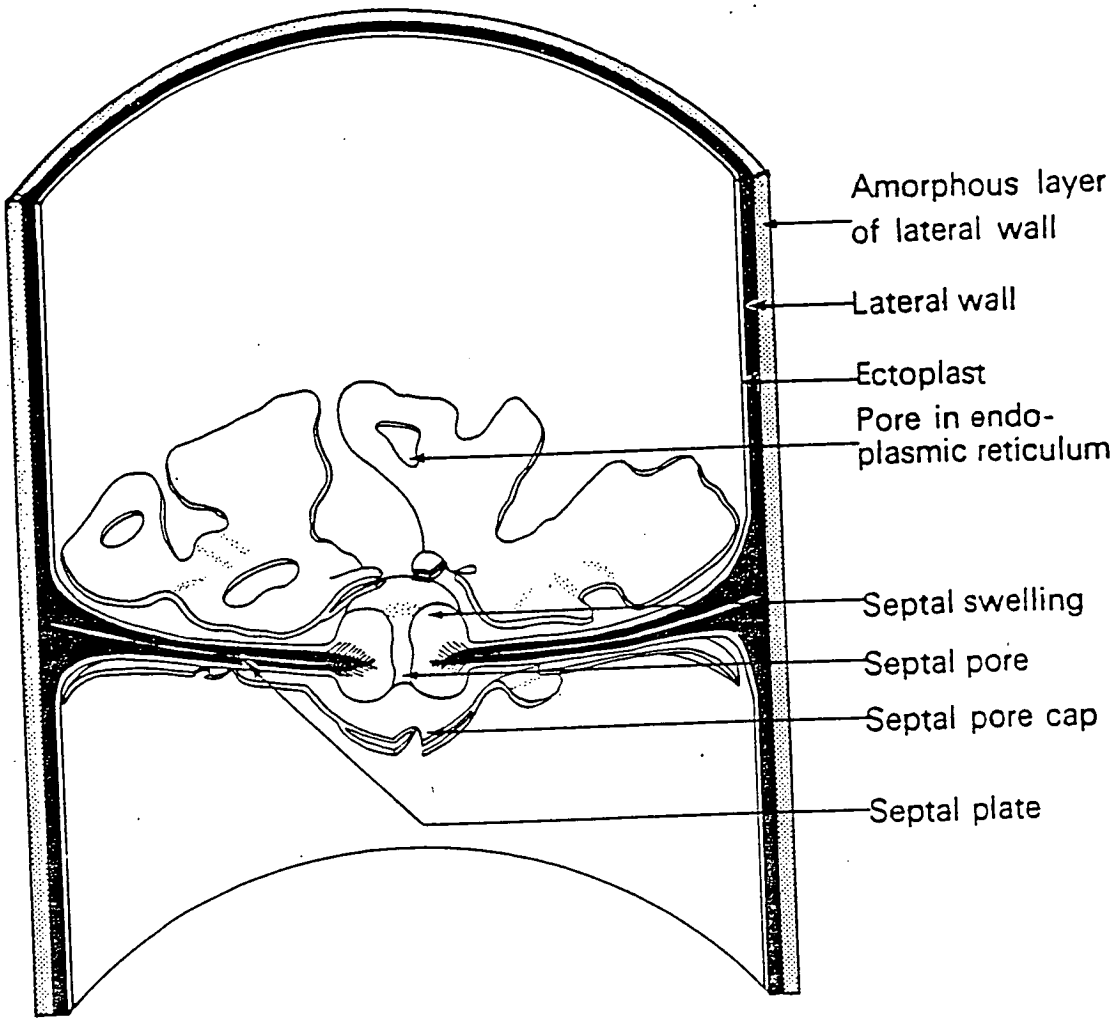


Figure 1.3. The dolipore septum of *Rhizoctonia solani.* Fine structure of a *R. solani* septum (reproduced from Butler & Bracker, 1963).

1.6.2. Physiology of *R. solani*

Nutrient requirements

As a species *R. solani* requires only a simple organic carbon source and inorganic salts for growth, but can also utilise a wide range of natural sources and synthetic media. *R. solani* is cellulolytic (Daniels, 1963) and can therefore utilise native cellulose as its sole carbon source. The fungus is equally able to metabolise pectin (Sherwood, 1966) but growth is slow on this substrate due to the production of acidic waste products.

Magnesium is essential for growth (Tyner and Sanford, 1935) and, for optimum growth, calcium is also required. Deficiencies in calcium cause arrested growth, loss of colour and decreased sclerotial formation (Sherwood, 1970). The addition of vitamins into the growth medium increases the initial growth rate but does not affect the overall dry weight produced. The composition of the media can however, influence vegetative characteristics such as thickness and colour of surface mycelia, abundance and colour of aerial mycelia and zonation of colonies (Flentje and Saksena, 1957) but the reasons for this are unknown.

Nutrient uptake and translocation

Uptake of nutrients by *R. solani* is dependent on the permeability of the mycelia allowing glucose, sorbose, phenylalanine and leucine to be imported (Obrig and Gottleib, 1966). Sugar uptake occurs as a two stage process. Initial transport across the cell membrane is energy dependent and is followed by active accumulation of the sugar in the cytoplasm (Sherwood, 1970). Cellulose uptake appears to be restricted to branched hyphae in older parts of the mycelium (Daniels, 1963).

The work of Sherwood (1970) shows that translocation of nutrients occurs from older hyphae to tip cells and is temperature dependent, i.e. optimum movement occurs at 20-30^{o}C. Slow-growing hyphae appear to translocate most efficiently, suggesting that translocation is a saturable process (Sherwood, 1970). Nutrients are thought to be soluble and transported by cytoplasmic streaming as this is unidirectional towards tip cells and the septal pore apparatus does not provide a mechanical barrier to streaming (Bracker & Butler, 1963).

1.6.3. Interactions of *Rhizoctonia solani* with plants

Isolates of *R. solani* exist as plant pathogens, symbionts and saprophytes, and the organism appears to adapt very successfully to its environment. Most anastomosis groups of *R. solani* are plant pathogens of economic importance. There is little known about the precise mechanisms of infection of the fungus although young plants and seedlings are most susceptible to *Rhizoctonia* disease.

Entry of *R. solani* into the plant host usually occurs via a natural opening or wound site, although there is also direct entry by individual hyphae. Infection is thought to be mediated by mechanical or enzymatic means, or a combination of both (Gonzalez & Owen, 1963). Once inside the plant the mycelia spread initially between cells. Indeed, in some plants, such as lettuce, the hyphae will never actually penetrate the cells but will always remain intercellular. In most cases, however, the hyphae will actually penetrate into the cells and a large build up of mycelia will form.

Infection is usually characterised by the host cells turning brown after hyphal penetration. Cellular collapse occurs after intracellular penetration (Chi & Childars, 1964) and sclerotia may be formed within dead host tissue. The plants defence system may be stimulated as a result of infection, causing necrosis of the tissue surrounding the infected cells (Flentje, 1959) and providing a barrier to further infection. It has been shown with soybean that metabolic by-products of the fungus are toxic to soybean roots and cause damage in the absence of the pathogen (Wyllie, 1962). However, toxic metabolites are not produced by all pathogenic strains of *R. solani* and so in some cases the necrosis must solely represent host damage due to cellular disruption after hyphal invasion.

Enzymes involved in R. solani pathogenicity

Enzymes play an important role in plant pathogenesis and *R. solani* is known to produce a variety of pectinases, cellulases, and proteases which are thought to have a role in aiding pathogenicity.

Pectin is a constituent of the middle lamellae of plant cell walls and acts as an intercellular cement. It is a polysaccharide of D-galacturonic acid linked in an α-1,4 arrangement to a glycoside. The breakdown of pectin causes tissue collapse as the

infrastructure of the plant is damaged. Pectic enzyme production is known to be important to various pathogens which cause soft rots, dry rots, wilts and leafspots. Even virus-infected tissues have been shown to undergo alterations in their extracellular pectic constituents (Weintraub & Ragetti, 1961). *R. solani* produces a battery of pectic enzymes, most of which have been identified and some of which have been purified (Marcus *et al.,* 1986). It is known that for successful invasion and maceration of plant tissues more than one pectic enzyme is needed (Bateman & Miller, 1966). Endopolygalacturonase, pectin esterase and endopectinolyase have all been purified from *R. solani* (Marcus *et al.*, 1986) and it has been noted that endopectinolyase was only detected in virulent strains of the fungus, the other enzymes being produced by both pathogenic and non-pathogenic strains. Endopectinolyase is only detected later in the course of infection and therefore appears to function primarily in aiding pathogen spread through host tissues.

Cellulose is the major structural component of the cell wall of higher plants. It is a polymer of β-1,4 linked glucopyranose which is found in the microfibrilar structure of the primary or secondary cell wall. *R. solani* is one of the relatively few organisms that can utilise native cellulose (Bateman, 1964). There is alteration and destruction of cellulose within infected hosts but cellulose breakdown is not known to be essential for initial pathogenesis by the fungus, although it may be involved in intracellular penetration and the ultimate collapse of the cells (Bateman, 1970).

R. solani can also utilise proteins as a growth substrate, so it must produce proteolytic enzymes. Furthermore, at least one known inducible extracellular protease has been shown to be produced by the fungus (Van Etten and Bateman, 1965). Proteases may be important in cell wall degradation and infected tissue appears to have a higher proteolytic activity than non-infected tissue.

Wyllie (1962) has also shown that *R. solani* is capable of producing toxic metabolites. These metabolic by-products of the fungus are thought to be involved in pathogenicity but production may be dependent on the individual fungal isolate. Most of these toxins are phenols and carboxylic acids (Aoki *et al.*, 1963).

1.6.4. Diseases caused by *R. solani*

As a species, *R. solani* is one of the most versatile plant pathogens, causing damping-off in at least 130 different plant types. It affects a wide variety of plants in diverse ecological niches and has also been reported as a parasite of other fungal species (Butler, 1957). It is impractical to describe in detail all the diseases caused by *R. solani* so only those associated with specific anastomosis groups will be mentioned.

Web blights, aerial blights and hypocotyl rots, such as sheath blight of rice, are caused by anastomosis group 1 strains of *R. solani* (Anderson, 1982).

Anastomosis group 2 isolates on the other hand more commonly cause cankers of root crops and root disease on crucifers (Anderson, 1982). However, this anastomosis group has been further divided into two groups. Anastomosis group 2-1 causes seed rot and damping off in radish. Seed rot is one of the most detrimental diseases caused by the fungus as *R. solani* will invade seeds while still in the fruit, or when planted in infested soil. The fungus will utilise the seed as a nutrient source thereby preventing germination (Baker, 1970). Anastomosis group 2-2 causes crown rots and root lesions in plants such as radish and carrot, in addition to seed rot and damping off (Anderson, 1982). Crown rot originates at the bases of young leaves and causes petiole disease. As new leaves form they are infected and the crown of the plant may eventually be killed. The fungus will then advance to the fleshy root causing a dry brown decay. Root lesions are not a major pathogenic feature of *R. solani* but a few crops such as lucerne (alfalfa), are devastated by this disease. Injurious cankers develop along the tap roots followed by sunken cankers along the secondary roots.

The pathogens of potato are mainly from anastomosis group 3. This causes stem cankers and stolon lesions. Stem cankers occur on fully mature plants, usually by contact of one of the plants leaves with soil, and spread throughout the plant. Black scurf of potato is one of the most commonly recognised *Rhizoctonia* diseases and lesions form on tubers of senescing plants or on the surface of the potato in storage. Although unsightly, the potato is not affected by the sclerotia formation on

its surface, which is the main economically disfiguring symptom of the disease (Anderson, 1982).

Anastomosis group 4 strains invade a wide variety of plant species. Seed rot , hypocotyl rot, stem lesions, crown rot, and aerial blights are all caused by anastomosis group 4, making it the most important pathogen in the northern United States (Anderson, 1982). Pathogenicity is not host specific and strains can infect a variety of commercial crops, for example carrot, radish, and cress, as well as flowers such as antirrhinum.

Anastomosis group 8 is associated with crater disease of wheat (Deacon & Scott, 1985) and barley stunt disease in Scotland. The disease is manifested by stunting of the plants in a given area and *R. solani* hyphae are found in bead like swellings on the roots or associated with brown, tapered roots where they have rotted.

1.6.5. *Rhizoctonia* symbiosis

The genus, *Rhizoctonia*, although best known for its pathogenic ability, forms a symbiotic relationship with orchids. In nature, orchids are closely associated with fungal mycelia. In most adult plants the absorbing organs are infected with intracellular fungi which form coils of hyphae within cortical cells. These coils are then digested by the orchid. The presence of the fungus, which obtains its nutrients by saprophytic activity in soil, allows the orchid to reach maturation by providing it with nutrients. The symbiotic strains were originally thought to be avirulent but Williamson & Hadley (1970) have shown that many pathogenic *Rhizoctonia* strains are also orchid symbionts.

1.6.6. Non-pathogenic *Rhizoctonia solani*

Until recently it was widely accepted that *R. solani* had a deleterious effect on plants with the exception of orchids (Warcup, 1985). However, a small number of studies have indicated that some strains of *R. solani* are non-pathogenic.

A series of experiments carried by Sneh *et al.* (1989) showed increased growth of plants colonised by non-pathogenic *Rhizoctonia solani*. A hypovirulent

isolate promoted growth in a wide range of species including radish, carrot, lettuce, cotton and wheat. It was also found to provide protection of seedlings against damping off (Ichielivich-Auster, 1985).

1.7. EXTRACHROMOSOMAL ELEMENTS OF *RHIZOCTONIA SOLANI*

1.7.1. Rhizoctonia decline

Degenerative diseases have been reported in *Helminthisporum victoriae* (Lindberg, 1960), *Cryphonectria parasitica* (Grente, 1965), *Helminthisporum maydis* (Bozarth *et al.,* 1972) and *Gaeumannomyces graminis* var *triciti* (Lapierre *et al.*, 1970). Each of these fungal species is a known plant pathogen and the disease symptoms in the fungus have been transmitted by hyphal anastomosis.

A degenerative disease of *Rhizoctonia solani* was observed by Castano & Butler (1978a) in an anastomosis group 1 isolate obtained during routine subculture of a healthy isolate. The diseased isolate was a white-tan colour, with irregular colony appearance. It had an extremely slow growth rate and produced very few sclerotia. The healthy isolate from which it was obtained was of uniform tan colour, rapidly growing, and produced many sclerotia. When tested on seedlings both in soil and on Petri dishes the diseased isolate was non-pathogenic (Castano *et al.*, 1978). It was further shown that addition of the diseased isolate to soil infested with the healthy isolate reduced post emergence damping-off caused by the latter (Castano & Butler, 1978b).

Subculturing of diseased cultures produced a constant phenotype, *i.*e. reduced pigmentation and reduced growth rate, or cell death; but in some cases a subculture from a healthy isolate resulted in the diseased phenotype. The diseased phenotypes could be separated into three categories depending on severity. In mild cases, isolates had reduced growth rate and sclerotia production. With the lethal phenotype, isolates failed to grow on subculture. Yet other strains had the debilitated phenotype but it was associated with stable cultures that remained viable.

The phenomenon was named Rhizoctonia decline as it was degenerative and the linear extension rate of diseased isolates dropped from approximately 40mm to 4mm per day (Castano & Butler, 1978a). Anastomosis enabled transmission of the decline from a diseased isolate to the healthy isolate from which it was obtained suggesting the presence of a cytoplasmically located element. The diseased isolate did not appear to be infected with bacteria and the disease could not be cured by antibiotics, heat treatment or acridine dyes, all of which are known to have a curative effect on plasmids. Nor was there evidence for toxin production by the strains. Attempts to transmit the disease to other healthy anastomosis group 1 strains via hyphal anastomosis failed so the disease was presumed to be isolate specific (Castano *et al.,* 1978).

1.7.2. Double-stranded RNA in *Rhizoctonia solani*

As there was no toxin production by the diseased isolates of *R. solani*, but the disease was transferable, investigations were carried out in an attempt to identify the cause. Upon examination of the fungal nucleic acids, double-stranded RNA was isolated from the diseased isolate, although no virus-like particles were detected in the strain using electron microscopy (Castano *et al.,* 1978). Three distinct segments of dsRNA of molecular weight 8.5kb, 2.3kb, and 1.8kb were consistently isolated. Upon conversion of a healthy isolate to the diseased phenotype, the co-association of dsRNA was detected. This indicates that dsRNA is correlated with Rhizoctonia decline, but the mechanism of decline and function of the dsRNA is unknown. Healthy isolates have also been found to contain traces of dsRNA of the same size and banding pattern to that found in the diseased isolates. This may be due to a quantitative relationship between the level of dsRNA per cell and the degree of symptoms observed. Four other anastomosis groups were also tested for the presence of dsRNA and it was found in 2 of 12 strains tested. Strains found to contain dsRNA were anastomosis group 2 strain C229 and group 1 strain S239. The molecular weights of these double-stranded RNA elements were unique to each strain (Castano *et al.,* 1978).

Unfortunately the anastomosis group 1 diseased isolate would not survive in soil for more than one month, suggesting that it was either converted to the virulent isolate following anastomosis, or it lacked environmental fitness. Biological control of the virulent isolate only occurred when both strains were actively growing and anastomosing (Castano *et al.*, 1978).

Castano and co-workers (1978) suggested dsRNA to be associated with Rhizoctonia decline, as in the association of dsRNA with hypovirulence in *C. parasitica.* This stimulated interest in the potential of dsRNA in *R. solani* for biological control and further investigations into the role of dsRNA in pathogenicity were carried out.

Zanzinger and colleagues (1984) isolated dsRNA from *R. solani* at a high frequency. Of 50 cultures tested, all of which were obtained from potato fields in Maine, USA, 49 contained dsRNA segments of sizes ranging from 0.6 to over 8.4kb. By allowing the field isolates to anastomose with tester strains from known anastomosis groups the isolates were shown to be distributed through anastomosis groups 1 to 5. They ranged from non-pathogenic to highly virulent within each anastomosis group. The only strain found not to contain dsRNA was from AG2 and, although it had an increased growth rate, pathogenicity was no different from that of any other AG2 isolate that contained dsRNA. A distribution of elements of the same size was found within and between anastomosis groups, to an extent conflicting with the work of Castano & Butler (1978b) which suggested the dsRNA was isolate specific (The existence of homology between dsRNA of similar sizes but from different isolates was not examined however).

The large number of isolates examined by Zanzinger and colleagues (1984) came from a small ecological niche. In order to obtain an overall view of dsRNA in *R. solani* it was necessary to examine a wide range of habitats. Finkler *et al.* (1985) re-examined the relationship between dsRNA and virulence by selecting specific criteria to define virulent and hypovirulent strains. These criteria were: hypovirulent strains should infect a wide variety of hosts but cause no damage to them; in all other respects the strains must be as fit as the virulent ones; virulent strains, on the other hand, should affect all tested hosts and cause severe damage.

Strains of *R. solani* were isolated from soil samples in Israel by Finkler and colleagues (1985). Samples were taken from diverse habitats in 26 locations. The strains isolated proved to be from anastomosis groups one to six. Pathogenicity of these isolates was tested on 11 host plants and 32 of 107 isolates were found to be essentially non-pathogenic on all hosts tested, based on the aforementioned criterion. These were hypovirulent (Ichielivich-Auster *et al.*, 1985). No special geographical location was noted among the hypovirulent strains. Four virulent AG4 strains, and four hypovirulent strains from anastomosis groups 1, 2 and 4, were further examined for the presence of dsRNA. These strains could not be distinguished morphologically or by growth rate. However, only virulent strains contained dsRNA (Finkler *et al.*, 1985). Three distinct banding patterns of dsRNA were identified by gel electrophoresis. Type I strains produced two segments of dsRNA of size, 2.2kb and 2kb. The second type contained three segments of dsRNA of size 2.4, 2.2 and 2kb. Finally, type III contains two segments of size 2.4 and 2.2kb. Repeated extractions of dsRNA over a period of one year from cultures maintained on potato-dextrose agar (PDA) showed some differences in banding pattern where smaller segments of dsRNA, of 2kb, were obtained from strains of type III (Finkler *et al.*, 1988). Such variations are common and also occur in *C. parasitica* (Shapira *et al.*, 1991a) and *G. graminis* (Buck *et al.*, 1981).

Attempts to transfer hypovirulence to a virulent isolate by pairing virulent and hypovirulent strains on agar using the method of Castano *et al.* (1978) failed to yield a hypovirulent isolate. However, hyphal tip subculturing from a virulent strain containing dsRNA resulted in loss of virulence in 1% of subcultures (Finkler *et al.*, 1985). These non-pathogenic cultures were weak and would not survive on PDA after 6 or 7 subcultures. Equally, attempts to isolate virulent strains from non-virulent hyphal tips were unsuccessful. Furthermore, loss of total or specific dsRNA segments was found to coincide with the recovery of hypovirulent strains from virulent strains (Finkler *et al.*, 1985) but no distinct correlation could be made between specific segments of dsRNA and virulence.

Cytoplasmic inheritance of virulence was also shown, using fungicide resistant-mutants, by heterokaryon formation between a hypovirulent strain resistant

to the fungicide Bayton, and a virulent Benomyl-resistant strain. The new cultures formed, which were resistant to both fungicides, were virulent but had the morphological characteristics of the hypovirulent strain. Double-stranded RNA extracted from the heterokaryons had an identical banding pattern to that of the virulent donor strain (Finkler *et al.*, 1985) .

The studies carried out by Finkler and co-workers (1985) are in direct disagreement with those of both Castano and colleagues (1978) and Zanzinger and colleagues (1984). The results also differ from the transmissible hypovirulence of *C. parisitica* in which dsRNA molecules suppress virulence. From the study by Finkler and colleagues (1985) the presence of dsRNA viruses in *R. solani* appears to be associated with the induction of virulence.

1.7.3. Characterisation of dsRNA from *R. solani*

Virus particles of 33nm in diameter have been detected in *R. solani* by electron microscopy (Zanzinger *et al.*, 1984; Finkler *et al.*, 1985). Purification of the particles from one AG4 strain, on a sucrose gradient (10-40%), showed the virions to be associated with the dsRNA particles. Two segments of dsRNA, of 2.2 and 2.0kb, were eluted from the purified virions. The virus particles have a major coat protein of 55,000Da and RNA-dependent RNA polymerase activity was found associated with the virions (Finkler *et al.*, 1985). This polymerase activity is found in most fungal viruses, such as those of *Ustilago maydis* (Ben-Zvi *et al.*, 1984) and *Penicillium stoloniferum* (Buck, 1975).

Analysis of viral encoded proteins was carried out, as previous studies suggested that dsRNA viruses may be involved in the regulation or production of virulence factors (Castano *et al.*, 1978; Finkler *et al.*, 1985). *In vitro* translation of purified ssRNA transcripts produced a major protein of 55,000Da and a number of smaller proteins. It is unclear whether the smaller proteins are non-structural or incomplete major protein products. However, as the minor proteins do not immunoprecipitate with antibody to the coat protein they are thought to be non-structural proteins. The major structural protein was confirmed as the coat protein by immunoprecipitation with antibodies raised to the virus. Furthermore, the protein

appeared to be very similar in strains containing different dsRNA particles. Peptide mapping of the coat protein by partial proteolysis with *Staphylococcus aureus* V8 protease gave the same banding pattern with the *in vitro* and wild type protein, further confirming that the 55,000Da protein is equivalent to the viral capsid (Finkler *et al.*, 1988).

In order to ascertain the coding potential of the viral genome, *in vitro* translation of the total dsRNA was examined. This gave similar gene products to the ssRNA transcripts. In addition, an extra protein of 60,000Da was produced from the dsRNA segments. This was thought to be the product of a 2.4kb segment which was extracted from cells but not found associated with virions. The 60,000Da protein did not immunoprecipitate with the antiviral antibody, further suggesting that the 2.4kb dsRNA is unencapsidated. The polypeptides identified exceed the coding capacity of the viral information so there may be use of overlapping reading frames or differential splicing.

1.7.4. Genetic relatedness of dsRNA from *R. solani*

Reports on the presence and association of dsRNA with virulence and hypovirulence have been conflicting. Three main factors may be involved.
(1). The complex genetics of *R. solani*. Anastomosis and vegetative incompatibility groupings mean that many different genetic backgrounds are present under one species name. (2). The lack of a bioassay for the introduction of purified dsRNA into suitable phenotypic strains. Although cured strains have been produced by hyphal tip subculture there is no direct transformation system for the selectable introduction of dsRNA into *R. solani*. (3). The genetic composition of the dsRNA isolated is varied.

The genetic diversity of the dsRNA was investigated by hybridisation studies. A large number of isolates from the USA, Japan and Israel were examined using specific viral transcripts as probes (Finkler *et al.*, 1988, Bharathan & Tavantzis, 1990). Genetic differences were expected as fungal viruses are segmented and mixed infections are possible.

The relatedness varied between anastomosis groups. For example, in AG4, dsRNA was isolate specific whereas AG5 contained interrelated dsRNAs (Bharathan

and Tavantzis, 1990). This variation may be due to the level of vegetative compatibility between isolates of AG4 which is much lower than for AG5. There was a lack of cross-hybridisation between anastomosis groups, emphasising that each group is genetically isolated. It was also noted that in AG2 hybridisation occurred between RNAs of different sizes. In this case the strain containing the larger dsRNA was virulent and the one with the smaller fragment was avirulent. It appears that a deletion may have occurred, changing the pathogenicity of the strain (Bharathan & Tavantzis, 1990). Therefore the dsRNA in *R. solani* may be associated with either virulence or the lack of virulence.

In general there is a closer genetic relatedness between isolates from the same geographic region than from disparate regions. However, even taking this into account there is still a great genetic diversity in the dsRNA of *R. solani* (Bharathan & Tavantzis, 1991).

1.7.5. DNA Plasmids of *Rhizoctonia solani*

Extrachromosomal DNA in *R. solani* has only been described by a single group working in Japan (Although in several reports). The DNA was isolated from AG4 strains which were slow growing and weakly pathogenic when applied to radish seedlings (Hashiba *et al.*, 1984). In addition to reduced virulence the strains produced oxalic acid which was secreted into the medium.

Agarose gel electrophoresis of total nucleic acid indicated the presence of a DNA element which electron microscopy showed to be linear. Characterisation showed the DNA to be 2.7kb in length, resistant to exonuclease III and λ exonuclease to digestion by protease and also to be easily renatured following treatment with alkali (Miyashita *et al.*, 1990). Restriction mapping indicated the presence of three plasmids of identical size which appeared to have different sequence. These plasmids were named pRS-64-1, -2 and -3. The cellular location of pRS-64 was determined as mitochondrial. Most linear DNA plasmids have terminally attached proteins which serve as primers for replication and the plasmids pRS-64 were shown by alkaline agarose gel electrophoresis to have hairpin loops at each end (Miyashita *et al.*, 1990).

Replication

Replication of the linear DNA plasmids of *R. solani* is thought to be similar to that proposed for Vaccinia virus (Baroudy *et al.*, 1983), rather than other linear DNA plasmids, as the plasmid of *R. solani* has inverted terminal repeats not terminal proteins to initiate replication (Figure 1.4).

Site-specific nicking has been proposed to occur in the terminal conserved region close to the terminal loop to form a 3' hydroxy end which serves as a primer for DNA replication. DNA synthesis will either proceed simultaneously from both ends or from a single end. It is unknown whether there is a single nicking enzyme or if more than one specific enzyme produces the breaks in the termini of the molecule or indeed if the nicking is mediated by proteins (Miyashita *et al.*, 1990).

Direct formation of new molecules will occur if DNA synthesis starts at both ends at the same time, but if there is only priming at one end there will be replication round the whole molecule and a dimer will be formed which must then be cleaved (Figure 1.4). Dimeric forms of pRS-64-1 were detected in gels, suggesting that replication of this plasmid is initiated from a single site. With the other two plasmids, pRS-64-2 and pRS-64-3, no dimeric forms were detected, suggesting that either initiation was from both ends simultaneously or the dimer was present for such a short time that it could not be detected (Miyashita *et al.*, 1990).

Genetic relatedness of R. solani DNA plasmids

In further studies, the same group isolated plasmid DNA from nine anastomosis groups of *R. solani* (Miyashita *et al.*, 1990). Forty two percent of isolates were found to contain plasmid DNA ranging in size from 2.2kb-7kb. Southern blotting analysis showed considerable sequence homology from isolates within the same anastomosis group but none between elements from different anastomosis groups. As the plasmids appear to be anastomosis group specific they may carry genes that determine host specialisation as many anastomosis groups are host specific.

It is known that some extrachromosomal genes share sequence homology with either the nuclear or mitochondrial genome of their host. For example, the

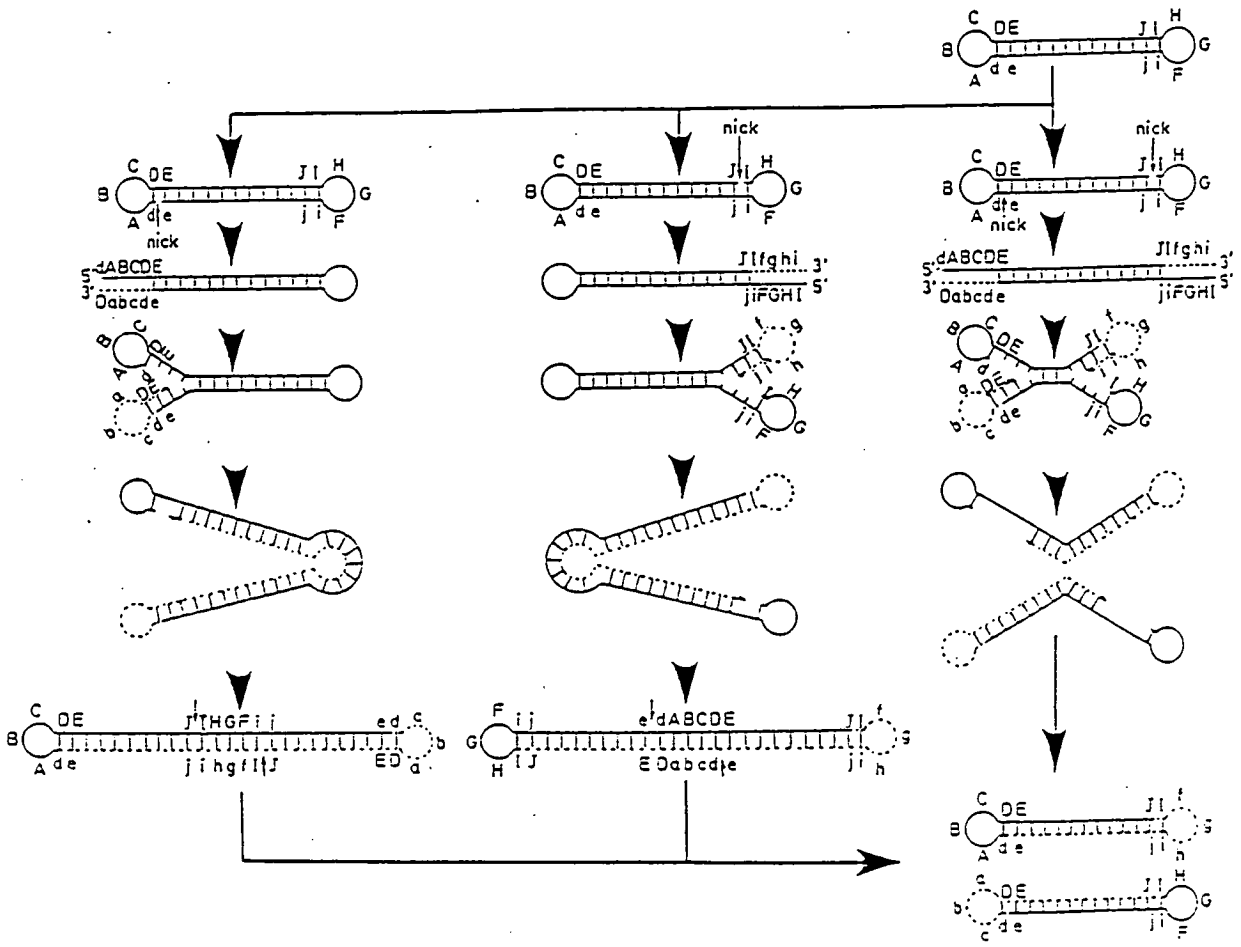


Figure 1.4. Replication of pRS-64. Possible models for replication of pRS-64 plasmid DNAs. Complementary sequences are indicated with capital and lowercase letters. The terminal hairpins are drawn as loops and designated ABC and FGH. The terminal homologous regions are designated DE and IJ (reproduced from Miyashita *et al.*, 1991).

mitochondrial DNA of maize shares sequence similarities with linear plasmids S-1 and S-2 (Kemble et al., 1983). However, southern hybridisation of full-length pRS-64 probes to chromosomal and mitochondrial DNA showed homology to chromosomal DNA only. There was also hybridisation to the chromosomal DNA of a non-plasmid-containing strain. This suggests that the plasmid may be stably integrated into the chromosomal DNA in some strains and is present there even when not detected extrachromosomally (Wako *et al.*, 1991).

At present, although extrachromosomal elements, both dsRNA and DNA, have been isolated from *Rhizoctonia solani* there is limited information available on both the elements and their ability to transfer in the environment.

1.8. AIMS OF THE WORK IN THIS THESIS

The soil fungus, *Rhizoctonia solani* is being used as a model to study the potential for gene transfer in the environment. *R. solani* is particularly useful for this as it forms a hyphal network in soil through which cytoplasm is transferred. As the fungus is known to contain extrachromosomal elements, these may be mobile and free to move within the hyphal network. There are barriers in *R. solani* which act against cytoplasmic mixing between strains of different anastomosis groups but the mechanisms preventing fusion and exchange (vegetative incompatibility) are largely unstudied, and the direction of transfer, whether unidirectional or bi-directional, after anastomosis is unknown. The aim is to further examine anastomosis microscopically and by specifically labelling organelles to monitor the potential for transfer after fusion. By monitoring organelles it is hoped to correlate their transfer with that of extrachromosomal elements.

There is much controversy as to the role of the extrachromosomal elements themselves, as very little is known about the DNA element and a wide variety of reports have obtained completely different conclusions with regard to the role of dsRNA elements. A second aim, therefore, was to look directly at the effect of dsRNA elements on *R. solani* by curing strains of the elements and comparing the

original dsRNA containing strains with their cured derivatives. Of specific interest are phenotypic traits which may be related to pathogenicity as these phenotypes could be attributed to the dsRNA.

In addition to dsRNA there is also a unique report of a dsDNA element in *R. solani*. Interest was stimulated in this by the fact that, during the present work, when curing a strain of dsRNA it was impossible to remove dsDNA by the same method. As direct removal of this element was impossible molecular characterisation of its structure was carried out in an attempt to elucidate its function. Characterisation of this element is useful as there is little known about linear DNA elements in fungi in general. Any information gained from this may be useful in determining its role in virulence and its potential use as a vector.

Overall, therefore, the aim of the work in this thesis is to examine the parameters of extrachromosomal gene transfer in *R. solani* and the direct effects of extrachromosomal elements on the fungus in order to gain more information on these elements and their potential for transfer in natural environments.

CHAPTER 2

MATERIALS AND METHODS

2.1. MATERIALS

All defined chemicals used were analytical grade (unless otherwise stated) and were purchased from Sigma (Poole, Dorset, UK), British Drug Houses (BDH) (Poole, Dorset, UK), Gibco-BRL (Paisley, Scotland), Fisons (Loughborough, UK), Pharmacia (Milton Keynes, UK) or Rathburn (Walkerburn, Scotland). Enzymes for nucleic acid manipulations were purchased from Boehringer Mannheim, as were X-gal (5-bromo-4-chloro-3-indolyl-ß-D-galactoside) and IPTG (isopropylthio-ß-D-galactoside).Membranes for hybridisation were purchased from Bio Rad (Zeta probe) or Amersham (Hybond N).

Radiolabelled nucleotides: α-^{32}P dCTP (3000Ci/mM, 10µCi/ml), γ-^{32}P dATP (55Ci/mM, 10µCi/ml), α-^{35}S dATP (400Ci/mM, 10µCi/ml) were purchased from Amersham.

Oligonucleotides were synthesised by the OSWEL DNA service at the University of Edinburgh.

2.1.1. Media

All media were sterilised by autoclaving at 20psi and 121°C for 15 minutes.

Fungal growth media

Potato dextrose agar (PDA)

Difco potato dextrose agar (15g) and Oxoid Agar No 1 (5g) was added to distilled water (dH_2O) to give a final volume of 1 litre and autoclaved before pouring into plastic petri dishes (approximately 20mls/petri dish).

Potato sucrose broth

Difco potato extract (4g) and sucrose (20g) were dissolved in dH_2O, with magnetic stirring, to give a volume of 1 litre.

Malt extract broth

Difco malt extract (3g) was dissolved in dH_2O to give a final volume of 1 litre.

Water agar

Oxoid Agar No 1 (20g) was added to dH_2O to give a final volume of 1 litre then the mixture was autoclaved before pouring into petri dishes (as above).

Pectic enzyme broth

$(NH_4)_2SO_4$ (2.64g), K_2HPO_4 (0.34g) and $MgSO_4.7H_20$ (0.14g) were dissolved in 500mls of dH_2O. Citrus pectin (10g) was added to the solution, with magnetic stirring, and dH_2O was added to give a final volume of 1 litre.

Minimal salts broth

$NaNO_3$ (5g), K_2HPO_4 (1g) and $MgSO_4.7H_20$ (2g) were added to tap water to give a final volume of 1 litre. After autoclaving, Biotin (10µg), Thiamine (100µg), $FeCl_3$ (1mg) and $ZnSO_4$ (0.9mg) were added.

Difco bacto agar and preparation of coated microscope slides

Difco bacto Agar (20g) was added to dH_2O to give a final volume of 1 litre. After autoclaving sterile microscope slides were thinly coated in the agar by immersing them in agar and allowing the excess to drip off before cooling.

Cellulose minimal agar

5g cellulose powder (Whatman CC31) was added to $NaNO_3$ (2g), KH_2PO_4 (1.23g), KCl (0.5g), $MgSO_4$. $7H_2O$ (0.5g), $FeCl_3.6H_2O$ (0.001g), $ZnSO_4.7H_2O$ (0.0009g), $MnSO_4.4H_2O$ (0.0004g) and Difco bacto agar (20g) in dH_2O to give a total volume of 1 litre.

Glucose-yeast extract agar

Glucose (30g), bacto yeast extract (5g) and Oxoid agar (15g) were added to dH_20 to give a volume of 1 litre. After autoclaving, the following filter sterilised solutions were added: adenine and uracil to provide 75mg of each supplement.

Bacterial growth media

Luria broth (LB)

Tryptone (10g), bacto yeast extract (5g) and NaCl (10g) were added to dH_2O to give a volume of 1 litre. After autoclaving of this, filter sterilised solutions of thiamine (35mg) and glucose (1g), prepared in dH_2O, were added.

Luria agar

The media was as above but with Oxoid agar No. 1 (15g).

M9 minimal medium

20ml of 20% Glucose (w/v) and 200ml of 5 x M9 salts were autoclaved separately. After autoclaving they were added along with 5ml filter-sterilised thiamine (0.2M) to 750ml of sterile distilled water. 5 x M9 Salts consists of $Na_2HPO_4.7H_2O$ (64g), K_2HPO_4 (15g), NaCl (2.5g) and NH_4Cl (5g) in 1 litre of deionised H_2O.

2.1.2. Solutions

Tris Cl

Tris base (tris (hydroxymethyl) amino methane) was dissolved in H_20, and the pH was adjusted to the required value by adding concentrated hydrochloric acid. H_20 was added to give a concentration of 1 molar.

EDTA

0.5M EDTA (ethylenediaminetetra amino acid, disodium salt) was prepared as a stock solution in H_20, the pH was adjusted to 8.0 with 10M NaOH.

TE

A solution of Tris.Cl (1M) and EDTA (0.5M) was used at pH 7.5 as a buffer solution for dissolving DNA.

TBE

TBE was prepared as a 10 fold stock solution and stored at room temperature. Tris base (108g), boric acid (55g) and 40ml of 0.5M EDTA were dissolved in dH_2O, with stirring, and diluted to 1-fold concentration immediately prior to use.

SSC

SSC was made as a 20-fold stock solution by adding NaCl (175g) and sodium citrate (88.2g) 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 20-fold stock solution by adding NaCl (5.84g), Tris (7.8g) and 2µl of 0.5M Na_2EDTA to dH_20 to give a total volume of 1 litre.

Phenol

Water saturated phenol was equilibrated to pH 8 for DNA manipulations. 0.5M Tris Cl (pH8.0) and 8-hydroxyquinoline (0.1% v/v) were added, with magnetic stirring, until the solution reached the appropriate pH. 8-hydroxyquinoline prevents oxidation of the phenol, inhibits RNAse activity and chelates metal ions (Kirby, 1956). Solutions are stored in the dark at - 20°C.

Chloroform-isoamyl alcohol

Chloroform was mixed with isoamyl alcohol in a ratio of 24:1 (v/v). The mixture was then used directly for nucleic acid manipulations.

Sodium acetate buffer

Sodium acetate (408.1g) was dissolved in 500ml dH_20 then the pH adjusted to 5.2 with the addition of glacial acetic acid (approximately 450mls). Distilled water was added to give a final volume of 1 litre.

Ethidium bromide

A stock solution was prepared by dissolving 1g of ethidium bromide in 100ml H_20 and the solution was then stored in the dark at room temperature.

Loading buffer for agarose gel electrophoresis

Loading buffer for agarose gel electrophoresis was prepared as a 6 x concentrate and consisted of 60% sucrose (w/v in H_2O), 6mM disodium EDTA and 0.025% bromophenol blue (w/v in H_2O) dissolved in 6 x TBE. The solution was stored at room temperature.

2.2. METHODS

2.2.1. Fungal methods

Strains

All strains used are listed in Tables 2.1 and 2.2. Cultures were grown at 25°C and maintained on potato dextrose agar or water agar for periods of up to 4 weeks.

For long term storage of cultures inoculum blocks were placed on sterile PDA slopes, or incubated in sterile water, at room temperature.

Strain	Anastomosis Group	Source.
SC220	1	R. Sherwood, USA
SC121	2	R. Sherwood, USA
HV1	2-1	R. Sherwood, USA
PS4	2-2	R. Sherwood, USA.
RH2/T	2-2	L. Burpee, USA.
C127	2-2	R. Sherwood, USA.
SC222	3	G. Papavizas, USA.
R41	3	P. van den Boogert, Netherlands.
PA1	4	G. Papavizas, USA.
I13	4	A. Finkler, Israel.
C233	4	R. SHerwood, USA
RH5/T	4	L. Burpee, USA.
GM1	5	R. Sherwood, USA.
RH6/T	5	L. Burpee, USA.
R470	5	R. Sherwood, USA
FIX1	6	Unknown
S3BS1	8	G. Murray, UK.

Table 2.1 *Rhizoctonia solani* strains

Species	Strain	Source
Ophiostoma novo-ulmi	H363	C. Brasier, UK.
Ophiostoma novo-ulmi	log 1/3 $8d^2$tol	C. Brasier, UK
Aspergillus foetoides	IMI 041871	C. Brasier, UK
Helminthisporum victoriae	B1	S. Ghabrial, USA.
Helminthisporum victoriae	HV83	S. Ghabrial, USA.
Helminthisporum victoriae	HV026	S. Ghabrial, USA.
Saccharomyces cerevisiae	1074	R. Whickner, USA.
Saccharomyces cerevisiae	1475	R. Whickner, USA.

Table 2.2. Fungal strains

Microscopy

All microscopy was carried out using a Lietz Orthoplan microscope, with Plano objectives (x10, x25, x70, x90) and Periplan (x10) eyepieces.

Video recordings of hyphal anastomosis were made using a colour Panasonic VW CL-350 video camera fitted to the microscope and attached to a Toshiba DV80B video recorder with a linked Sony Triniton 1460UB 14" colour monitor. Black and white video pictures were obtained with a Mitsubishi video copy processor.

Fluorescence microscopy was carried out using the same microscope equipped with a Ploemopak 2.1 fluorescence vertical illuminator which supplied ultra-violet light from an HBO-200 mercury vapour lamp. For fluorescence observations Leitz filter blocks A and H, incorporating a BP 330-385nm exciting (ultra-violet) filter or a BP 390-490nm exciting (ultra violet and blue) filter respectively, were used in conjunction with the appropriate suppression filters which were K400 and K460, respectively. Photographs were taken on Kodak Extrachrome P800/1600 film using a Leitz Orthomat fully automated 35mm camera.

Fluorochromes

The following dyes were obtained from Molecular Probes, Inc., Oregon, USA: fluorescein diacetate (FDA, mixed isomers; C-195), 5 (and 6)-carboxyfluorescein diacetate (CFDA CellTracker Blue; C-1361), 5-chloromethylfluorescein diacetate (CMFDA CellTracker Green; C-2925), 7-amino-4-chloromethyl coumarin (CMAC CellTracker Blue; A-2110), Flourescein-s-isothiocyanate (FITC; F-143), dihydroethidine (hydroethidine; D-1168), Lucifer Yellow CH, lithium salt (L-453), Rhodamine 123 (R-302), $DiIC_{18}$ (3) (1, 1-dioctadecyl 3, 3, 3', 3'-tetramethylindo carbocyanine perchlorate; D-282), $DiOC_{18}(3)$ (3,3-dioctadecyloxacarbocyanine perchlorate; D-275) and fluorescein isothiocyanate (FITC, F-143).

Dyes obtained from Park Scientific Ltd, Moulton Park, Northampton, UK., were as follows: Cellufluor (disodium salt of 4,4'-bis[(4-anilino-6-[bis(2-hydroxyethyl) amino]-s-triazin-2-yl) amino]-2,2'-stilbene; 17353), fluorescein diacetate (00615) and Nile Red (9-diethylamino-5-H-benzo-phenoxazine-5-one; 17997)

Ethidium Bromide (2,7-diamino-10-ethyl-9-phenyl-phenanthridinium bromide; E-8751), 4,6-diamidino-2-phenyl indole (DAPI; D-9542) and Acridine Orange (3,6-

bis[dimethylamino] acridine; A-6014) were obtained from Sigma Chemical Co., Poole, Dorset, UK.

Stains were dissolved in the appropriate solvents (Table 2.3). Stock solutions were diluted in sdH_2O to give working concentrations which were determined for *R. solani* (Table 2.3) by testing a range of concentrations and selecting for optimal staining with viability .

Incubation of R. solani with fluorochromes

A 1cm diameter agar block of mycelium was inoculated in potato sucrose broth or 1/4 strength potato sucrose broth for 2-3 days at 25°C. Hyphal tips were removed, using sterile forceps and placed in an empty Petri dish and then covered with 500µl of the appropriate fluorochrome. The solution was incubated for 1 hour at room temperature in the dark as many of the fluorescent compounds are light sensitive. The mycelia were washed well in distilled water and teased out to form a monolayer. The hyphae were placed on a slide containing a drop of water and examined microscopically under ultra-violet light for uptake of the fluorochrome and its ability to stain the appropriate organelles.

Anastomosis

A sterile microscope slide, 76 x 26mm (Chance Propper Ltd), was coated very thinly with water agar and placed on water agar within a Petri dish. Inoculum blocks of each of two different isolates of *R. solani* was placed on either end of the slide (see Figure 2.1). Slides were incubated at 25°C for 15-20h and examined microscopically for interactions.

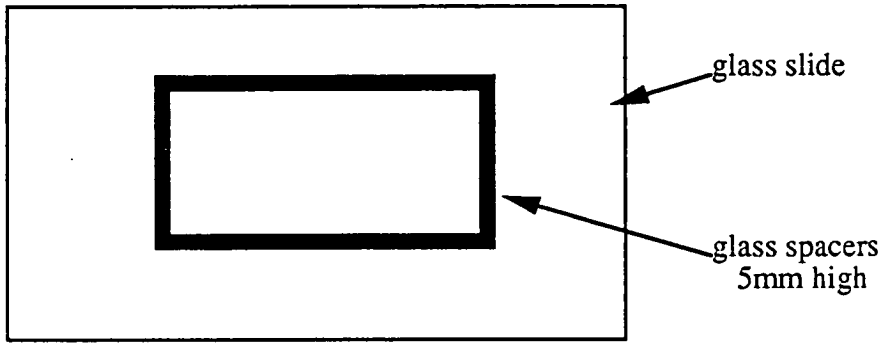
An enclosed soil system was used to study anastomosis in natural environments. Three strips of cellulose film (Rayophane PU525) each 1cm in width, and previously autoclaved in dH_2O, were placed in a square plastic dish half, filled with air dried soil (Figure 2.2). An inoculum disc of *R. solani* was placed on either end of the cellulose strip. To aid recovery of the cellulose a piece of gauze was placed over it. The box was then completely filled with soil brought to 50% saturation with water.

Growth rate in soil was also measured using this system. In this case only one end of the strip was inoculated. Growth of *R. solani* from the cellulose film through

Stain	Solvent	Stock Solution mg/ml	Working Solution µg/ml
Cellufluor	water	1	100
Nile Red	ethanol	1	50
FDA	acetone	1	50
CFDA	DMSO	2	10
CMFDA	DMSO	2	50
CMAC	DMSO	2	50
DAPI	water	1	10
FITC	DMF	1	10
DiI	ethanol	2	50
DiO	DMF	2	50
Ethidium Bromide	water	1	50
Acridine Orange	ethanol	1	10
Rhodomine 123	DMF	1	50
Hydroethidine	DMSO	1	50
Lucifer Yellow	DMSO	1	50

Table 2.3 Fluorochromes used in this study. The solvent is the solution used to dilute the original powdered form to make the stock solution. All fluorochromes were subsequently diluted in water to form the working concentration. DMF, Dimethyl formamide. DMSO, Dimethyl sulphoxide

Top View.



For observation a coverslip is placed on top of the spacers.

Side View.

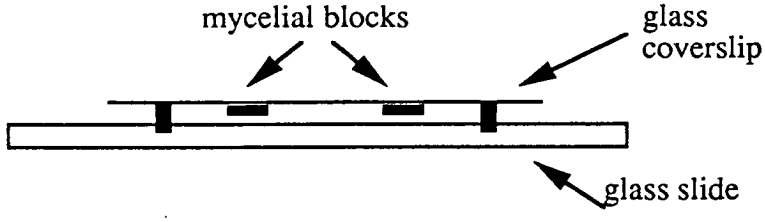
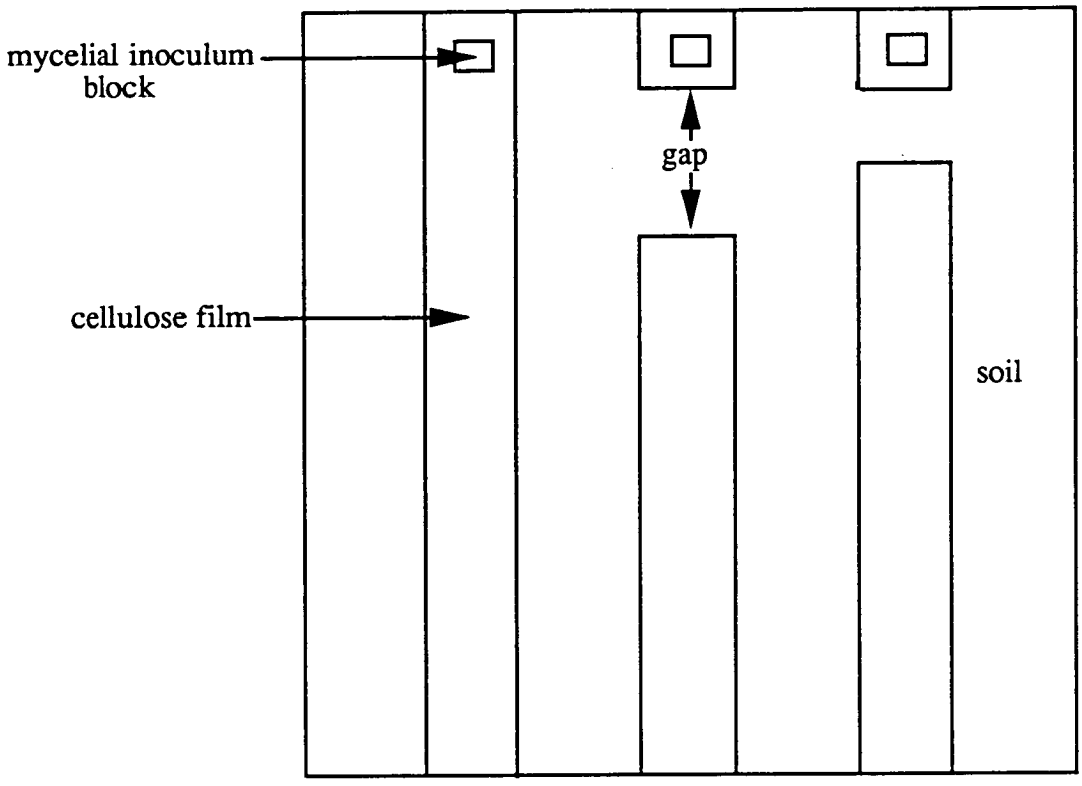
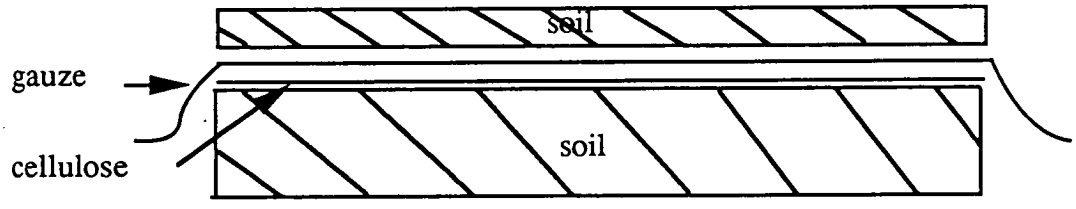


Figure 2.1. Enclosed microscope examination chamber.



A. Aerial View.



B. Side View.

Figure 2.2. Model system to study growth of *R. solani* on or between strips of cellulose film in soil.

soil was investigated by leaving gaps of 1-3 cm in the cellulose, starting at a distance of 1 cm from the inoculated end of the strip. Cellulose strips were examined microscopically for growth, anastomosis and bridging on retrieval after 1-5 days.

Curing of dsRNA in R. solani

Curing strains of dsRNA was carried out by repeated subculture of mycelial hyphal tips, at 2 day intervals over a period of 8 weeks. Cultures were grown at 25°C on PDA. Hyphal tips were removed from the colony margin of 2 day old colonies using fine forceps, and the tip cells inoculated onto fresh PDA and allowed to regenerate by incubation at 25°C.

Growth rates of Rhizoctonia solani

Linear extension rates (as colony diameter) were recorded as daily growth increments on PDA at 25°C.

Mycelial dry weights were measured from cultures grown in potato dextrose broth. After incubation at 25°C, cultures were filtered, under vacuum, using a buchner funnel, onto 0.45μm Millipore membrane filters. Filters with mycelia were dried at 80°C overnight then weighed. Five filters without mycelia were also dried as controls. Dry weights were measured and the weight of the filters subtracted to give mycelial dry weights.

Plant pathogenicity tests

Pathogenicity tests were carried out on a variety of host plants. A plastic drinking cup of 200ml capacity which was pierced to give free flow of water through the bottom. Water-saturated perlite was added to the cup until it was 2/3 full. Four 1cm diameter inoculum blocks of mycelia on PDA were laid on the perlite and a surface sterilised seed was placed on each block. The cups were then filled completely with perlite, covering the seeds. Control tests were carried out using agar without the addition of fungal mycelia. 50 replicates were used for each treatment. Seeds were incubated for 7-10 days at 25°C after which time they were screened for symptoms of damping-off.

TCA precipitation of proteins

100% trichloroacetic acid (TCA) was added to 1ml of culture supernatant of *R. solani* grown in pectin containing broth to give a final concentration of 10%. The mixture was incubated at 4°C for 30 minutes then the proteins were pelleted by centrifugation at 20,000*g* in a microfuge. Each pellet was resuspended in approximately 10µl gel running buffer.

Pectic zymograms

Pectic enzyme detection on pectin acrylamide gels was based on the methods of Cruickshank & Wade (1980) and Sweetingham and co-workers (1986). A 1cm diameter mycelial covered PDA block was inoculated into 5ml of pectic enzyme broth and incubated at 25°C for 7 days. Sephadex G-150 superfine was then added to a concentration of 50 mg/ml, to add weight to the sample so that it entered the wells of the polyacrylamide gel, and 30µl applied to a polymerised pectin acrylamide gel (10.25% acrylamide, 2.5% bis-acrylamide, 0.1% citrus pectin), buffered to pH 8.7 with citric acid buffer (0.525g citric acid, 4.598g Tris, 1 litre dH_2O, pH8.7). Polymerisation of the gel was carried out by the addition of TEMED (0.1ml) and ammonium persulphate (0.25g). Electrophoresis was carried out at 4°C at 70 volts until the sample dye (0.05% bromophenol blue) had migrated 5cm. Following electrophoresis, gels were incubated in 0.1M malic acid, pH2, for 2 hours at room temperature. The malic acid reduced the pH of the gel gradually from 8 to 3 in a period of approximately 90 minutes and allowed the pectic enzymes to break down the pectin in the gel at their optimum pH. The gels were then stained in 0.02% (w/v in H_2O) ruthenium red for 2 hours at 4°C and destained in distilled water for 12-16 hours.

Degradation of enzyme on the pectin was visualised as follows: pectin esterase produced zones of staining darker than background, polygalacturonase produced colourless or cleared zones in the stained gel and pectin lyase changed the colour of the gel to yellow, or produced clear zones surrounded by a yellow outline.

Pectic enzyme assays

A 1/4 oz. bijou bottle (5ml capacity) containing 2ml of pectin broth was inoculated with an 8mm diameter PDA block of *Rhizoctonia solani* and the culture was incubated at 25°C.

On day 5 after inoculation, a 500 µl aliquot of culture supernatant was removed and added to an equal volume of pectin lyase buffer (50µM Tris-Cl, pH 8.3, 0.01M $CaCl_2$ containing 1% citrus pectin) (Pitt, 1988) or polygalacturonase buffer (45mM sodium acetate buffer, pH5, containing 1% sodium polypectate) (Ayres *et al.*, 1966).

After mixing, a 100µl sample was removed and boiled to inactivate the enzymes. The rest of the sample was incubated at 25°C. Further 100µl samples were removed from the mixture at daily intervals and boiled on each of the following 5 days.

Further 500µl samples of culture supernatant were also removed on days 6-10 after inoculation of *R. solani* into the pectin broth and added to the appropriate buffer. Enzyme levels were then assayed over a period of 5 days for each culture sample, as before (i.e. by assaying 100µl samples). Pectic enzyme production was measured by assaying the boiled samples using the thiobarbituric acid assay described below.

Thiobarbituric acid assay

The thiobarbituric acid assay was based on the method of Warren (1960) and used to measure 4,5 unsaturated oligogalacturonides produced by the cleavage of pectin or sodium polypectate by the appropriate pectic enzyme.

100µl of enzyme and buffer mixture were boiled for 5 minutes to inactivate the proteins and 125µl of Reagent A (200ml of concentrated sulphuric acid, 18M, and 0.535g periodic acid made up to 100ml in dH_2O) was added. The mixture was incubated at room temperature for 10 minutes then 250µl of Reagent B (5ml concentrated HCl, 11.6M, 2g sodium arsenite made up to 100ml in dH_2O) was added and incubation was continued for a further 2 minutes. 1ml of Reagent C (0.3g thiobarbituric acid in 100ml dH_2O) was added to the tube which was subsequently 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 549nm against

a reference cuvette which contained the same reagents as the experimental cuvettes but in which no fungal mycelia has been added to the pectin broth.

As there is no standard available for this assay a calibration curve cannot be produced therefore the readings are arbitrary. Following the approach of previous workers, one unit of activity was designated as that amount of enzyme causing a change in absorbance of 0.01 under the conditions of the assay (Pitt, 1988).

Measurement of cellulase activity

This assay is based on the method of Garrett (1966). Groups of five circles of Whatman number 3 filter paper (7cm diameter) were weighed accurately and placed in 250ml conical flasks with 12ml mineral salts broth. The flasks were autoclaved and inoculated with an agar block of *R. solani*, 8mm in diameter, at the filter paper margin. Cultures were then incubated at 25°C for 4 weeks. After incubation the flask contents were removed and oven dried at 80°C overnight. Uninoculated controls were treated similarly. From comparison of the weight change of uninoculated and inoculated flasks it was possible to determine the amount of weight loss of cellulose caused by the fungus.

Isolation of double-stranded RNA

Isolation of fungal dsRNA was carried out using a modification of the methods of Morris and Dodds (1979) and Hoch and co-workers (1985).

Cultures (14 days) grown in 300ml potato sucrose broth or malt extract broth were filtered using Whatman No.3 filter paper and the filtrate was discarded. 10g, fresh weight, of mycelium was chilled and homogenised in a blender for 5 minutes in a solution composed of 15ml GPS buffer (0.1M Na_2HPO_4, pH9.5 containing 0.6M NaCl and 0.2M glycine), 10ml water-saturated phenol, 10ml chloroform-isoamyl alcohol (24:1 v/v) 100µl mercaptoethanol and 1% SDS. The mixture was centrifuged at 20,000*g* for 10 minutes. The supernatant was retained and ethanol was added to a final concentration of 15% (v/v) in water. Cellulose powder was added to the supernatant at 1g/40 ml of liquid and the mixture was stirred for 2 h at 4°C. The slurry was centrifuged for 10 minutes at 20,000*g* and the pellet resuspended in 10ml STE buffer (0.1M NaCl, 0.05M Tris, 0.001M EDTA, pH7) containing 15% ethanol.

The cellulose and nucleic acid mix was added to a glass column of 1cm diameter by 30cm length. The single stranded RNA and all DNA were eluted by washing the column with 200ml of STE buffer containing 15% ethanol (v/v). The dsRNA was subsequently eluted in 5ml STE buffer without ethanol. This eluate was then ethanol precipitated in the presence of sodium acetate (see section 2.2.2).

Isolation of total fungal nucleic acid

Mycelium blocks of agar were inoculated into 300ml of PS broth or malt extract broth and incubated for 14 days. Cultures were filtered using Whatman No. 3 filter paper and the filtrate discarded. 10g of mycelium was chilled and homogenised for 5 minutes in a solution containing 15ml SDS extraction buffer (0.08M Tris pH7 containing 0.5M NaCl, 10mM EDTA, 1% SDS (w/v)), 10ml water-saturated phenol, 10ml chloroform-isoamyl alcohol 24:1 (v/v) and 100µl mercaptoethanol. The mixture was centrifuged at 20,000*g* for 10 min. The supernatant was removed and ethanol precipitated in the presence of sodium acetate (as described in section 2.2.2).

Isolation of fungal plasmids

The protocol for isolation of total fungal nucleic acids was followed initially. After ethanol precipitation and resuspension of the pellet in 500µl sdH_20, 20 units of DNAse-free Ribonuclease A was added and the sample was incubated at 37°C for 30 min. Samples were then run on a 1% agarose gel with TBE and the DNA band was cut out. The DNA was purified and concentrated using the Geneclean II Kit (Bio101, Inc.).

Exonuclease III digestion of fungal plasmid DNA

Exonuclease III digestion of the 3' ends of *R. solani* linear plasmids was carried out using the method of Kikuchi and co-workers (1984). The enzyme digests the 3' terminus of double-stranded DNA with blunt ends or with a protruding 5' terminus. 1 unit produces 1nmol of acid-soluble nucleotide from sonicated DNA in 30 min at 37°C. Plasmid DNA (15µg) was incubated at 37°C in exonuclease III buffer (0.66M Tris HCl, pH8, containing 66mM MgCl) and 40 units of exonuclease III was added to give a total volume of 90µl. After incubation for 0, 2, 5, 10, 20 or 30 minutes,

15µl aliquots were removed and heated to 70°C for 5 minutes to inactivate the enzyme. Samples were electrophoresed on a 1% (w/v) agarose gel.

λ exonuclease digestion of fungal plasmid DNA

Lambda exonuclease digestion of the 5' ends of *R. solani* linear plasmid DNA was carried out using the method of Kikuchi and co-workers (1984). 1 unit of λ exonuclease produces 10nmol of acid soluble DNA from double-stranded DNA in 30 min at 37°C. 15µg of plasmid DNA was incubated at 37°C in lambda exonuclease buffer (100mM Tris HCl, pH7.5 containing 60mM $MgCl_2$, 60mM mercaptoethanol and 100mM NaCl) and 12 units exonuclease. After incubation for 0, 2, 5, 10, 20 or 30 minutes, 15µl aliquots were removed and heated to 70°C for 5 minutes to inactivate the enzyme. Electrophoresis was carried out using a 1% (w/v) agarose gel in TBE.

Tailing of fungal plasmid DNA

A poly A tail was added to *R. solani* plasmid DNA using terminal transferase. This DNA polymerase catalyses the addition of dNTPs to the 3' hydroxy termini of DNA (Chang & Bollum, 1986). 10µg of 'Genecleaned' *R. solani* plasmid DNA was denatured at 85°C for 5 minutes in a water bath, and then added to 4µl of a solution containing 5 x tailing buffer (500mM potassium cacodylate, pH 6.8 containing 5mM $CoCl_2$ and 0.5mM DTT), 1µl of 2mM ATP, 1µl terminal transferase and 4µl dH_2O. The mixture was incubated for 10 minutes at 37°C and then the enzyme was inactivated by heating to 85°C for 5 minutes. The tailed DNA was used in the polymerase chain reaction.

Amplification of nucleic acids by the polymerase chain reaction (PCR)

Amplification of tailed fungal plasmid DNA was carried out in a Hybaid Omnigene programmable dry block, using the method of Scherf and colleagues (1986). An internal plasmid primer (P2 or P3), Table 2.4, of 24 nucleotides, including a 6 nucleotide restriction site and a polyT adapter primer, Table 2.4, with a 24 base linker containing 5 restriction sites were used for the amplification of the tailed fungal DNA. The reaction mixture consisted of 20µl of tailed DNA (the total

Primer	Sequence	Comments on Use
T7 (736-720)	5'-AATACGACTCACTATAG-3'	Sequencing across *BamHI* region of pMCC1.
T7 reverse (823-808)	5'-AACACGTATGACCATG-3'	Sequencing across *BamHI* region of pMCC1.
P1 (111-129)	5'-TTGCACGCCAGCTCAGCC-3'	Sequencing overlap region of pMCC1.
P2 (195-618)	5'-CCTTCTGAGATGATTAGGCACGTA-3'	PCR from complementary strand of I13 extrachromosomal DNA.
P3 (9-36)	5'-GGCTCGAGGTTCTATGAACTGATG-3'	PCR from I13 extrachromosomal DNA.
Poly T Adapter	5'-TTTTTTTTTTTTTTTTT CCTAGGCTTACGTCGACTAGT-3'	PCR of sequence complementary to polyA tailed I13 extrachromosomal DNA.

Table 2.4. Oligonucleotide primers. The nucleotide position of the T7 and T7 reverse primers (as shown in brackets) correspond to those of published sequences (Short *et al.*, 1988). The nucleotide positions of primers P1, P2 and P3 correspond to the nucleotide positions of the sequenced I13 DNA fragment of the I13 DNA fragment as shown in Figure 6.5.

sample from the tailing reaction), 8µl of 2.5 mM dNTPs (2.5mM of each of dATP, dGTP, dTTP and dCTP), 0.5µl of Taq DNA polymerase (2.5 units), 10µl of Taq incubation buffer (100mM Tris HCl, pH8.3 containing 500mM KCl and 1mg gelatine ml^{-1}) 6µl of each primer (23pM) and 56µl of sterile dH_2O. 50µl of mineral oil was placed on top of the mixture to prevent evaporation. Initial denaturation of the DNA was carried out at 95°C for 5 minutes. Amplification of the DNA was obtained by a process of 35 cycles of denaturation at 94°C for 2 minutes, annealing at 55°C for 2 minutes and elongation from the primers at 72°C for 3 minutes. To complete the reaction the mixture was heated to 72°C for 10 minutes. A 20µl aliquot of each of the products was electrophoresed on a 1% agarose gel.

2.2.2. Bacterial manipulations

Bacterial strains and plasmids

All procaryotic strains used in this study are listed in Table 2.5. Liquid cultures were routinely grown in 5ml of Luria broth with shaking, at 37°C. The antibiotics ampicillin and kanamycin were added at 50µg/ml when appropriate. A sterile inoculating loop or a sterile toothpick was used to remove a single bacterial colony from solid medium for inoculation.

Plate cultures were grown overnight as streaks on Luria agar at 37°C and then stored at 4°C for periods of up to 3 weeks. For long term storage, 1ml of an overnight liquid culture, grown in Luria broth, was mixed with 70µl of 100% DMSO and held at -70°C.

Plasmids used in this study are listed in Table 2.6.

Small scale plasmid preparations

Bacterial plasmid minipreps were carried out using the method of He and colleagues (1991). 1.5 ml of an overnight culture grown in LB broth supplemented with ampicillin (50µg/ml) was harvested by centrifugation in a microfuge (20,000*g* for 1 minute) and resuspended in 800µl of TELT solution (50mM Tris HCl, pH7.5, containing 62.5mM Na_2EDTA, 2.5M LiCl and 0.4% (w/v) Triton X100). 40µl of freshly resuspended lysozyme (50µg/ml in TELT) was added and the mixture was incubated at room temperature for 2 minutes. Then the mixture was boiled for 2

Table 2.5 Bacterial strians

Strain	Genotype	Source
Escherichia coli NM522	*supE, thi Δ(lac⁻ , proAB) Δhsd(r⁻m⁻)* F'[*proAB*⁺*lac*I ᑫZΔM15]	Gough & Murray (1983)
Escherichia coli JM109	F' *t*[*ra*D36 *pro*AB⁺*lac* Iᑫ lacZ] ΔM15 *rec*A1 *end*A1 *gyr*A96 *thi hsd*R17 *sup*E44 *rel*AI Δ(*lac pro*AB) *mcr*A.	Yanisch-Perron *et al.* (1985)
Erwinia caratovora E1039	Wild Type	J. Chard. Edinburgh University

Table 2.6. Plasmids

Plasmid	Relevent Features	Source
pBluescript (M13⁺)	ColE1 like replicon Apᴿ carries lacZ with a multiple cloning site.	Short *et al.* (1988)
pMC1	pBluescript derivative containing cloned DNA from *R. solani* I13 extrachromosomal DNA	This Thesis

minutes and incubated on ice for 10 minutes. After incubation, cell debris was removed by microcentrifugation at 20,000*g* for 15 minutes. The supernatant was precipitated by addition of 0.6 volumes of isopropanol on ice for 2 minutes and centrifugation was carried out at 20,000*g* for 30 minutes. The resulting pellet was washed in 70% ethanol and resuspended in 50µl sdH_2O.

Plasmid DNA was digested by restriction endonucleases and examined by agarose gel electrophoresis.

Large scale plasmid preparations

All large scale plasmid preparations of DNA were carried out using an alkaline lysis method based on the procedure of Birnboim & Doly (1979).

Cells were grown in 200ml cultures in LB and harvested by centrifugation at 7,500*g*, using a JA-14 rotor, for 5 minutes. The cells were resuspended in 5ml of ice cold Solution 1 (25mM Tris HCl, pH8, containing 50mM glucose, 10mM EDTA and freshly dissolved lysozyme (2mg/ml final concentration)). 20ml of Solution 2 (0.2M NaOH solution, containing 1% SDS (w/v)) was added and the mixture was left on ice for 5 minutes. 10ml of ice cold Solution 3 (5M potassium acetate buffer, pH 4.8) was then immediately added and the mixture was vortexed and left on ice for 15 minutes. The precipitate was pelleted by centrifugation at 9,800*g* in a JA-14 rotor for 5 minutes. The supernatant was removed by filtering through nylon wool and the DNA was precipitated by the addition of 0.6 volumes of isopropanol at room temperature. After centrifugation at 9,800*g* for 5 minutes, the pellet was dried and resuspended in 4ml of TE (10mM Tris solution, pH7.8, containing 1mM EDTA). Caesium chloride was added at a concentration of 1g/ml together with 250µl ethidium bromide (10mg/ml). The mixture was spun at 3,000*g* on a benchtop centrifuge and the particle-free supernatant was loaded into a 11.5ml Sorvall "QUICKSEAL" tube. This was crimped and spun at 200,000*g* for 18 hrs in a TV865 vertical rotor in a Sorvall OTD 50B ultracentrifuge with a controlled breaking programme. Afterwards, the super-coiled plasmid band (the lower of 2 discrete bands) was removed with a needle and syringe. Ethidium bromide was removed by repeated extraction (3 or more times) with water-saturated butanol and then the remaining caesium chloride was removed by repeated dialysis in several changes of water and finally of TE buffer.

Following ethanol precipitation in the presence of sodium acetate, the pellet was dried and resuspended in 200µl of TE buffer.

Extraction of proteins from nucleic acid by phenol and chloroform

Phenol was mixed with an equal volume of DNA (suspended in water or TE) in order to extract proteins from DNA and the sample was spun for 2 min (20,000*g*). The upper aqueous phase was removed into a fresh tube, taking care not to collect any proteins located at the interface. An equal volume of chloroform was then added to the solution, the sample was mixed and spun for 1 minute (20,000*g*) and the upper fraction was decanted into a fresh tube. Extraction with chloroform was repeated and then the nucleic acid-containing phase was precipitated with ethanol and sodium acetate, as described below.

Precipitation of nucleic acids with ethanol

Standardly, DNA was precipitated from solution by addition of 0.1 volumes of 3M sodium acetate buffer, pH4.8-5.2, and 2.5 total volumes of absolute ethanol. The solution was mixed thoroughly and allowed to stand at either -70°C for 15 min or on dry ice for 5 minutes. DNA was pelleted by centrifugation for 15 min. The resulting pellet was washed in 70% ethanol, further spun for 5 min and then dried under vacuum for 2-10 min or until no visible traces of ethanol remained. The DNA was resuspended in a small volume of TE.

Agarose gel electrophoresis

DNA was separated in 0.7-2.0% (w/v in TBE) agarose gel (electrophoresis grade) containing 0.5µg/ml ethidium bromide, in 1 x TBE buffer (0.089M Tris solution, containing 0.089M boric acid and 0.002M EDTA). Prior to loading, DNA samples were mixed with 1/6th volume of sample buffer. Bacteriophage lambda DNA cut with *Hind* III was used as a size marker. DNA was visualised by UV illumination (302nm) and photographed using a UVP camera and Mitsubishi video copy processor.

Purification of DNA fragments from agarose gels

DNA fragments were purified from agarose gels using the Geneclean II kit (BIO 101, Inc.). The desired band was cut from the gel while being illuminated by ultra-violet light (wavelength 302nm). The gel slice was then placed in an eppendorf tube containing 4.5 volumes of 6M sodium iodide and 0.5 volumes of TBE modifier (a proprietary mixture of concentrated salts). Tubes were heated to 55°C for 2-3 min in order to melt the agarose and 5µl of Glassmilk was added to the solution, which was then vortexed. The mixture was incubated on ice for 5 min, to allow binding of the DNA to the Glassmilk and then centrifuged at 20,000*g* for 5 sec. The pellet was washed 3 times with 300-700µl of New Wash (a solution of NaCl, Tris, EDTA, ethanol and water) centrifuging for 5 seconds each time. DNA was eluted from the Glassmilk by resuspending the pellet in 20µl dH_2O, heating to 55°C for 2-3 minutes and centrifuging at 20,000*g* for 30 sec. The DNA was contained in the dH_20 supernatant and was decanted for further use.

Restriction digests

Restriction digests were carried out over 1-16 h at 37°C with commercially available enzymes, using the supplied buffers. 15µl of plasmid DNA (approximately 15µg) was digested in a total volume of 20µl containing 1 x restriction enzyme buffer, dH_20 and 10 units of restriction enzyme. When more than one digest was necessary digests were carried out simultaneously in the same buffer, or sequentially in different buffers, restricting with the enzyme which cuts in buffer of the lower salt concentration first.

Kinase and klenow of PCR products for blunt ended ligation

23µl (~10ug) of PCR product was incubated with 5 units T_4 polynucleotide kinase and 2.5µl of 10 x incubation buffer (50mM Tris-HCl, pH 7.5, containing 1mM DTT, 0.1mM EDTA, 1µM ATP and 50% glycerol (v/v)) for 45 min at 37°C, to phosphorylate the DNA. 1µl of klenow polymerase and 5µl of 2.5mM dNTPs were added and the sample was incubated at room temperature for 30 mins to facilitate the addition of nucleotides to the recessed 3' ends. The mixture was extracted with

phenol and chloroform to inactivate the enzymes and the aqueous phase was then ethanol precipitated to concentrate the samples, before ligation.

Ligation conditions

Purified DNA fragments (approximately 100ng) were ligated to vector DNA (50-100ng) in a 20µl volume consisting of 2µl of 10x T_4 ligase buffer (660mM Tris Cl, pH 7.5, containing 50mM $MgCl_2$, 10mM dithioerythritol and 10mM ATP) and 1µl (1 unit) of T_4 DNA ligase. Distilled water was used to adjust the final volume to 20µl.

Generation of competent E. coli cells

An overnight culture of strain NM522 or JM109 was diluted 100 fold in LB medium and grown for 1.5 h at 37°C with shaking. The culture was spun in a benchtop centrifuge at 3,000*g* for 10 minutes, the supernatant was discarded and the cells were resuspended in 5 ml of autoclaved 0.1M $CaCl_2$ solution prepared in sdH_2O. Cells were incubated on ice for 2 hours. After a further spin, the cells were resuspended in 1ml of autoclaved 0.1M $CaCl_2$ solution and allowed to stand overnight at 4°C to improve competence (Dagert & Ehrlich, 1979). The cells were then placed into tubes and stored frozen in 0.1M $CaCl_2$ solution, containing 30% glycerol at -70°C.

Transformation protocol

Cells (100µl) were mixed with up to 10µl of the desired DNA or TE buffer and left on ice for 1 hour. The cells were then heat-shocked at 42°C for 2 min. 900µl of LB was added and the mixture was incubated at 37°C for 30-45 min to allow expression of the selectable marker, before spreading on LB agar supplemented with ampicillin, X-gal and IPTG at the concentrations stated above.

2.2.3. Nucleic acid radiolabelling

Random primed labelling of DNA

This method was first described by Feinberg & Vogelstein (1983, 1984). Labelling was carried out using a "Random Primed DNA Labelling Kit" (Boehringer Mannheim). Random hexanucleotides bind to the DNA fragment and DNA polymerisation is initiated from these by the klenow fragment of DNA polymerase I. One of the nucleotides incorporated in the reaction is radiolabelled so that the newly synthesised DNA is radiolabelled.

50-100ng of linear DNA in a total volume of 9µl was heat denatured by boiling for 10 minutes. After cooling on ice, the DNA was mixed with 2µl of reaction mix (hexanucleotide mixture in 10 x concentrated reaction buffer), 1µl of each of dATP, dTTP, dGTP and α-^{32}P dCTP (50µCi; 3000mCi/mmol) and 1µl of *E. coli* DNA polymerase 1 Klenow fragment (2 units/µl). The reaction was allowed to proceed for 1h at 37°C and was stopped by adding 2µl of 0.2M Na_2EDTA (pH 8.0).

5' end labelling

5' end labelling of DNA and dsRNA was carried out using a γ-ATP forward reaction (Sambrook *et al.*, 1989). Bacterial T_4 polynucleotide kinase catalyses the addition of the γ-phosphate of ATP to a 5' terminal phosphate of DNA or RNA.

5µl (approximately 0.5µg) of the nucleic acid to be labelled was added to a solution comprising of 1.5µl of 10 x reaction buffer (500mM imidazole, pH6.4 containing 180mM $MgCl_2$ and 4.5mM DTT), 1µl of T4 polynucleotide kinase, 0.8µl of 1M DTT, 5µl of ^{32}P γ-ATP and 10.5µl of dH2O. The mixture was vortexed and then incubated at 37°C for 30 min to allow the reaction to occur. The enzyme was inactivated using 2µl of 0.5M Na_2 EDTA (pH8).

3' end labelling

3' end labelling of DNA was carried out by replacement synthesis of the single stranded 3' recess using T_4 DNA polymerase (Sambrook *et al.*, 1989). 0.2-0.5µg of DNA was added to 2µl of 10 x T_4 DNA polymerase buffer (0.33M Tris-acetate, pH8, containing 0.66M potassium acetate, 0.1M magnesium acetate, 5mM DTT and 1mg/ml bovine serum albumin (fraction V, Sigma)) and 1µl of a solution containing

2mM dATP, dTTP and dGTP. 10μCi (1μl) of ^{32}P α-dCTP and 2.5 units (1μl) of T_4 DNA polymerase were added and the mixture was incubated at 37°C for 1 hour. 1μl of a 2mM solution of dCTP was added and the mixture was incubated for a further 15 min. The reaction was stopped by heating the sample to 70°C for 5 min.

Purification of labelled nucleic acid

Radiolabelled DNA was separated from unincorporated radionucleotide using a sephadex G-50 (Pharmacia) matrix size exclusion column. Sephadex G-50 consists of small porous beads. Small fragments (< 20 nucleotides) enter the holes in the beads and are retained, whereas larger DNA fragments cannot enter the holes and pass through the column between the beads. Labelled DNA will therefore be separated from unincorporated radionucleotide by passage through the column.

The column consists of a 1ml plastipac sterile syringe with nylon wool packed into the bottom. Sephadex was poured into the syringe to the 1ml mark. The syringe was suspended in a 50ml Falcon tube with a hole in the lid. An eppendorf was placed in the bottom of the tube to collect the eluent. The labelled sample was diluted in water to 100μl and then applied to the top of the column matrix. The column is spun in a bench top centrifuge for 2-5 min and the labelled DNA or RNA collected in an eppendorf tube.

Measurement of percentage incorporation of probe

1μl of labelling reaction mixture (after incubation) was spotted onto a GT/C filter (Whatman), and allowed to dry. The total counts of ^{32}P, were measured by a scintillation counter. The filter was washed in 10% TCA to remove the unincorporated label, and the incorporated counts measured. Percentage incorporation was calculated and incorporated counts divided by total counts.

5.2.4. Nucleic acid hybridisation

Southern blotting

Total fungal DNA (10μg) or plasmid DNA (15μg) was separated by electrophoresis on a 1% agarose gel (w/v) in TBE buffer, containing 0.5μg/ml ethidium bromide. DNA was electrophoresed for 12-15 hours at 1V/cm and

photographed. The method used for transfer of DNA from gels to nylon membranes was an adaptation of the method of Smith & Summers (1980) which itself was a modification of the original procedure described by Southern (1975). Firstly, the gel was soaked in denaturing solution (0.5M NaOH, 1.5M NaCl in H_20) for 40 min with gentle shaking. The gel was rinsed in distilled water and then neutralised in 0.5M Tris.HCl (pH 7.4) and 1.5M NaCl for 40 min. The DNA was transferred onto nylon membranes by capillary transfer (see below).

Capillary transfer of nucleic acids to membranes

For transfer, a tray was filled with blotting buffer (20 x SSC) and a platform was placed in the tray covered with a wick made from two sheets of Whatman 3MM filter paper which was saturated with blotting buffer. The gel was placed on the wick carefully to avoid trapping air bubbles beneath it. The gel was then surrounded with cling-film to prevent the blotting buffer being absorbed directly into the paper towels above. A sheet of nylon membrane, cut to the exact dimensions of the gel, was soaked in dH_2O and placed on top of the gel again avoiding bubbles being caught between the membrane and the gel. Two sheets of 3MM Whatman paper were cut to gel size and wetted in 20 x SSC and were then placed on top of the membrane. A stack of absorbent paper towels were placed on top of the 3MM paper (approximately 7cm in height) and a glass plate was placed on top of the paper towels. Finally, a 2kg weight was placed on top and transfer was allowed to proceed overnight. After blotting the apparatus was dismantled and the membrane was briefly washed in 2 x SSC, at room temperature. The DNA was fixed to the blot by drying at 80°C for 10 min followed by UV crosslinking for 2 min in a UV Strata linker 1800 (Stratagene) at wavelength 254nm.

Hybridisation

Membranes were placed in rollers containing 150-170ml of prehybridisation solution per cm^2 (0.12M $Na_2 HPO_4$, pH 7.4, containing 0.25M NaCl and 7% (w/v) sodium dodecyl sulphate) (Church & Gilbert, 1984) and incubated for 1 hour at 65°C in a Techne Hybridisation HB-1 oven. The prehybridisation solution was replaced by an equal volume of the same solution containing 0.5% (w/v) dried skimmed milk and

the radiolabelled was probe added directly to the roller. Hybridisation was carried out overnight at 65°C for stringent blots. For lower stringency, with Zeta probe membranes only, hybridisation was carried out overnight at 42°C in the presence of 50% formamide and no dried skimmed milk was added to the solution. The hybridisation solution was subsequently removed, the membranes were washed in 2 x SSC for 2 min and then rinsed successively in 2 x SSC solution containing 0.1% SDS in 0.5 x SSC solution containing 0.1% SDS and finally in a 0.1 x SSC solution containing 0.1% SDS, for periods of 15 minutes. Filters were ultimately removed from the rollers and wrapped in Saran wrap prior to autoradiography.

5.2.5. DNA sequencing

Preparation of DNA for double-stranded sequencing

Plasmid DNA was prepared by the TELT method of He and co-workers (1991) followed by ribonuclease A digestion, phenol:chloroform extraction and ethanol precipitation as standardly performed, or by purification on a CsCl gradient. DNA (5 μg) was diluted in dH_2O to 100μl in then denatured by adding 25μl of 1M NaOH containing 0.2M EDTA and incubating at 37°C for 30 min. The DNA was ethanol precipitated as previously described, resuspended in 7μl dH_2O and sequenced directly as described below.

Preparation of DNA for single stranded sequencing

Single stranded DNA was prepared using the method of Vieira and Messing (1987). Cells were incubated by shaking at 37°C in 5ml Luria broth containing 20μg/ml ampicillin and M13 KO7 bacteriophage ($2x10^7$ plaque forming units/ml) until they reached an OD_{600} of 0.8, after which time kanamycin was added, at a concentration of 25mg/ml, to kill the non-infected cells. The cells were then grown overnight and single stranded DNA was isolated as follows. 1ml of the overnight culture was placed in an eppendorf tube and spun for 5 min at 20,000*g*. The M13 was then precipitated from the supernatant in 2.5M NaCl containing 20% polyethylene glycol (molecular weight 6000) at room temperature over a period of 15 minutes and then spun for a further 5 min at 20,000*g*. The pellet was resuspended in 100μl of TE and the DNA purified by phenol:chloroform extraction and ethanol

precipitation as described above. The pellet was finally resuspended in 7µl sterile dH_20 and used directly for sequencing.

Sequencing protocol

Sequencing reactions were carried out using SEQUENASE™ Version 2.0 (United States Biochemical). The DNA (7µl) and primer were MIXED by adding 2µl 5 x Sequenase reaction buffer and 1µl primer (1pM/µl) to the 7µl of DNA. The mixture was incubated at 65°C for 2 min then allowed to cool to room temperature over a period of 30 min to allow the primer to anneal. Extension was carried out from the annealed primers by adding 1µl of 0.1M DTT, 2µl of dGTP labelling mix (a 1:4 dilution of a solution containing 7.5µM each of dGTP, dCTP and dTTP in sterile water), 0.8µl of α-^{35}S dATP (50µCi/µl; 400Ci/mM) and 2µl of Sequenase TM (1.75 units/µl in 10mM Tris HCl, pH 7.5, containing 5mM DTT). The reaction mixture was incubated at room temperature for 2-5 min. The reaction was then terminated by the addition of 3.5µl of the mixture to 2.5µl of one of the four termination mixes. The termination mixes consist of 80µM of ddATP, ddTTP, ddGTP or ddCTP with 80µM dNTPs in 50mM NaCl. The termination reaction was allowed to proceed for 5 minutes at 37°C and was then stopped by the addition of 4µl of stop solution (95% formamide solution in water, containing 20mM EDTA and 0.05% bromophenol blue). Samples were further treated as described below before loading on a sequencing gel.

Sequencing gels

Electrophoresis of the DNA was carried out on a Bio Rad Sequi-Gen Nucleic Acid Sequencing Cell of IPC size 38 x 50cm. A 6% denaturing polyacrylamide gel composed 52.5g urea, 12.5ml 10 x TBE, 25ml 30% acrylamide (w/v), which contains acrylamide:bisacrylamide at relative concentrations of 19:1 was made up to 125ml with dH_20 to give a final concentration of 6%. The gel was polymerised by the addition of 300µl each of TEMED and an ammonium persulphate solution (25% w/v in dH_20) immediately prior to pouring. Sequencing samples were heat-denatured by boiling for 2 minutes and then 2.5µl was loaded per well. Electrophoresis was carried out in TBE buffer for 1.5 to 7 hours at 2200 volts, following a pre-run of 30 min, or

until the temperature of the gel had reached 50°C. The gel was fixed in methanol:acetic acid:water (1:1:18) for 15 min, dried under vacuum, and autoradiographed as stated below.

Sequence analysis

Database analysis of the sequence was carried out using the Genetics Computer Group (1991), Program Manual for the GCG Package, Version 7, April 1991; 575 Science Drive, Madison, Wisconsin, USA 53711.

Autoradiography

Membranes, sealed in Saran Wrap, and vacuum-dried gels were placed in contact with Cronex or Agfa curix X-ray film in cassettes. For the detection of ^{32}P, cassettes were placed at -70°C and intensifying screens were used. For the detection of ^{35}S, cassettes were placed at room temperature without intensifying screens. Exposure times varied from 1 hour to 7 days. Films were developed using an X-ograph compact X2 automatic developer.

CHAPTER 3

ANASTOMOSIS IN *RHIZOCTONIA SOLANI*

3.1. INTRODUCTION

Gene transfer is of potential interest as a means to exploit recombinant DNA technology, to introduce new and useful genes into existing species and thereby change defined characteristics of organisms, or to disarm pathogens. But it also has potential detrimental effects in the environment, if newly introduced genes or gene combinations escape into natural populations with unforeseen and uncontrollable consequences. Thus, the ability of organisms to spread genes within, and between, populations must be investigated.

In bacteria the potential for gene transfer has been examined quite extensively and there are three well established transfer mechanisms. (1) Transformation, in which DNA is taken up directly by new strains. (2) Transduction, where a viral bacteriophage is used to transfer the DNA between cells. (3) Conjugation, where DNA transfer occurs during direct contact between host and recipient strains. With all of these bacterial systems the critical conditions *in vitro* have been optimised, making DNA transfer in the laboratory a very efficient process for a number of organisms. However, the rate of natural transfer in the environment is presumed to occur at a much lower frequency.

In filamentous fungi gene transfer systems have been developed to a lesser degree for laboratory use, but transformation systems, such as electroporation, have enabled the direct uptake of DNA in some species. Without transfer mechanisms, transfer of genes is often limited to a very small subset of the population, usually strains within species that are closely related to one another. With *Rhizoctonia solani* this is known to be the case as genetic exchange of subcellular components will occur following the hyphal fusion process, anastomosis. The hyphal network formed by this fungus in soil is very stable and allows efficient transport of nutrients and perhaps even organelles throughout the network. As there is cytoplasmic mixing following anastomosis it can be assumed that many of the cytoplasmic components, including plasmids and viruses, are equally able to move between hyphae as in *C. parasitica* (Anagnostakis & Day, 1979). Although the fusion mechanism is known to occur very efficiently, the mechanism itself, and specific signals involved in it, are

unknown. Nor is there much information on post-fusion events, such as direction of transfer of cytoplasmic components.

In order to understand the potential for gene transfer in the environment, specifically with regard to *Rhizoctonia solani*, this chapter investigates the mechanism of anastomosis, in defined laboratory conditions, and looks at the potential for a labelling mechanism to monitor specific organelle transfer between hyphae of *R. solani* after fusion.

3.2. RESULTS

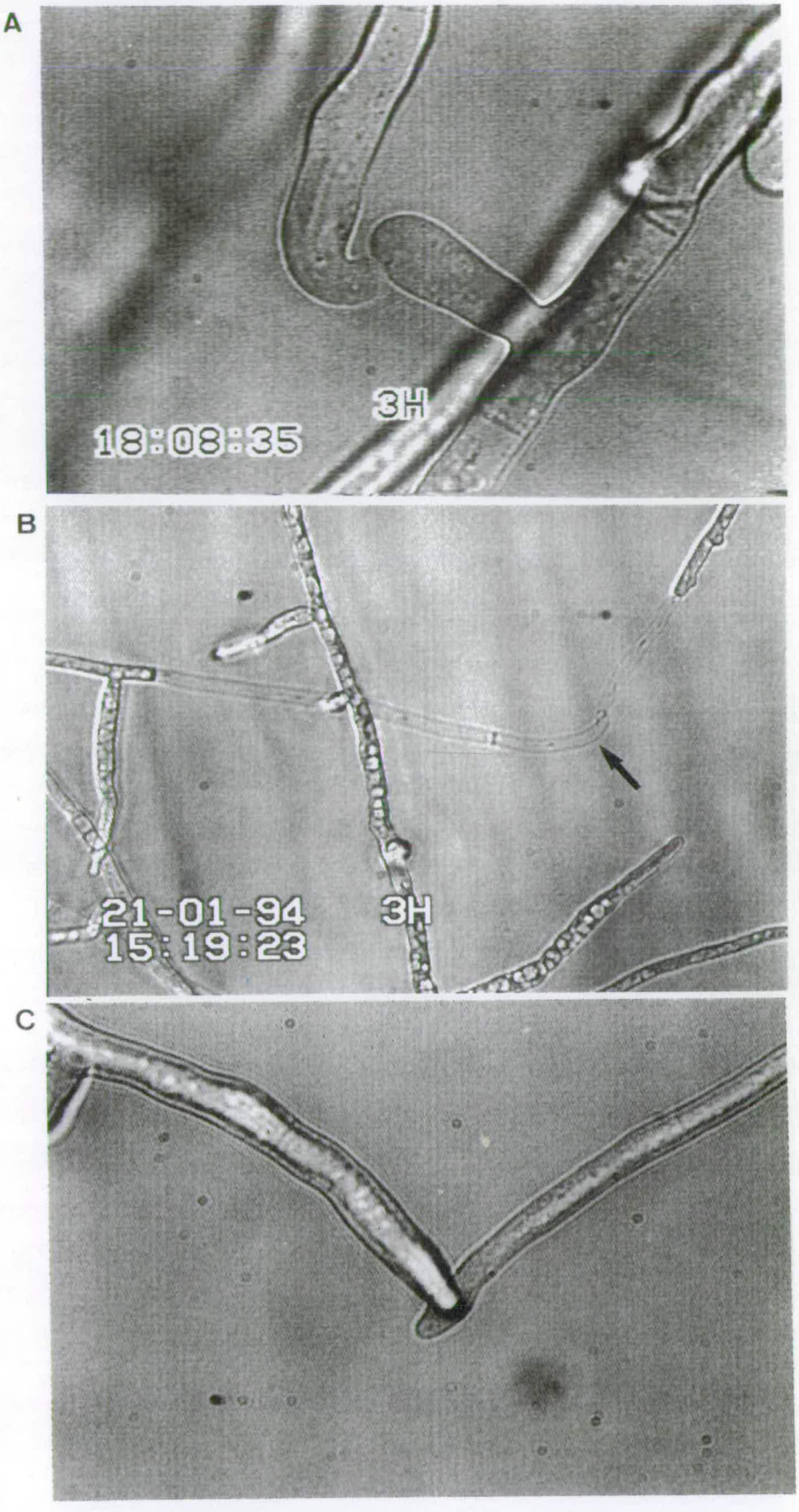
3.2.1. Microscopic analysis of anastomosis in *Rhizoctonia solani*

Anastomosis is the mechanism of cell fusion, allowing variation and gene exchange, in *R. solani* (Menzies, 1970; Anderson, 1982). Initial studies were carried out to clarify the anastomosis groups listed in Table 2.1. Different strains were paired on water agar slides and microscopically examined for their interaction, as described in Chapter 2. The hyphae either grew past each other without contact, or contact occurred resulting in either complete fusion and cytoplasmic mixing, or localised cell death after fusion. From these observations the anastomosis groups in Table 2.1 were confirmed and some characteristics associated with anastomosis were noted.

When two colonies of AG4 strain Rh5/t were opposed on water agar, they anastomosed freely and compatibly, as would be expected of a self-self interaction (Figure 3.1A). Cell fusions of the two hyphae resulted in the formation of a single cell, allowing cytoplasmic streaming and transfer of nutrients between the hyphae. Often, after a period of time, a side branch was produced from the fused cells, or from one of the main hyphae adjoining the fused cells, and a septum was formed to delimit this branch.

Pairing of hyphae from different strains of the same anastomosis group on water agar produced cellular fusion but anastomosis led to a vegetative incompatibility reaction. Figure 3.1B shows such a case between two strains from anastomosis group five, AG5 GM1 and AG5 Rh6/t. Within the overlap region of the

Figure 3.1. Examples of types of interaction between hyphae in zones of overlap between colonies of *Rhizoctonia solani* on water agar films. Hyphae were observed with a x70 objective on a microscope attached to a video camera. Photographs were produced from video copy processor prints. **A.** The interaction between two hyphae of *Rhizoctonia solani* strain AG4 Rh5/t results in complete anastomosis and compatibility allowing the formation of a single cell joining the two hyphae. **B.** Anastomosis between AG5 Rh6/t and AG5 GM1 results in cell fusion followed by a vegetative incompatibility reaction causing localised cell death in the fused and surrounding cells which is visualised as degradation of contents (arrow). **C.** Hyphae from AG4 RH5/t and AG5 GM1 did not attempt to interact and always grow past each other without recognition.


A
3H
18:08:35
B
21-01-94
15:19:23
3H
C

two colonies, fusion occurred readily, but an incompatibility reaction subsequently occurred causing cytoplasmic coagulation and localised cell death around the fusion area extending to one or two cells on either side of the fusion.

In contrast to the cases above, there was not even attempted anastomosis between strains from different anastomosis groups. Figure 3.1C shows hyphae from AG5 strain GM1 and AG4 strain Rh5/t. Throughout the contact zone of the colonies there was no single instance of attempted anastomosis, although further back within each colony self anastomosis was observed, indicating that conditions for anastomosis were favourable.

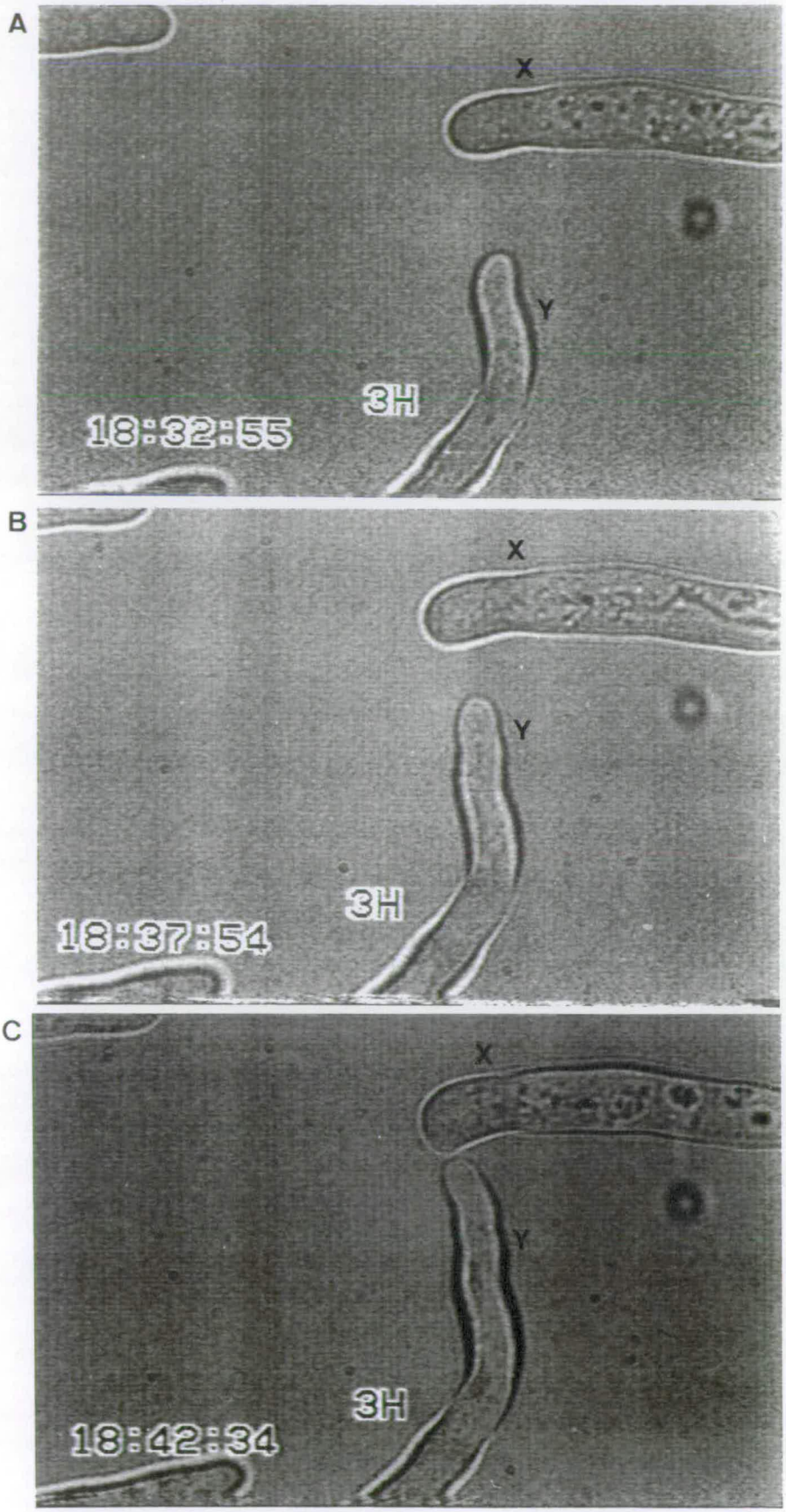
During the examination of these reactions some general observations were made. The process of anastomosis was limited to hyphal tip cells. Of approximately 25 anastomosis reactions observed in detail, fusion between one tip cell and the side of a branch or main hypha was never observed, nor was anastomosis attempted between lateral contacts of hyphae. Furthermore, anastomosis only occurred between the tips of cells from side branches. Main runner hyphae did not fuse with each other or with branches. Thus, cross bridge formation only occurred via interaction between the tips of two branch cells between two main hyphae.

In older cultures where hyphal interaction was allowed to proceed for 36-48 hours it was noted that there were localised pockets of anastomosis. Large areas of hyphal network along the overlapping zone between two colonies contained no fused cells, but where anastomosis was observed there was regularly a large number of fused cells in a very small area. This may be due to the requirement for a specific signal to invoke hyphal tip fusion.

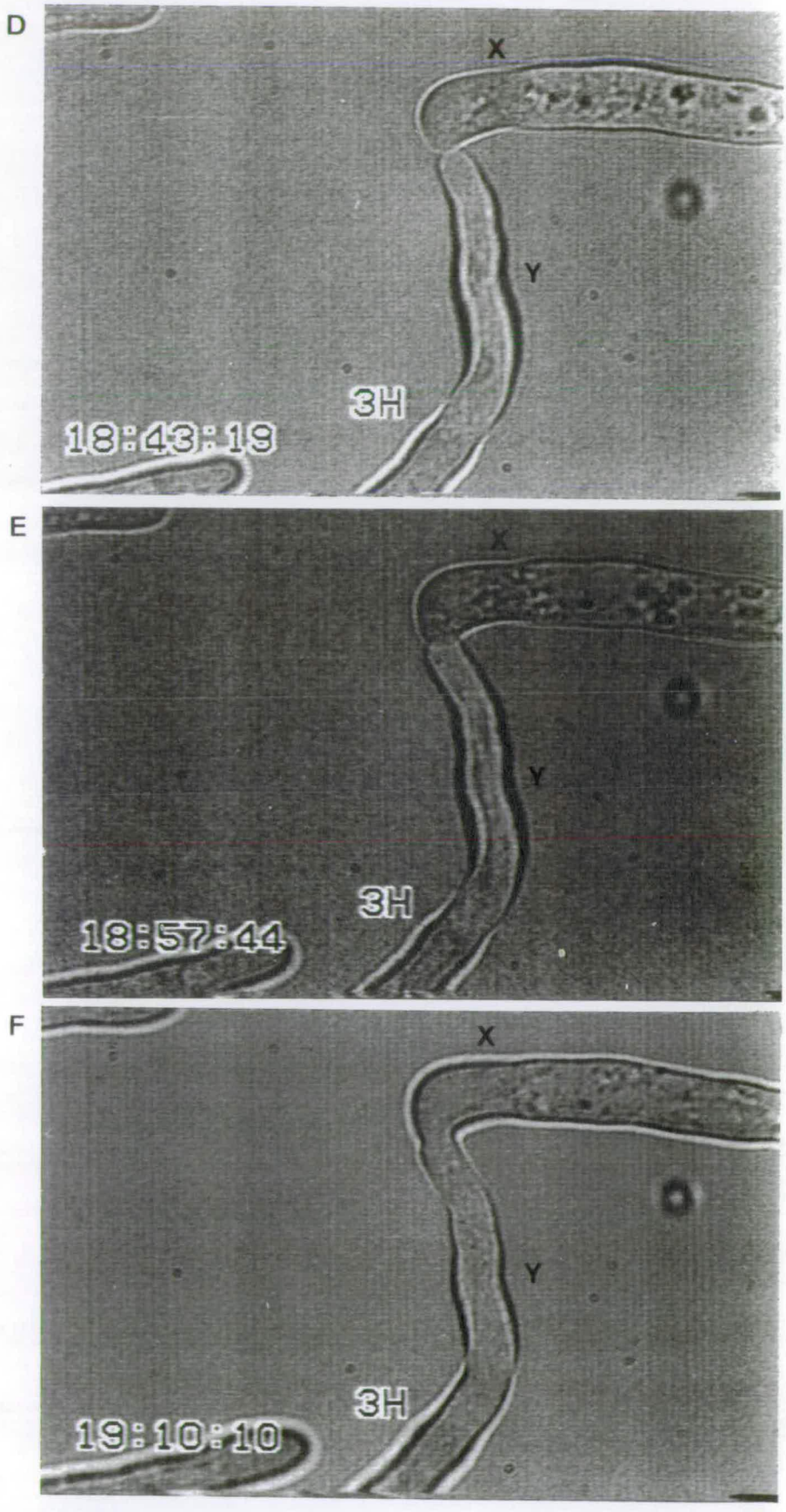
3.2.2. Video analysis of anastomosis

The self-self anastomosis of AG4 strain Rh5/t was followed in detail (Figure 3.2 A-F). The interaction described below involves two hyphal tip cells, one of which is 7μm diameter (X), the other 9.5μm diameter (Y). The two hyphae were observed to be in the same focal plane, on the surface of the agar film. Both cells are from young side branches, each only one cell in length, with a septum close to the point of branch initiation. Actual time of a 24-hour clock (hours:min:sec) is shown on each

Figure 3.2. Video microscopic sequence of anastomosis in *Rhizoctonia solani.* A-F represent sequential stages, over 38 min, of anastomosis of two tips of *Rhizoctonia solani* strain AG4 RH5/t. **A.** At the start of observation (18:32:55) hypha Y shows tropism towards hypha X. **B.** Five min later hypha Y has increased its growth rate and is moving rapidly towards the tip of hypha X which is not extending but has begun to form an apical bulge. **C.** Approximately 5 min later, hyphal tip contact has occurred, and a small lateral branch tip has developed from hypha X (continued overleaf).


A
X
Y
3H
18:32:55
B
X
Y
3H
18:37:54
C
X
Y
3H
18:42:34

D. Contact of hyphal tips. Hypha X shows considerable cytoplasmic movement, evidenced by changes in positions of optically dark organelles (compare with below). **E**. 14 min later, both hyphal tips have stopped growing and have slightly overlapped. Although there is no extension in either hypha there is a lot of intracellular movement. **F**. 13 min later, the tip cell walls have broken down such that fusion is complete and a single cell is observed. There is no obvious cytoplasmic movement of organelles in either direction but cytoplasmic streaming (of groundplasm) was observed to occur from hypha X to hypha Y.


D
X
Y
3H
18:43:19
E
X
Y
3H
18:57:44
F
X
Y
3H
19:10:10

video frame; the symbol "3H" indicates that recording was made in real-time, not time-lapse.

At the start of observation (Fig 3.2A), the tip of one hyphal branch (Y) showed tropism towards the other branch (X) from a distance of 34μm, such that it reoriented to grow perpendicular to branch X. The tip of branch X has stopped extending at this time but showed no cytoplasmic abnormality, its cytoplasm showed normal cytoplasmic motion. Initially hypha Y was growing at a rate of 1.2μm min^{-1} (averaged over 4 minutes), but as it grew towards hypha X its rate increased to 2μm min^{-1} and it was seen constantly to make minor changes in direction of growth (Fig 3.2B). Hypha X was not extending at the tip but at 5-10 minutes after the start of observations it was seen to develop a bulge at the tip (Figure 3.2C) and growth then continued laterally from this bulge (Figure 3.2D). When the two tips met they continued to grow and overlap by ca. 2μm, then growth of both tips stopped for 15 min (Figure 3.2E). During this time there were high levels of intercellular movement especially in hypha X. Localised cell wall breakdown then occurred, resulting in complete fusion of the cells (Figure 3.2 F). But the precise time of fusion could not be assessed because there was no sudden movement of cytoplasm to indicate that continuity had been achieved once fusion had occurred. Cytoplasmic streaming was observed from hypha X to hypha Y and there was some detectable flow of small organelles, but the nature of these could not be discerned. This example, described in detail, was typical of many instances of anastomosis involving self-self or self-non-self strains. In all cases, an attempted anastomosis was predicted by the orientation of two hyphal tips towards one another, and often associated with localised branch tip formation by one hypha as another hypha approached it. Anastomosis was never 'unilateral', involving growth of only one hypha towards another; in these cases the hyphae might meet, even at their tips, but if they had not shown pre-contact tropism towards each other then there was no anastomosis.

3.2.3. Potential for anastomosis of *Rhizoctonia solani* in soil

Having established that successful anastomosis can be followed by light microscopy, the study was extended to examine the behaviour of the hyphae in a soil

based system. *Rhizoctonia solani* is known to form a hyphal network in soil which is stable over a long time (Papavizas, 1970), so it may be assumed that anastomosis will occur, as on water agar especially as soil is largely considered to be deficient in readily available organic nutrients (Lockwood, 1977).

Initial studies were carried out to determine the reproducibility of the method, and compare the growth rates of different strains in the soil-based system (see Chapter 2). Cellulose strips were placed along the full length of soil in a square Petri dish and each strip was inoculated with a $1cm^2$ mycelial block of *R. solani*. At daily intervals for 4 days, strips were removed and the extent of growth from the inoculum was measured. Figure 3.3 shows that each of 5 isolates, from three anastomosis groups grew almost linearly throughout the 4 day period. There was no replication in this test so interstrain comparison must be made with caution. But, it is evident that the strains differed considerably in extension rate, ranging from <2 to nearly 12mm $24h^{-1}$.

Growth of R. solani AG5 R470 in a soil based system

As AG5 R470 was the fastest growing of the strains tested in soil, further studies were carried out on its growth rate and survival. The same model system was used as above (Chapter 2), but each soil dish contained (1) complete cellulose strips covering the length of the dish, (2) strips with a 1cm 'gap' removed and (3) strips with a 2cm gap. The gaps were located at 1cm from the edge of the film, i.e. immediately next to the inoculum block (Figure 2.2). The aim was to determine the ability of the fungus to grow through soil by translocating nutrients from an established colony on a cellulosic substrate to an adjacent cellulosic substrate. Table 3.1 shows, that during 5 days, strain AG5 R470 grew progressively across the complete strips of cellulose film. At corresponding times it had grown less far across the strips of film with gaps in them. The first evident growth over the 1cm gap was after 3 days, when the film distant from the gap showed only 11mm extension of *R. solani* (i.e. 21mm total, including the gap). The first evident growth over the 2cm gap was after 4 days when the distal part of the cellulose film was colonised by 8mm growth (28mm total including the gap). Nevertheless, the experiment demonstrates

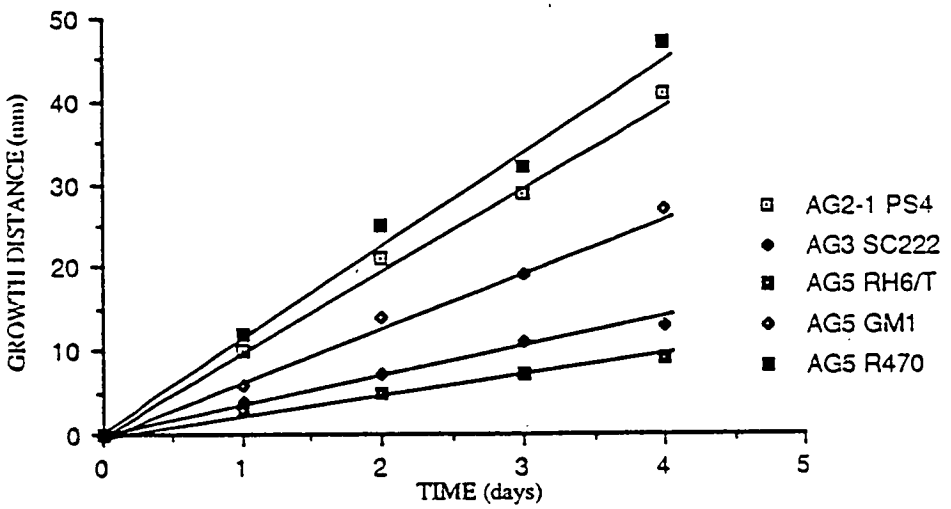


Figure 3.3. Linear growth of isolates of *R. solani* in soil along continuous strips of cellulose film, as assessed microscopically daily retrieval of strips inoculated with 1cm^2 agar blocks of 5 different isolates. AG2-1 PS4 (y = 0.2 + 9.9x; r = 0.998; P<0.001). AG3 SC222 (y = 0.4 + 3.3x; r = 0.994; P<0.001). AG5 RH6/t (y = 0.52 + 2.15x; r = 0.995; p<0.001). AG5 GM1 (y = -0.2 + 11.4x; r = 0.997; P<0.001). AG5 R470 (y = 0.4 + 11.4x; r = 0.995; P<0.001)

Day Sampling	**Growth (mm)**		
	Complete strips	Strips with 1cm gap	Strips with 2cm gap
0	0	0	0
1	5 +/- 0.5	-	-
2	13 +/- 0.8	-	-
3	35 +/- 2.4	21 +/- 0.8	-
4	45 +/- 3.2	31 +/- 3.4	28 +/- 4.1
5	64 +/- 6.6	37 +/- 2.8	33 +/- 6.8

Table 3.1 Growth of *R. solani* AG5 R470 from an inoculum block along strips of cellulose film in soil when the cellulose film was complete or contained a 1cm or 2cm gap located immediately next to the inoculum block. The experimental system is shown in Figure 2.2. Figures are mean +/- S.E.M. for 3 replicates. A different strip was used for each assessment day; - = not assesable

that this strain of *R. solani* could grow at least 2cm through soil from an inoculum base and then continue to colonise cellulose film, presumably translocating nutrients to support hyphal growth through soil.

3.2.4. Development of a labelling system to monitor anastomosis

Conventional light and video microscopy provided insights into anastomosis but it was difficult to assess the movement of cytoplasm or specific organelles across anastomosis bridges. To monitor transfer of subcellular components more specifically, a method of staining was needed which could distinguish between the two hyphae involved in an interaction. The ideal properties of a stain for this purpose were (1) that it must be transported passively into the living hyphae and not cause hyphal damage, (2) that upon internalisation, the two hyphae in an interaction should be easily identifiable, (3) that cytoplasmic and organelle movement following anastomosis must be detectable and (4) that the stains must be stable enough to be detected up to 48 hours after pre-loading of the fungal inocula.

Of several vital dyes that are available, the fluorochrome group of compounds show promise as tracers in animal and plant cell growth and development (Moon *et al.*, 1986; Safrayos *et al.*, 1987) and some have been used to visualise fungal propagules and identify specific cell components. Fluorochromes are dyes which fluoresce when excited by ultra violet or visible light. They are either transported into cell organelles or bind to specific residues inside or on the cell. However, there have been few reports of the use of fluorochromes as markers to trace the growth of fungi, or the movement of organelles, although the compounds have been used for many years as probes for fungal cell components. Their potential for use with *R. solani* was investigated here.

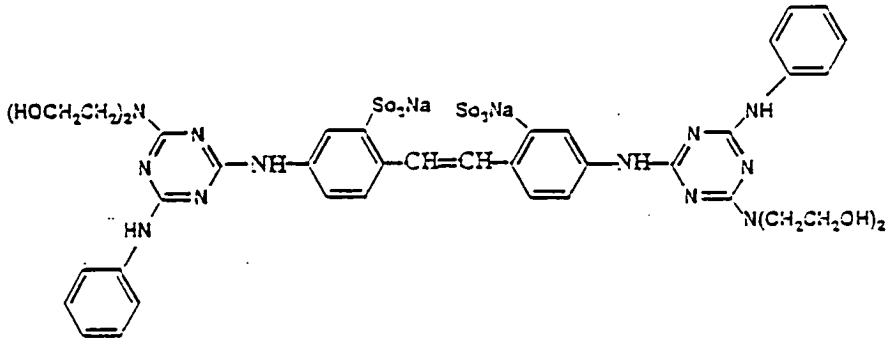
Fluorescent labelling of R. solani organelles

Initial studies were carried out to investigate the efficiency of uptake of fluorochromes by *R. solani* and the stability of fluorescence from these compounds under excitation. A summary of the specificity and spectral properties of the dyes is listed in Table 3.2 and their chemical structures are shown in Figure 3.4. Studies

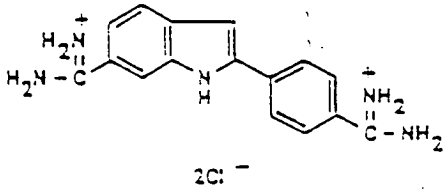
Stain	Specificity	Absorbtion (nm)	Emission (nm)	Colour
Cellufluor	β–glycans	340-400	400-440	blue
Nile Red	neutral lipids	450-500	520-560	yellow
FDA	esterases	490-510	515-535	green
CFDA	esterases	490-515	515-535	green
CMFDA	glutathione-s-transferases	490-510	515-535	green
CMAC	glutathione-s-transferases	354	469	blue
DAPI	DNA	350-390	400-440	blue
FITC	cytoplasm	450-490	520	green
DiI	membranes	484	501	red
DiO	membranes	550	565	green
Ethidium Bromide	nucleic acids	450-490	525-535	orange
Acridine Orange	RNA/DNA	450-490	515-535	red/green
Rhodamine 123	mitochondria	450-470	520-560	green
Hydroethidine	DNA/cytoplasm	370	400-440	red/blue
Lucifer Yellow	aliphatic aldehydes	420-450	520-560	yellow

Table 3.2. Reported staining specificities and spectral properties of fluorochromes used in this study. Data from various sources (see Haughland, 1992). FDA, Fluorescein diacatate; CFDA, Carboxyfluorescein diacatate; CMFDA, Chloromethyl fluorescein diacatate; CMAC, Aminochloromethylcoumarin; FITC, Fluorescein isothiocyanate; DiO, DiOc18(3); DiI, DiIc18(3)

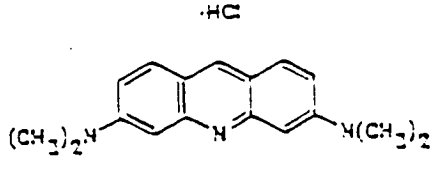
Figure 3.4. Chemical structures of fluorochromes used in this study



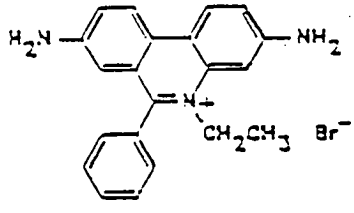
Cellufluor



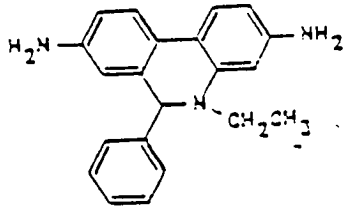
DAPI



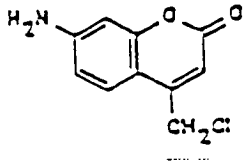
Acridine Orange



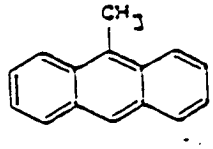
Ethidium Bromide



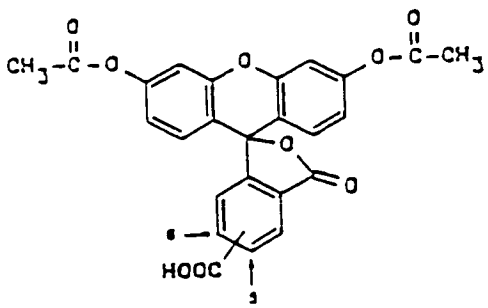
Hydroethidine



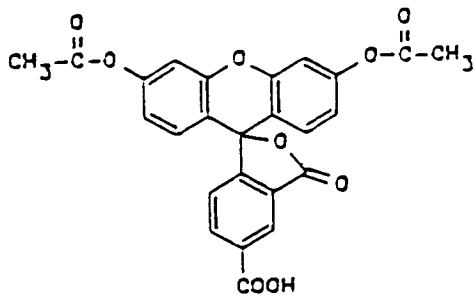
Amino chloro methyl coumarin



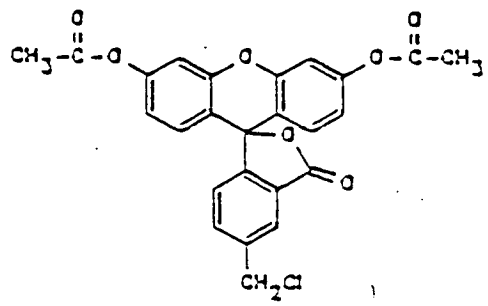
Nile Red



Fluorecein diacetate



Carboxy fluorecein diacetate



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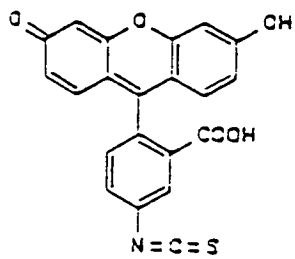
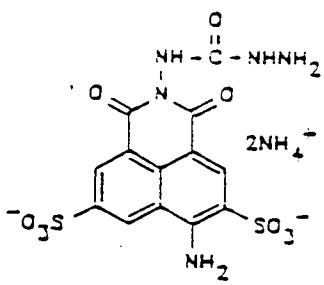
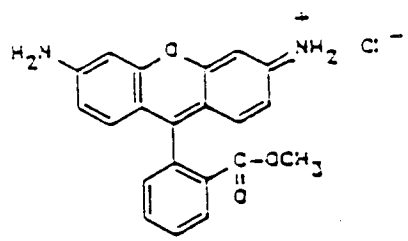


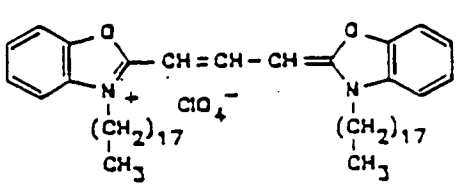
Fluorescein isothiocyanate



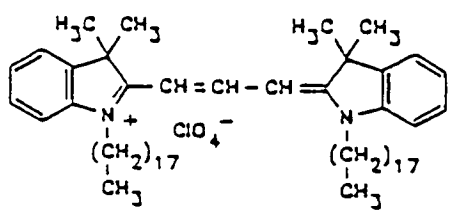
Lucifer Yellow



Rhodamine 123



DiOc18(3)



DiIc18(3)

were carried out with AG4 strain Rh5/t as its mycelia are light in colour, so cell wall pigmentation did not hinder visualisation of fluorescence.

The staining of *R. solani* was tested in both liquid and solid media (Chapter 2) and the findings are summarised in Table 3.3. The uptake of fluorochrome by *R. solani* was stain dependent and did not appear to be greatly affected by whether the cells were grown in fluorochrome containing liquid media or agar. In general there was either very efficient staining of mycelia after incubation in either medium such as with Cellufluor (Figure 3.5A), or no uptake such as with hydroethidine. With some of the stains such as carboxyfluorescein diacetate (CFDA), fluorescein isothiocyanate (FITC) and ethidium bromide, there was uptake in both types of medium but visualisation of the hyphae and efficiency of staining were better with liquid medium. This was often because stain within the agar gave a high background level of fluorescence. Dye binding by agar was especially high for fluorescein diacetate, giving a totally green background. When liquid culture was used the mycelia could be washed in distilled water to remove any external stain. So subsequent experiments were always carried out using hyphae grown in fluorochrome-containing liquid medium, and the mycelia were washed twice in distilled water prior to observation.

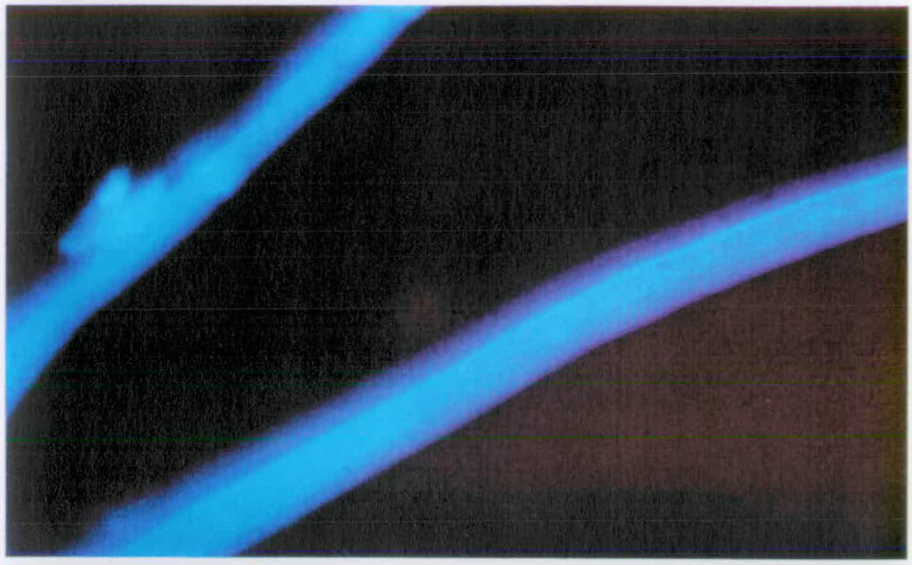
The mycelia of *R. solani* often did not stain uniformly. Mature regions of the hyphae had thick walls and in many such cases the fluorochrome could not be observed in older cells and was only detected in young hyphae, especially tip cells. Pigmentation of the mature cells may also have prevented visualisation of the fluorochrome. This was typical of Nile Red and FITC where preferential staining of the hyphal apical cells was observed with few older cells internalising the stain. Cellufluor gave the best overall staining as an even blue fluorescence was observed throughout the hyphal network (Figure 3.5A). Additional complications were found with some of the stains; for example fluorescein diacetate (FDA) gave good initial fluorescence of the cytoplasm (Figure 3.5B) but the fluorescence faded within 5 seconds of continuous excitation. Whilst Nile red produced an intense yellow-gold fluorescence of lipid globules with a dull orange fluorescence of the groundplasm (Figure 3.5C), staining was very uneven and only approximately 5% of the cells

Stain	Uptake		Linear Extension	
	Liquid	Agar	Liquid	Agar
Cellufluor	++	++	+	+
Nile Red	+	+	-	-
FDA	+	+	+	+
CFDA	+	-	+	+
CMFDA	++	+	+	+
CMAC	++	+	+	+
DAPI	+	+	+	+
FITC	++	+	+	+
DiI	-	-	nt	nt
DiO	-	-	nt	nt
Ethidium Bromide	++	+	+	+
Acridine Orange	+	+	+	+
Rhodamine 123	+/-	+/-	-	-
Hydroethidine	-	-	nt	nt
Lucifer Yellow	-	-	nt	nt

Table 3.3. Fluorochrome uptake by *R. solani* in liquid and solid media. Uptake was measured as the ability of the fluorochrome to react with the appropriate target site in the cell and was recorded as: ++, excellent staining; +, good staining; +/-, weak staining; -, no staining. Linear extension was measured as diameter of growth of mycelia: +, extension as for an unloaded control; - no linear extension; nt, not tested.

Figure 3.5. Fluorescent labelling of *Rhizoctonia solani* hyphae. Figures 3.5 A-I show hyphae of *R. solani* AG4 RH5/t stained with different fluorescent dyes. In all cases a mycelial covered agar block was inoculated into potato sucrose broth containing the working concentration of the appropriate fluorochrome (Table 2.3) and incubated at 25°C in the dark for 24-48 h to allow cell growth and uptake of the fluorochrome. The mycelial mat was then removed from the broth, washed 3 times in sterile distilled water and teased out to form a monolayer on a microscope slide. Observation of stained cells was carried out microscopically using appropriate barrier filters for visualisation of the stains (Section 2.2.3.) Magnification was x700 and photographs were taken using a Leitz Orthomat fully automated 35mm camera. **A.** Cellufluor staining of *Rhizoctonia solani* cell wall. Cellufluor is a vital stain which is absorbed onto extracellular β-glycans in fungal cell walls, producing an intense blue fluorescence (Maeda & Ishida, 1967). **B.** Fluorescein diacetate staining of *R. solani* cells. Fluorescein diacetate is a vital stain which is hydrolysed by esterases within living cells to release fluorescein. Fluorescein cannot be exported therefore accumulates in the cytoplasm producing a bright green fluorescence when illuminated with blue light (Rotman & Papermaster, 1966). **C.** A cell of *R. solani* stained with Nile red, a phenoxazine dye which is highly specific for neutral lipids. Fluorescence is diminished when not associated with the lipids, and is visualised as red background staining of the cell. In association with lipids the fluorescence is substantially enhanced to a bright yellow (Greenspan *et al.*, 1985).

A



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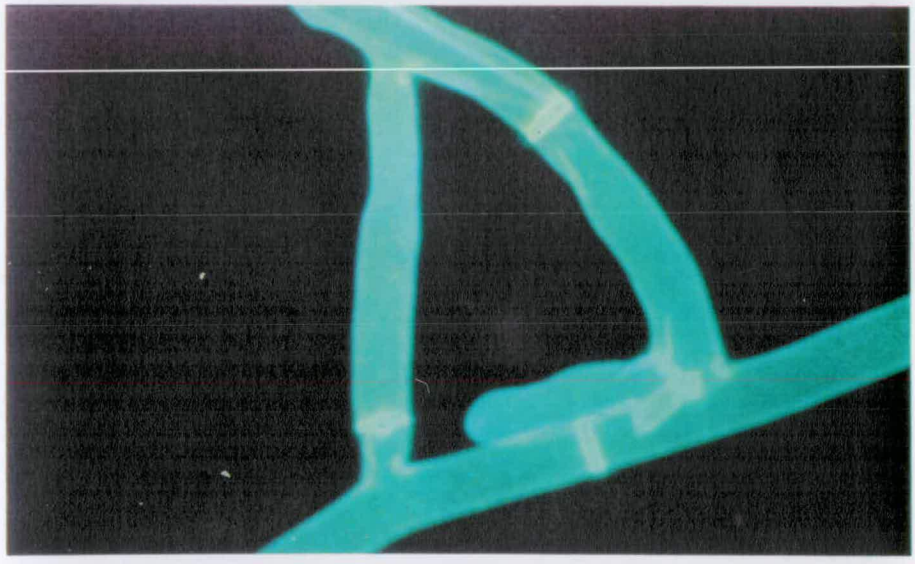

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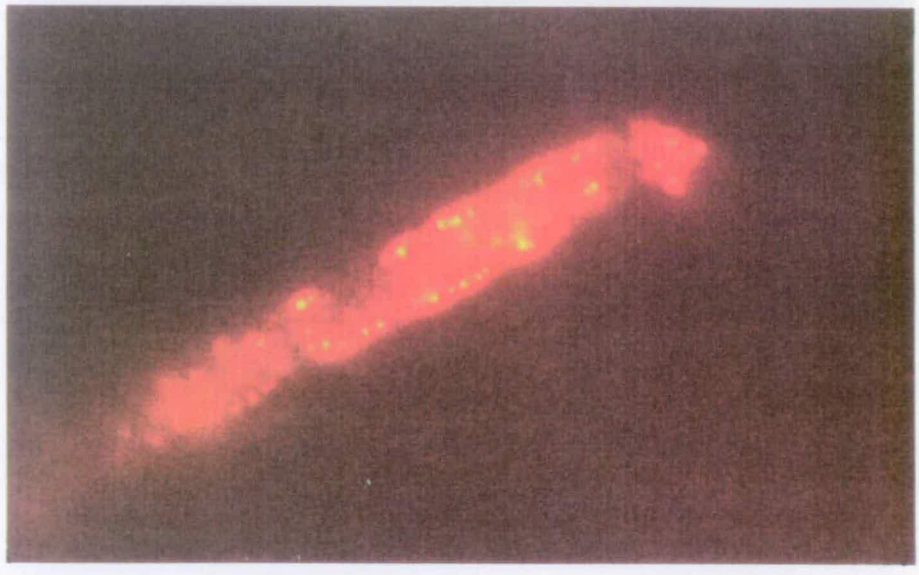
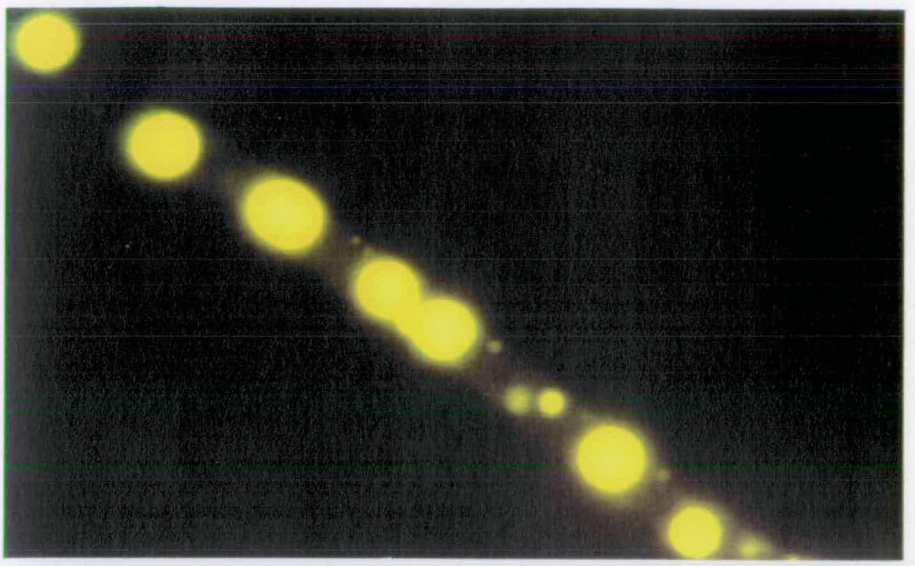


D. Vacuoles of *R. solani* stained with chloromethyl fluorescein diacetate resulting in yellow-green fluorescence. Chloromethyl fluorescein diacetate is a membrane-permeant fluorochrome with reactive chloromethyl groups. Once inside the cell they undergo a glutathione-S-transferase mediated reaction to produce a very photostable, cell-impermeant fluorochrome-thio-ester complex. The reaction product requires enzymatic cleavage to release the green fluorescent product (Baron-Epel *et al.*, 1988). **E**. Internalisation of fluorescein isothiocyanate into vacuoles of *R. solani* resulting in a bright green fluorescence. Fluorescein isothiocyanate reacts selectively with amino groups, although it is also known to react reversibly with thiols. Upon entering the cell the isothiocyanate reacts with the amines of proteins (Haughland, 1992). Although good fluorescence is obtained by this probe, it fades extremely quickly. **F**. Aminochloromethyl coumarin is visualised in *R. solani* as bright blue fluorescence of vacuoles upon excitation with ultra-violet light. Aminochloromethyl coumarin is a membrane permeant fluorochrome with reactive chloromethyl groups which undergoes enzymatic cleavage by a glutathione-S-transferase mediated reaction. Upon excitation with ultra violet light a blue fluorescence is produced (Haughland, 1992).

D



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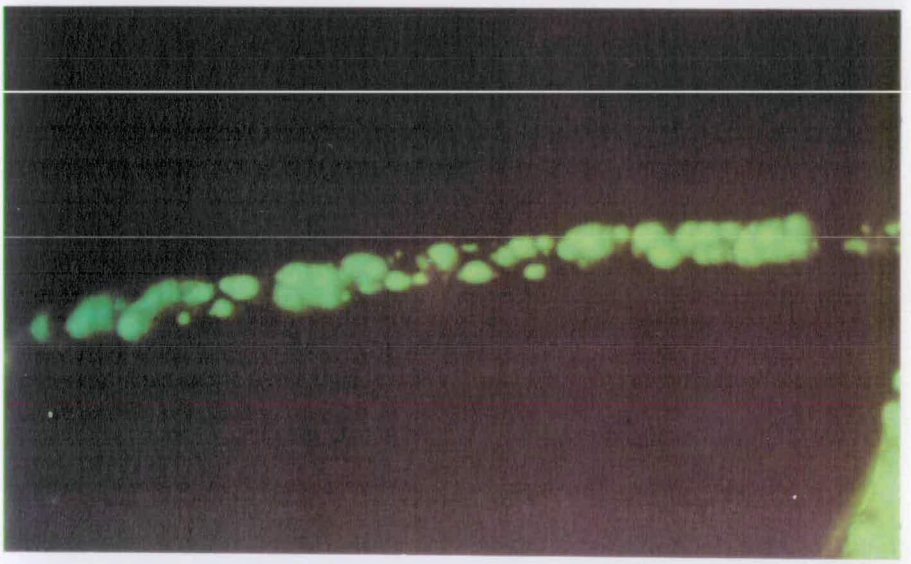

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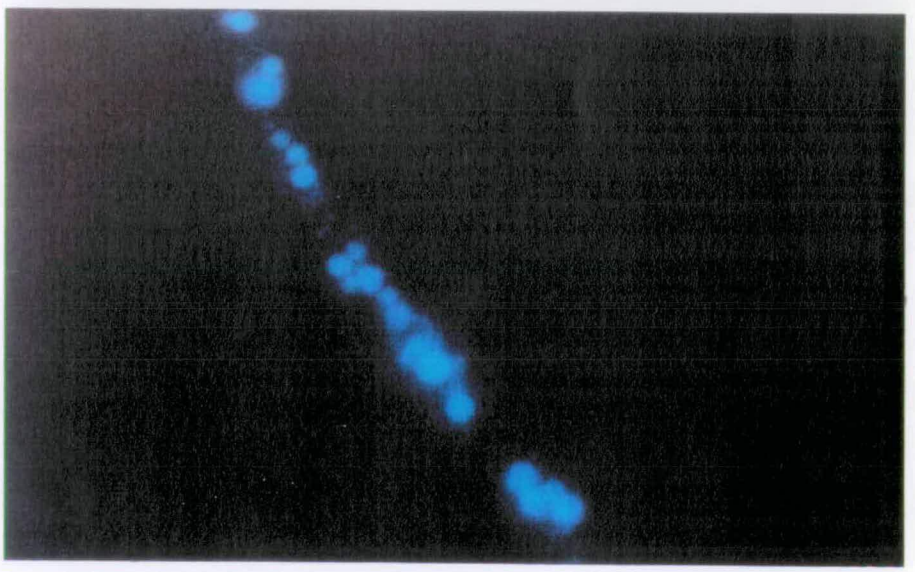
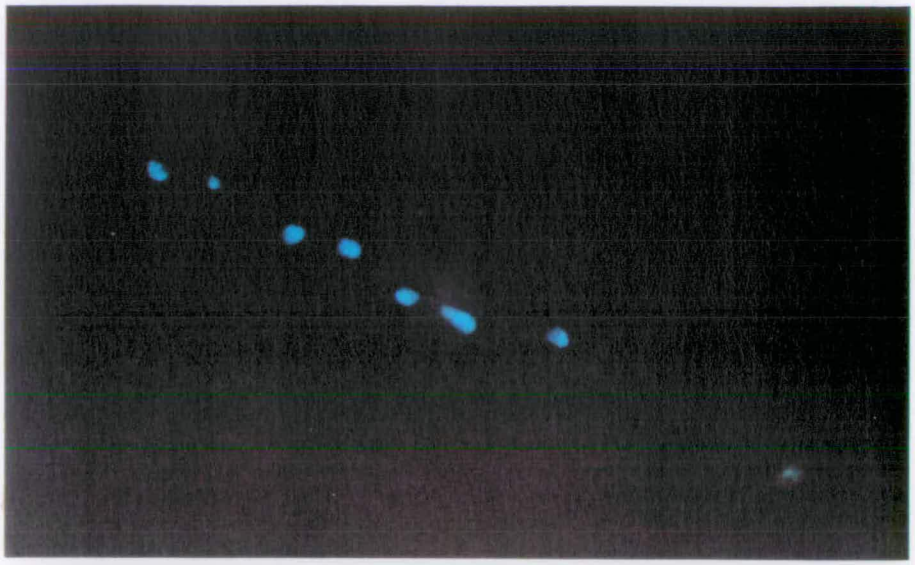


G. Hyphae of *R. solani* stained with DAPI which is a membrane permeant dye which specifically stains chromatin in the nucleus. It binds preferentially to AT-rich regions in the minor groove of the DNA double helix (Haughland, 1992). A bright blue fluorescence is emitted upon excitation with ultra violet light. **H**. *R. solani* stained with ethidium bromide which is a membrane impermeant phenanthridium intercalater which has no base pair preference. Upon excitation with ultra-violet light a bright orange fluorescence is observed (Haughland, 1992). **I**. Acridine orange staining of *R. solani.* Acridine orange is a cell permeant nucleic acid stain which interacts with DNA and RNA by intercalation or electrostatic attractions. On interaction with DNA a green fluorescence if observed which is similar to that seen with fluorescein. With RNA the emission is higher ,giving a red fluorescence (Haughland, 1992).

G



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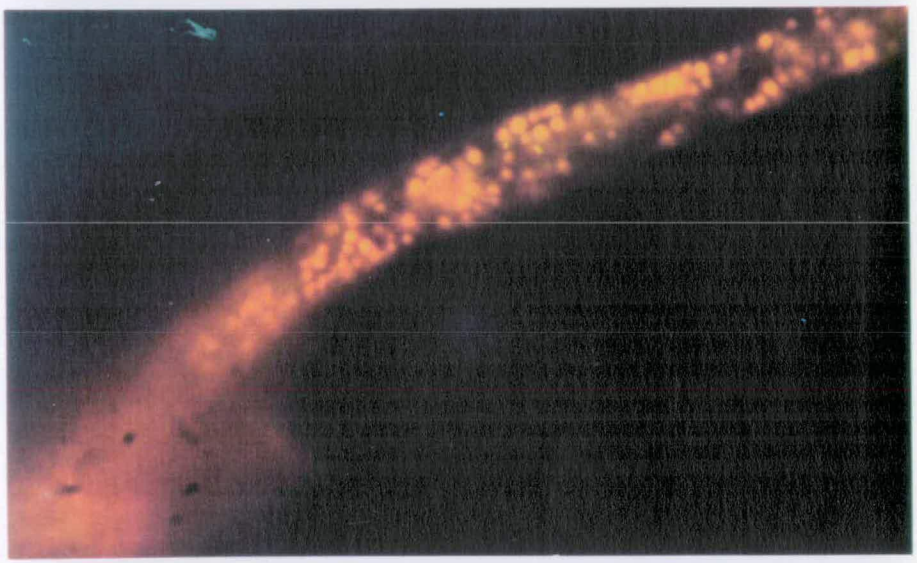

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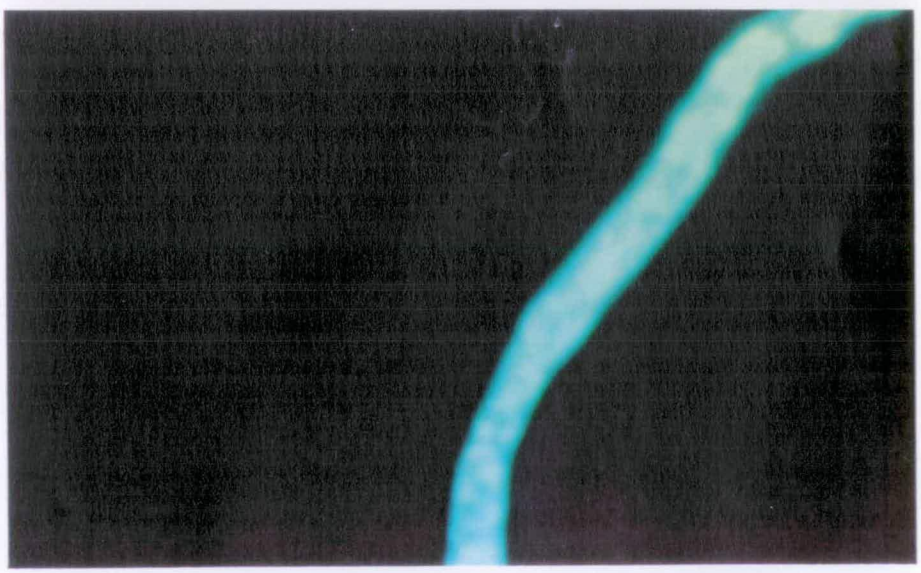


stained. Vacuolar staining with FITC and CMAC showed a notable difference in size of vacuoles between old and young cells. Figures 3.5E and 3.5F show FITC and amino chloromethyl coumarin (CMAC) staining of young cells close to the hyphal tip. Small vacuoles presumably either fuse during hyphal ageing, as seen in the case of large vacuoles stained by CMFDA in Figure 3.5D.

Staining of nucleic acid was especially of interest, as it was hoped to stain nuclei to monitor their movement during anastomosis. The internalisation and stability of a variety of nucleic acid specific stains were tested with varying degrees of success. Total nucleic acid was visualised using ethidium bromide (Figure 3.5H) which fluoresces bright orange upon interaction with both DNA and RNA (Haughland, 1992). Although excellent fluorescence was observed, the lack of specificity of the stain made it impossible to distinguish between types of nucleic acid. The differential stain acridine orange was used to distinguish between DNA, which fluoresces green, and RNA, which fluoresces red (Haughland, 1992). Staining with acridine orange gave excellent contrast and easy identification of the different nucleic acid types but definition and colour sensitivity of the strains was not preserved in the photograph (Figure 3.5I). Repeated attempts to capture the colours using different exposure times and film speeds were unsuccessful. For more specific nuclear staining DAPI produced a bright blue fluorescence upon interaction with DNA (Figure 3.5G). However, cellular uptake of DAPI was very uneven with less than 50% of cells staining. As with Nile red and FITC, preferential staining of tip cells occurred. Often at high magnification (x 700) tiny specks of stained mitochondrial DNA could be observed, especially at the tips. These were easily distinguishable from the nuclei due to their size.

As labelled hyphae must grow towards each other and fuse, it was important that the stains did not significantly reduce growth of the mycelia. Linear extension in both liquid media and on agar was therefore monitored in the presence of fluorochromes by measuring diameter growth of hyphae in petri dishes. Growth occurred as normal with most of the stains (Table 3.3). There were two significant exceptions to this, with Nile red and Rhodamine 123. In both cases, when fluorochrome containing media were inoculated with agar blocks of *R. solani*, many

aerial mycelia were produced from the blocks but there was little linear extension across the media. As Rhodamine 123 was difficult to detect in *R. solani* this stain was not studied further. Nile red produced good fluorescence of lipids so tests were carried out using lower concentrations of the compound. When the concentration was reduced from 50 to 10μg/ml, hyphal growth rate was as normal with little reduction in staining efficiency.

Dual staining of R. solani hyphae

The overall aim of this study was to visualise fused hyphae, to identify subcellular components before and after fusion, so it was important to be able to selectively stain different isolates. This could theoretically be achieved by labelling each strain with a different fluorochrome prior to fusion such that they were easily distinguished.

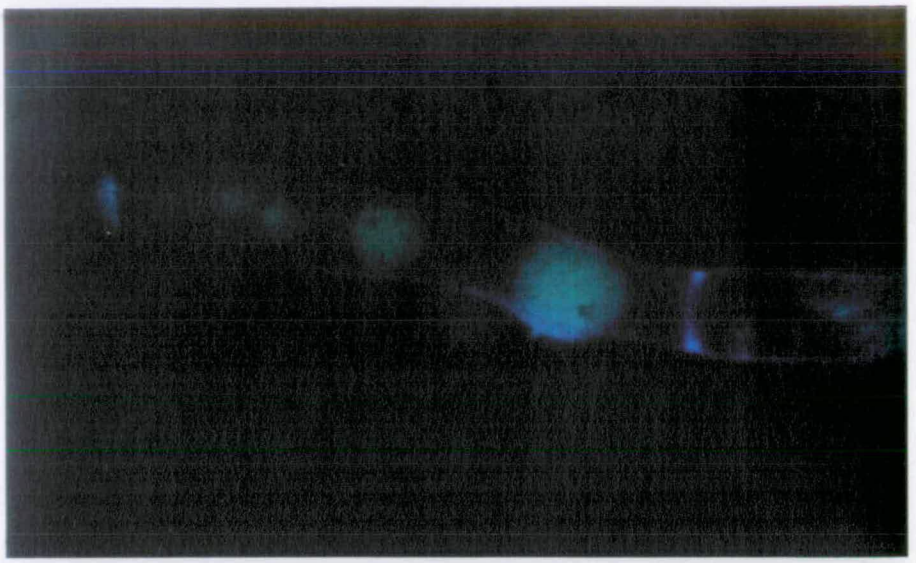
In order to test that more than one fluorochrome could be visualised in a single cell, dual staining was carried out in liquid medium as described in Chapter 2, except two fluorochromes were added simultaneously to the mycelia. Table 3.4 gives an overview of the fluorochrome combinations that were tested and the ability to distinguish between them. Although with nine of the combinations tested one could distinguish between the strains, the quality of fluorescence was not nearly as good as with the single stains. Fluorochromes could only be visualised under a single wavelength at any one time so, often at least one of the fluorochromes did not emit in distinct colour and was visualised as off-white. Figure 3.6 shows three of the better fluorochrome combinations when viewed with a single filter combination; only the combination of CMFDA and DAPI gave an image in which the distinctive colours of both fluorochromes could be seen (Figure 3.6B). In Figures 3.6B and C the blue barrier filter was used to visualise Cellufluor; Nile red (Fig 3.6B) appears less bright than usual, and fluorescence of acridine orange (Fig 3.5C) is almost totally suppressed by Cellufluor appearing as a non-distinct pale orange/white colour. In general, dual staining of hyphae was not as successful as single staining, and although it would be possible to change from one filter combination to another to

Fluorochrome A	Fluorochrome B	Identification of both stains
Cellufluor	Nile Red	+
	FITC	+
	CFDA	-
	CMFDA	+
	DAPI	+
	Acridine Orange	-
Nile Red	FITC	+
	CMFDA	+
	CMAC	-
	DAPI	-
	Acridine Orange	-
CMFDA	DAPI	+
	Acridine Orange	+
	CMAC	-
DAPI	CFDA	-
	CMAC	-
	Acridine Orange	+
	FITC	-
Acridine Orange	CMAC	-
	FDA	-
	CFDA	-
	FITC	-

Table 3.4. Differential staining of *R. solani* hyphae. The table shows the results of various combinations tested in single cells of *R. solani* (AG4 RH5/t) on water agar films. i.e. combinations of fluorochrome A with each of those listed as fluorochrome B. + shows that both fluorochromes could be detected and distinguished from each other. - shows that there was only identification of one of the fluorochromes; either due to similarities in colour or target site, or different emmision wavelengths resulted in lack of visualisation of one of the stains under any given filter combination.

Figure 3.6. Differential staining of *Rhizoctonia solani* hyphae with two distinct fluorochromes. Dual staining of *R. solani* hyphae was carried out by direct addition of the fluorochrome to the mycelial mat. *R. solani* was incubated in potato sucrose broth for 48 hours to allow good growth of the mycelia. A portion of the mycelial mat was removed, washed and placed in an empty Petri dish. 500µl of each of the appropriate fluorochromes, which had been diluted to the appropriate working concentration in sterile distilled water, was added directly to the mycelial mat. Petri dishes were then incubated for 2-3 hours in the dark to allow uptake of the fluorochromes. The mycelial mat was then rinsed in sterile distilled water to remove any external dye and teased out to form a monolayer. Microscopic observations were carried out at a x700 magnification and photographs were taken using a Leitz Orthomat fully automated 35mm camera. A. Hyphae stained with DAPI and CMFDA. The pale green vacuoles of CMFDA are localised in a single cell, and DAPI has stained one nucleus within the same cell. DAPI also highlights the cell wall and septum. **B.** Hyphae stained with Cellufluor and Nile Red. The Cellufluor staining is the strongest with Nile red visualised as a pale almost white fluorescence. The blue staining of the cell wall masks the red cytoplasm seem in Figure 3.4E. C. Hyphae stained with Cellufluor and acridine orange. Visualisation under the blue filter once more enhances the staining of Cellufluor while reducing that of acridine orange which could only be detected as an indistinct diffuse white cytoplasm.

A



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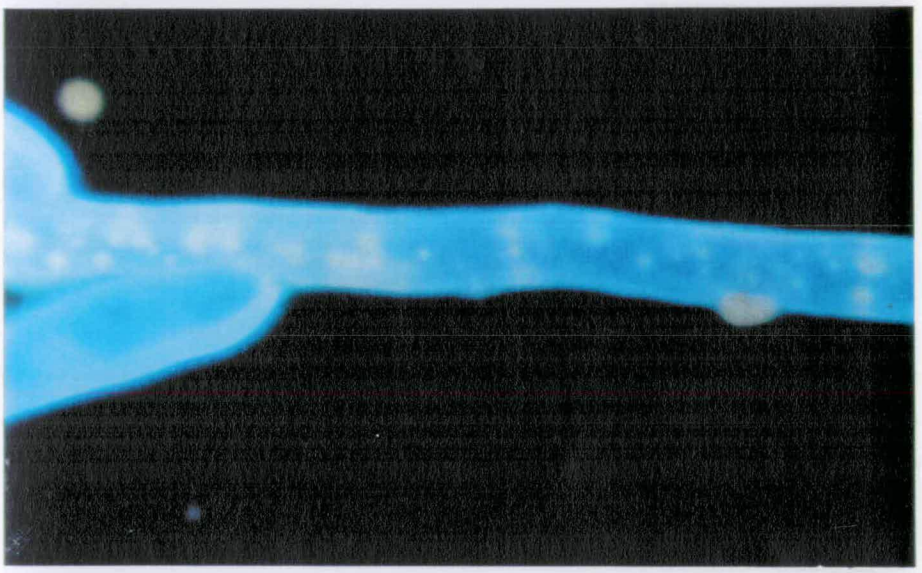

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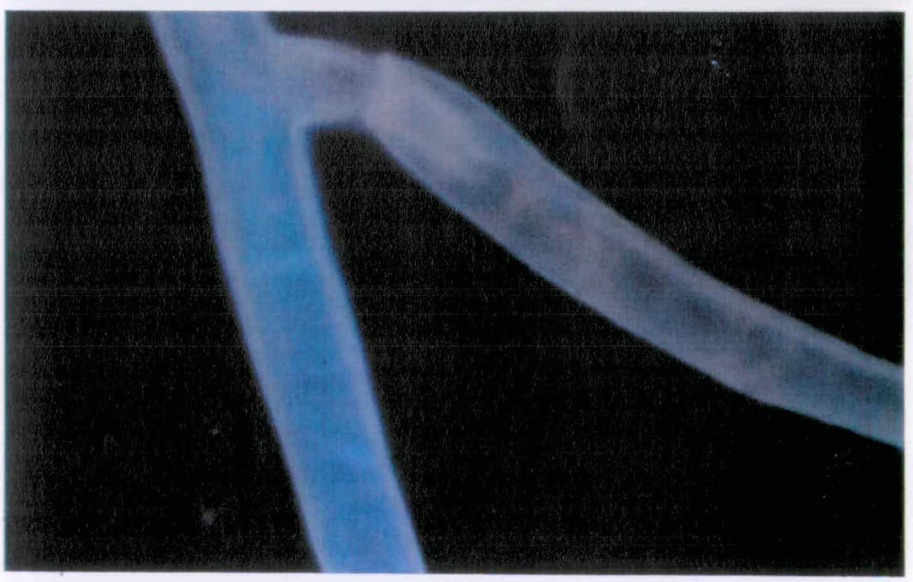


visualise two separate fluorochromes, this was not always feasible because of the rapid loss of fluorescence of some dyes during excitation.

Anastomosis of fluorochrome stained hyphae

Stewart & Deacon (1994) showed the ability of pre-loaded fluorochromes to be translocated into new growth. However, the distance of translocation which could be detected was quite small due to dilution of the stains during elongation of the cells. These workers found that the maximum detectable levels of movement with Cellufluor was approximately 8.4mm, when studied in *Fusarium oxysporum;* Nile red could only be detected in new growth up to 3.2mm, in *Pythium oligandrum*; CMFDA was detected in new growth up to 9.2mm, in *F. oxysporum,* and CMAC was detected in new growth of up to 6.2mm, in *Botrytis cir. erea.*

As *R. solani* had shown good uptake of fluorochromes, and some differential staining, with relatively little effect of fluorochromes on growth rate the key anticipated problem for using fluorochromes to study anastomosis might be dilution of the stains during hyphal extension. A variety of methods were therefore tested to optimise hyphal staining when the hyphae were in close proximity. Figure 3.7 shows some of these methods diagrammaticaly. Initial experiments were carried out with labelling of one of the mycelia only. It was hoped that upon anastomosis there would be transfer of stained organelles into the unstained hyphae. The mycelium-colonised agar blocks were inoculated onto a cavity slide (Figure 3.7A) and the cavity was filled with diluted fluorochrome, such that the mycelium from one block would grow through the fluorochrome-filled chamber and then encounter the unstained hyphae on the further side of the cavity. Using this method only unidirectional transfer could be monitored, but an indication of organellar transfer might be obtained.

Initial problems arose due to the inefficiency of staining of the hyphae. When tested with FITC, less than 15% of the tip cells had internalised the fluorochrome and where there was staining it was faint and faded very quickly. To try and circumvent this fading, video microscopy was used so that hyphae may be exposed to excitation wavelengths for a minimum period. Two hyphae which appeared to be attracted to one another and about to fuse were illuminated by ultra violet light just long enough

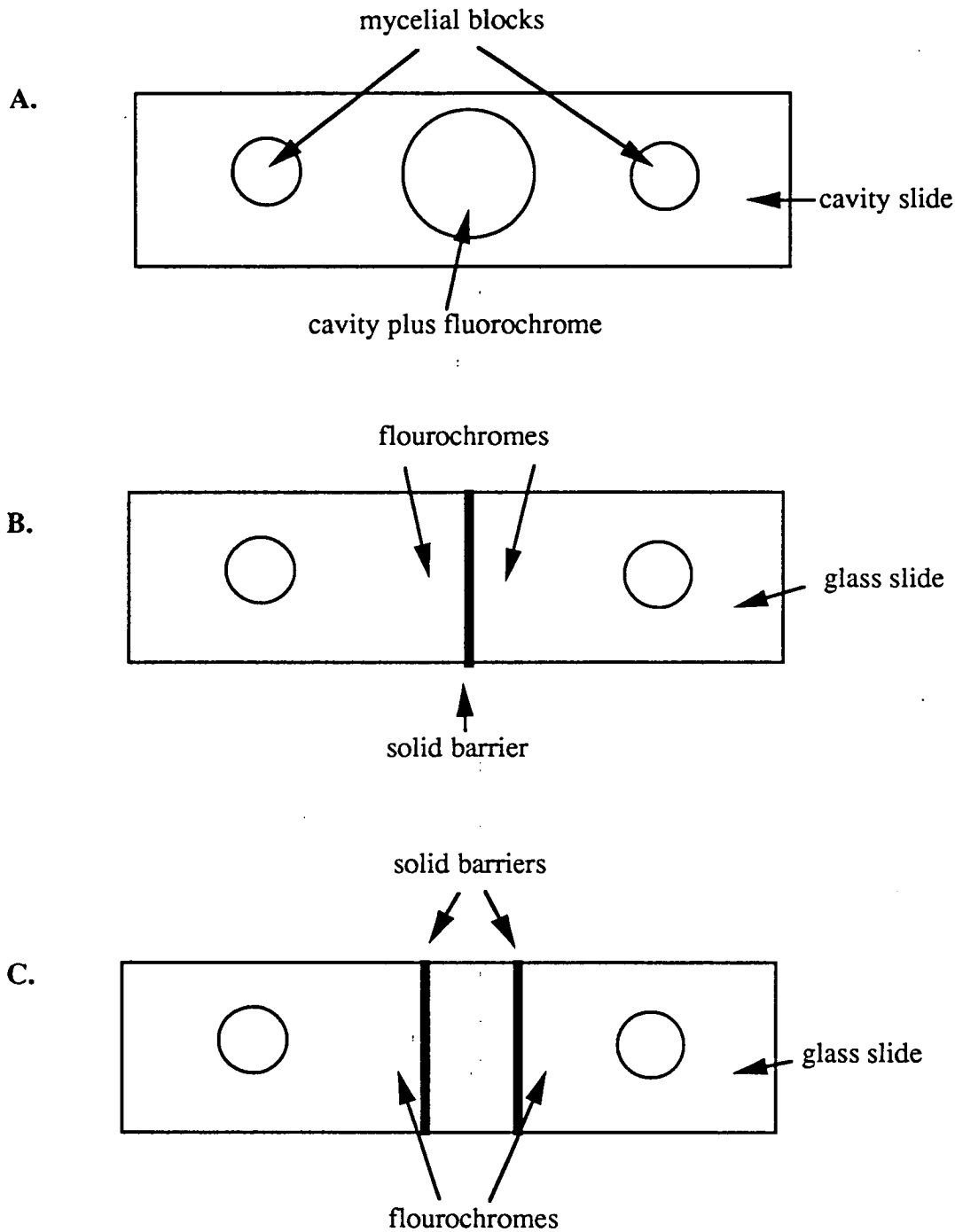


Figure 3.7. Methods used to detect anastomosis of *R. solani* using fluorochrome stained hyphae.

to check that one of the tip cells was stained, then anastomosis was followed by conventional brightfield microscopy. Just prior to anastomosis the cells were illuminated again with UV light to visualise the stained organelles and anastomosis was again continued under brightfield microscopy. In many cases the fluorescence was so faint by this stage that the vacuoles could not be identified. In a single case the vacuoles still fluoresced enough to be recorded but, after anastomosis the stain could not be visualised due to fading. Therefore in this case organellar transfer could not be monitored, but the study confirmed that, without ultra violet light, the fluorochromes caused minimal damage to the fungus and their presence did not prevent anastomosis.

As single fluorochrome staining did not give an indication of movement after anastomosis, dual hyphal staining was carried out by placing a solid barrier of vinyl polysiloxane elastomeric impression material (Kerr UK Ltd, Peterborough) across the middle of the slide (Figure 3.7B). After growth of the separate mycelia the barrier was removed, allowing anastomosis. Problems were encountered with this method due to fluorochrome mixing after removal of the barrier. So, using this method one could not unambiguously identify the source of the hyphae by staining. To overcome the problems with fluorochrome mixing, two barriers were placed on a slide in close proximity, approximately 5mm apart (Figure 3.7C), and the fungi allowed to grow from the mycelial blocks, through the fluorochrome and over the barrier. Anastomosis would be expected to occur between the barriers, thus ensuring that fluorochrome stained hyphae from different sources could easily be identified. As before, however, only limited success was achieved by this method, due to the low proportion of both cellular staining and anastomosis which occurred between stained cells. Very few hyphal tips which showed tropism were both stained with fluors, therefore examination was only carried out post-fusion to eliminate fading. However, although fusion and mixing of stains was identified, photography was ineffective due to faint staining from the dilute fluorochromes in the region of new growth between the barriers.

In further attempts to circumvent the above complication, video and fluorescence microscopy were performed in unison to follow the movement of

fluorochromes during anastomosis. Using the barrier method of Figure 3.7C, mycelial blocks were inoculated onto the slide, and grew through the fluorochrome and over the barrier. Upon hyphal overlap the area between the barriers was scanned for tip cells with different stains which appeared to be about to anastomose. Approximately 20 such cells were examined but no anastomosis was observed. When examined under ultra-violet light hyphal extension was not seen to occur. As previous observations had shown that the presence of fluorochrome did not greatly affect the growth of the hyphae, further studies were carried out on the effect of ultra-violet light on stained and unstained hyphae.

Firstly, the effect of ultra-violet light on unstained mycelium was tested. As a control the rate of hyphal growth of *R. solani* under bright field microscopy was measured over a period of 10 min. The average growth rate was 9.5 to 10μm per min. Hyphae growing at a similar rate were recorded for 5 minutes then exposed to ultra-violet light (Figure 3.8). The linear extension rate was observed to slow to such an extent that within 5 min exposure to ultra-violet light there was no growth in any of the irradiated hyphae. Indeed, often the growth rate had diminished by half after as little as 1 min of exposure. This observation was consistent for all hyphae tested. Although no growth was observable there was still cytoplasmic movement within the tip cells and they appeared as normal. Removing the source of ultra-violet light for periods of up to 10 min did not restore growth of the hyphae.

As the fluorochrome without ultra-violet light did not affect growth of the hyphae, and ultra-violet without fluorochromes prevented hyphal growth but did not kill the cells within 10 min, observations of hyphae stained with a variety of fluorochromes under ultra-violet light were made for a maximum period of 5 min. In all cases, where stained and unstained hyphae were exposed to ultra-violet light, there was no extension of the hyphal tips. The unstained (control) tip cells remained viable, with visible organellar movement even after 5 min. However for fluorochrome-stained hyphae the cytoplasm often coagulated rapidly (Fig 3.9). Of the four fluorochromes tested in this way, all of which targeted different cytoplasmic and organellar locations, only cells stained with Cellufluor did not show any coagulation within the recorded time period.

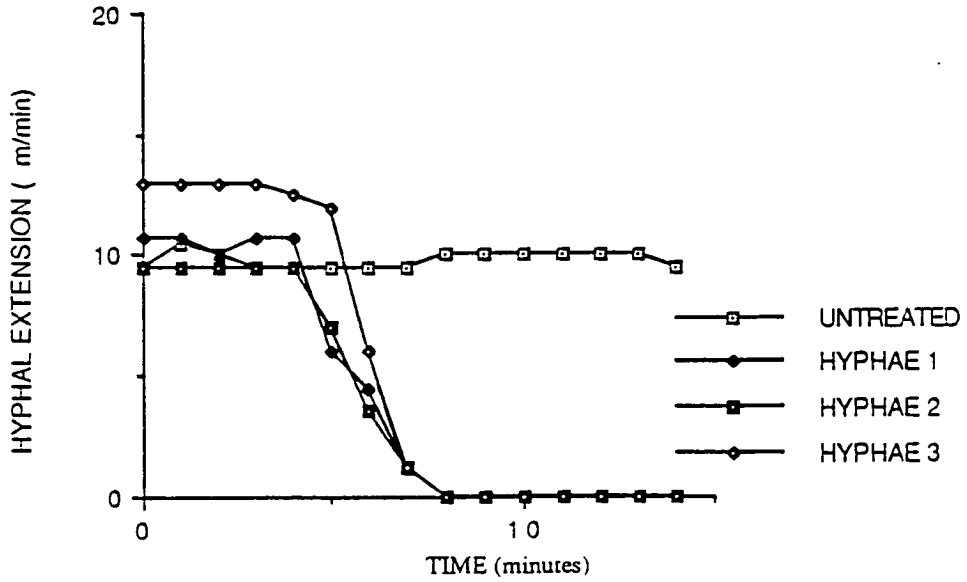


Figure 3.8. The effect of ultra-violet irradiation on the linear extension rate of unstained hyphae of *Rhizoctonia solani.* An agar block of mycelium was inoculated onto a water-coated agar slide and incubated at 25°C, on water agar in a Petri dish for 24 hours. The slide was then inverted onto a microscope examination chamber and growth of tips was measured using an eyepiece graticule. With unstained hyphae under normal brightfield microscopy an average extension rate was calculated for 6 replicates. Hyphae 1, 2 and 3 were followed for 5 min in these conditions then subjected to ultra-violet light and continued observation of their growth rate was made.

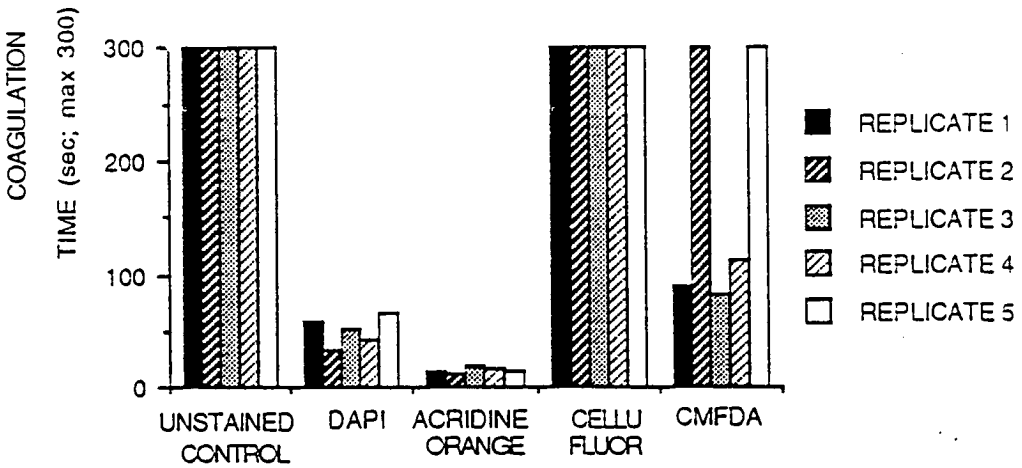


Figure 3.9. Cytoplasmic coagulation in fluorochrome-stained hyphae of *R. solani.* An agar block of mycelium was inoculated onto a water agar slide and incubated at 25°C in a Petri dish containing water agar for 24hrs. The slide was then inverted onto a microscope examination chamber and subjected to ultra-violet irradiation for up to 5 min during which it was examined microscopically (x700) for cytoplasmic coagulation in tip cells. The tests were repeated five times, for a single hyphal tip each time.

The most dramatic effect of UV-irradiation was on hyphal tips containing the nucleic acid stains, DAPI and acridine orange. These tips coagulated in less than 1 min exposure to ultra-violet irradiation. Acridine orange caused faster coagulation than did DAPI, perhaps due to the fact that both DNA and RNA were bound by acridine orange. The effect of CMFDA on cell viability was more variable. Only 3 of 5 hyphal tips showed coagulation within 5 min. This variability appeared to be due to vacuolar size because tip cells which had smaller vacuoles survived up to 5 min exposure to ultra-violet light whereas those with larger vacuoles coagulated quickly.

The loss of viability of fluorochrome-stained cells following exposure to ultra-violet irradiation explained the lack of growth and anastomosis in *R. solani* cells in the experiments mentioned earlier. Thus, the ultra-violet sensitivity of the hyphae limited the value of this technique for monitoring anastomosis.

3.3. DISCUSSION

The recognition of anastomosis groups has been very successful in aiding species identification in *Rhizoctonia solani* but, although anastomosis is a very useful tool, there is very little information available on the mechanism itself. This chapter set out to examine some of the parameters of anastomosis using light and fluorescence microscopy in order to examine the process of anastomosis and organelle transfer *in vivo,* without artefactual problems which may occur during sectioning and fixing of hyphae for electron microscopy.

Using light microscopy, anastomosis could be monitored and observation of successful anastomosis resulting in a compatible reaction was recorded. One of the time limiting factors with this system was the high frequency of hyphal approaches to one another where the hyphae either stopped growing as they approached one another, or continued growing past one another without contact. Initially, as the type of fusion mechanism was unknown, many hyphal tips growing towards the side of another hypha were monitored. Previous reports of anastomosis in *Schizophyllum commune* Fr. showed the majority of successful fusions to be tip to side (Todd &

Aylmore, 1985) and initially it was presumed to be the same in *R. solani.* This was shown not to be the case with fusion only occurring between two tip cells and always being preceded by an apparent attraction signal so that hyphal tips readjusted their growth direction as they 'homed' on one another. With both *R. solani* and *S. commune* a very low frequency of anastomosis was observed and most tip cells, even after showing some form of attraction, would not fuse. This may be due to availability of nutrients in the medium because it seems that only nutrient depleted hyphae produced, or were succeptible to, the signal for fusion. Also, in common with *S. commune* and *Coriolus versicolor* (L.) Quelet (Aylmore & Todd, 1984), examination of the mycelial mat showed localised areas of high frequency of anastomosis. The existence of these 'multifusion' areas suggest that release of an attraction signal might stimulate a similar response in other hyphae in the surrounding area.

The hypothesis that a diffusable attractant is the initial stimulus for anastomosis was supported by the observation that, prior to contact, at least one of the hyphae shows tropism towards the other. There appears to be only localised release of this substance, but some workers have debated whether a chemical diffuses from the cell into the surrounding medium or whether the attractants remain associated with the hyphal surfaces (Dickerson & Baker, 1979, Palmer *et al.,* 1983). Localisation of extracellular substances at the hyphal surface would be advantageous to the fungus as, if production was specific to the hyphal apex, the attracted fungus would be in the correct position for fusion. Either the presence of small amounts of the substance in the agar are sufficient for attraction and subsequent anastomosis by surrounding hyphae, or a very low level of attractant acts as a positive regulator of production of attractant by other hyphae, stimulating many hyphae within an area to produce attractants. A second indication that the substances are not attached to the hyphal wall is that hyphae may be attracted from distances as large as 100μm (Ogoshi, 1987) and change their direction of growth such that it is towards the signalling hypha. It is unknown whether the second hypha will then send a signal back, as there appears to be tropism in both hyphae, or whether it is the same as the initial signal and hyphae will grow along an increasing concentration gradient where

fusion occurs at the highest concentration of substance. The substances also appear to be anastomosis group specific (Ogoshi, 1987) as there is no recognition or tropism between hyphae from different anastomosis groups. As hyphal growth is of priority to the cell and it must economise on production of unnecessary substances, there must be regulatory and structural mechanisms placed on the production, secretion and activity of such substances (Priest, 1983). Response to environmental signals by fungi is, in part, mediated by second messenger pathways, and the mechanisms of recognition and adaptive response appear to be fundamentally similar in all eucaryotes (Kincaid, 1991). In many signalling pathways a surface receptor transmits the external signal via a GTP binding protein through the plasma membrane which in turn activates a protein phosphorylation cascade. Target proteins are then activated altering cellular activities in response to the stimulus (Janssens & Van Haastert, 1987). A number of molecules have been recognised as important mediators of extracellular signals, such as cyclic nucleotides and calcium ions. In filamentous fungi, cyclic AMP has been indicated in the expression of many phenotypic characteristics, which include restricted growth of mycelia (Yarden *et al.,* 1992), altered colony morphology (Robson *et al.,* 1991), conidiation (Rosenberg & Pall, 1979) and changes in enzyme activities (Terenzi *et al.,* 1992). But although cAMP has been shown to be an important regulator of development, the mechanism is far from completely understood.

Among the few tropic factors that have been characterised in fungi are the sex hormones (pheromones) of Oomycetes and Zygomycetes. For sexual reproduction in these species hormones are produced which are specifically regulated, spatially, temporally and by environmental factors, and which elicit a specific set of responses at low concentrations. The best characterised hormones are steroids in *Achlya*, an Oomycete, and terpenoic acid in *Mucor,* a Zygomycete.

In *Achlya* female cells of both *A. bisexualis* Cocker & Couch and *A. ambisexualis* J.R. Raper secrete antheridiol, a biologically active metabolite to which male cells respond by producing many branched antheridial hyphae and secreting oogoniol, which in turn causes female cells to produce antheridial hyphae. These antheridial hyphae grow towards each other, developing an oogonium and become

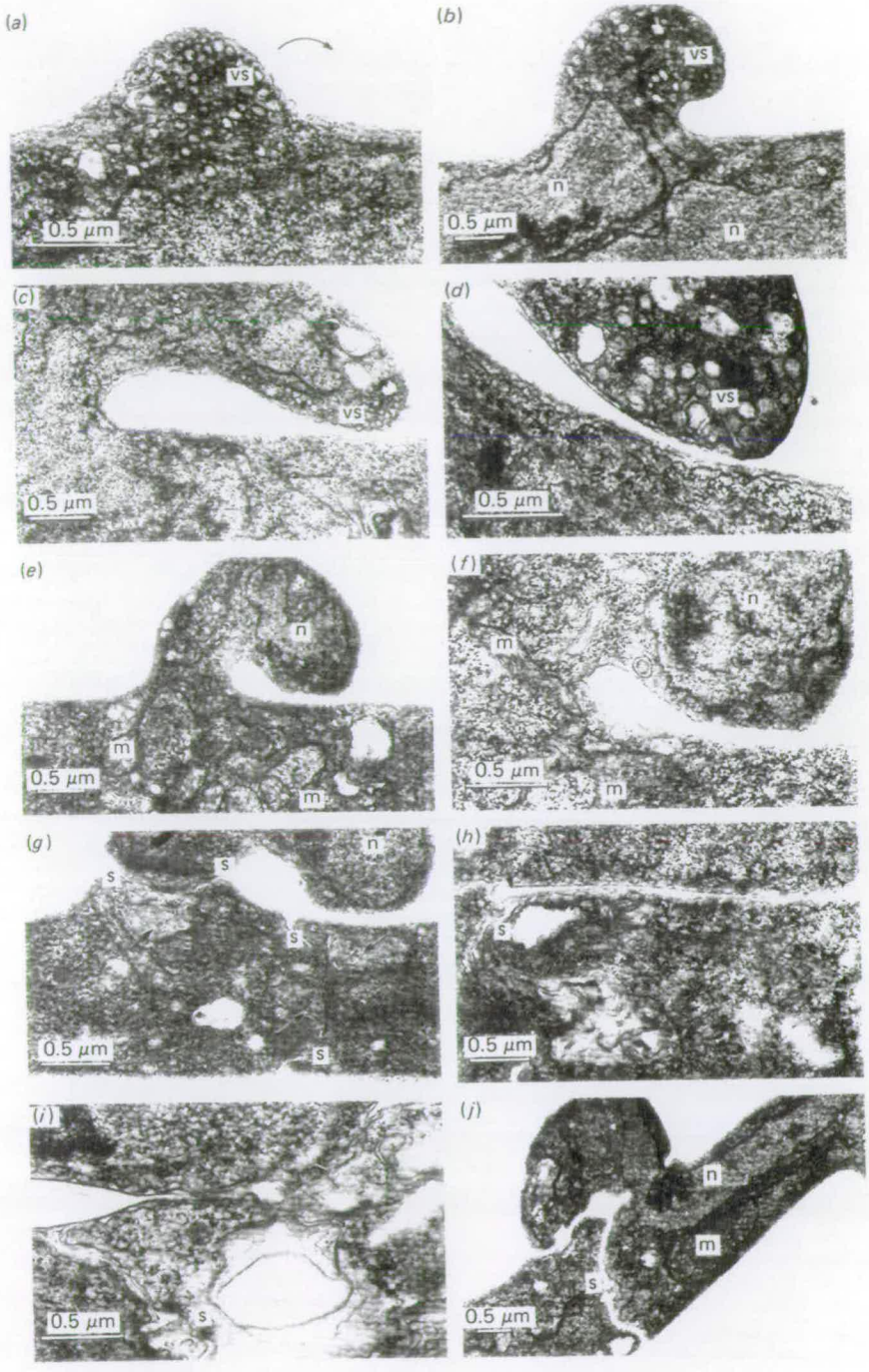
appressed to it. Multinucleate antheridia are determined from which nuclei migrate to fertilise the female gametes (Gooday & Adams, 1993).

In Zygomycetes, such as *Mucor* sp. sexual reproduction is a result of the fusion of cells of the opposite mating types (+ and -). In the absence of an opposite mating type vegetative cells differentiate to form large numbers of asexual sporangiospores. In the presence of an opposite mating type trisporic acid regulates the formation of zygophores, which formed from sexual hyphae, and fuse in mated pairs to form a large resistant sexual spore, the zygospore. Trisporic acid is known to be responsible for sexual mating as, in the absence of the compound, unmated cells of both mating types form sporangiospores, but in the presence of trisporic acid zygophores are formed (Gooday & Adams, 1993).

The attractant substances formed in the aforementioned species for sexual reproduction may be analogous to those produced during anastomosis in *R. solani.* Although with *R. solani* the interaction is asexual, there must de a specific chemical signal produced, probably at low concentrations, which results in attraction between hyphae of the correct anastomosis group.

Following the initial attraction of hyphae, fusion must occur to produce a single cell in which organellar transfer occurs. Formation of a single cell requires localised cell wall breakdown and synthesis, events which must be tightly regulated. Examination of anastomosis in *R. solani* by light microscopy did not give an insight into the interactions of the cell walls of the two hyphae. Todd & Aylmore (1985) examined the fusion process in *Schizophyllum commune* by electron microscopy. Preparation and fixing of samples was carried out at different stages in the anastomosis process to try to follow the cellular interactions. As with *R. solani* there were problems in anticipating successful fusions, and these workers looked at many hyphae during the early stages of attraction which did not undergo anastomosis. To overcome this problem, as *S. commune* is dikaryotic, they examined clamp connection formation as this anastomosis event could be guaranteed. To monitor the early stages of the fusion process electron microscopy of clamp connection formation was carried out (Figure 3.10). During clamp connection formation there is an outgrowth of the cell which is thought to be similar to the early stages of production

Figure 3. 10. Clamp connection formation in *Schizophyllum commune*. **(a).** The apical region of the clamp cell outgrowth resembles that of an extending vegetative hypha, containing numerous discrete membrane-bound vesicles. There is an apparent displacement of vesicles in the direction of future curvature (arrowed). **(b).** A more mature clamp initial. Vesicles occur in the extending apex. Two nuclei are present in the hypha. Vesicles are only present at a stage, before mitosis, where the walls have just touched, where they accumulate at the point of contact. **(c), (d).** Clamps fixed soon after contact with the parent hyphae, just before the onset of mitosis. Vesicles (vs) are present, accumulated in the apex at the point of wall appression. **(e), (f).** Specimens fixed just after mitosis and entry of the nucleus (n) into the clamp. Vesicles are now absent from the apex in the region in contact with the main hypha. Note the numerous mitochondria (m). **(g).** Septa (s) forming by annular ingrowth. Note the regions of amorphous material bounded by double-membrane (arrowed) associated with these structures. The nucleus (n) is present in the clamp. No vesicles are evident in the clamp apex and there is no sign of wall lysis. **(h).** As in (g), septa are forming. Note that the region of contact between the clamp and wall of the main hypha (between arrows) shows no sign of lysis. Vesicles are absent from this region. **(i).** With dolipore septum fully formed (s), wall lysis and clamp fusion have occurred at the point arrowed. **(j).** Migration of the nucleus (n) through enlarging fusion pore. Note the forming dolipore septum (s) and enlarged mitochondrion (m) in the penultimate cell.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of a side branch. This then bends such that it fuses with the side of the cell wall from which it has grown. In this way clamp cell formation is analogous to tip to side anastomosis. As *R. solani* only undergoes tip to tip fusion the physical contact point will be different but a similar mechanism of cell wall fusion should occur in *R. solani* self-anastomosis.

In the initial outgrowth of the clamp branch there are vesicles present in the tip (Figure 3.10a) which are then displaced such that they are localised in the direction of future curvature (Figure 3.10b). These vesicles may represent the Spitzenkorper, an electron dense body which has been observed in the apex of actively growing hyphae. Close examination of the Spitzenkorper showed it to consist of many vesicles and the direction of hyphal growth could be correlated with prior changes in the position of the Spitzenkorper, so it appears to be important for growth (Bartnicki-Garcia, 1975). The Spitzenkorper is common in both basidiomycetes and ascomycetes and appears just before hyphal elongation, disappearing again once growth has ceased. The vesicles observed in *S. commune* (Figure 3.10), also appear to be involved in growth of the cell as they are only present until the walls have just touched (Todd & Aylmore, 1985), and are always located at the point of contact (Figure 3.10d). Upon contact, many mitochondria enter the clamp suggesting that the process of wall dissolution and formation is energy-consuming (Figure 3.10e). After mitosis, a daughter nucleus enters the clamp branch and before wall dissolution there is formation of a septum by annular ingrowth at the base of the clamp branch. At this point, although the cells are separated the clamp and recipient cell walls appear to be intact, as yet, there is no sign of cell lysis (Figure 3.10h). Only when the septum is fully formed does lysis of the wall of the clamp branch tip and recipient cell occur. Lysis results in the formation of a pore which, as it enlarges, allows migration of the nucleus through to the recipient cell.

Clamp formation is similar to anastomosis in that it involves the attraction between two hyphae, localised cell wall lysis and the enlargement of a single fusion pore (Figure 3.10j). As *R. solani* is multinucleate there may not be mitosis first, prior to fusion, but simple nuclear mixing may take place. Alternatively there may be mitosis of all nuclei prior to fusion.

In the accepted model for hyphal extension (Bartnicki-Garcia, 1975; Wessels, 1990) a role for the apical vesicles is proposed which involves them in cell wall lysis. This is feasible as there is discharge of the contents of the vesicle shortly before dissolution (Harvey, 1975; van der Valk & Marchant, 1975) which provides circumstantial evidence that the apical apparatus contains wall lytic enzymes. The model proposed by Bartnicki-Garcia is outlined in Figure 3.11 and is a unitary model for cell wall growth. Hyphal elongation has been shown by autoradiograph studies to occur at the apex of hyphae, not further back in the cell. The model allows discharge of both cell wall material, by fusion, and lysis enzymes from the vesicles. By light microscopy it was impossible in the present study to validate the mode of hyphal wall breakdown and synthesis, but the studies of Todd and Aylmore (1985) with clamp connections show that wall synthesis occurs and a dolipore septum is formed before any cell wall lysis, preventing bursting or cytoplasmic leakage from the cell. The process must be very tightly regulated and vesicles are thought to play an important role in this regulation as cell wall components are synthesised *in situ*. Using the model of Bartniki-Garcia (1975) the presence of vesicles is needed for both cell wall lysis and synthesis so control of vesicle production controls hyphal extension and anastomosis. There are two subsequent events of cell fusion. Post-fusion cytoplasmic mixing and the formation of a single cell, or post-fusion incompatibility.

As an alternative to the model for apical growth proposed by Bartnicki-Garcia (1975), a more recent model has been proposed by Wessels (1990). As there is no evidence for net synthesis of polymers in the wall, they are all added to the wall from the inside by apposition. Wessels has therefore proposed a steady-state theory for apical wall growth. This assumes that there is constant secretion of a plastic wall material into the apex of the cell, which is continuously displaced both backwards from the apex and outwards, such that it migrates through the wall. The newly added material is stretched and becomes cross-linked as it moves throughout the wall, thereby developing resistance to turgour pressure. The outer layers of the wall are the oldest and have the greatest degree of cross-linking. With time cross-linking will spread through the whole wall forming a rigid structure. To form a steady state, in

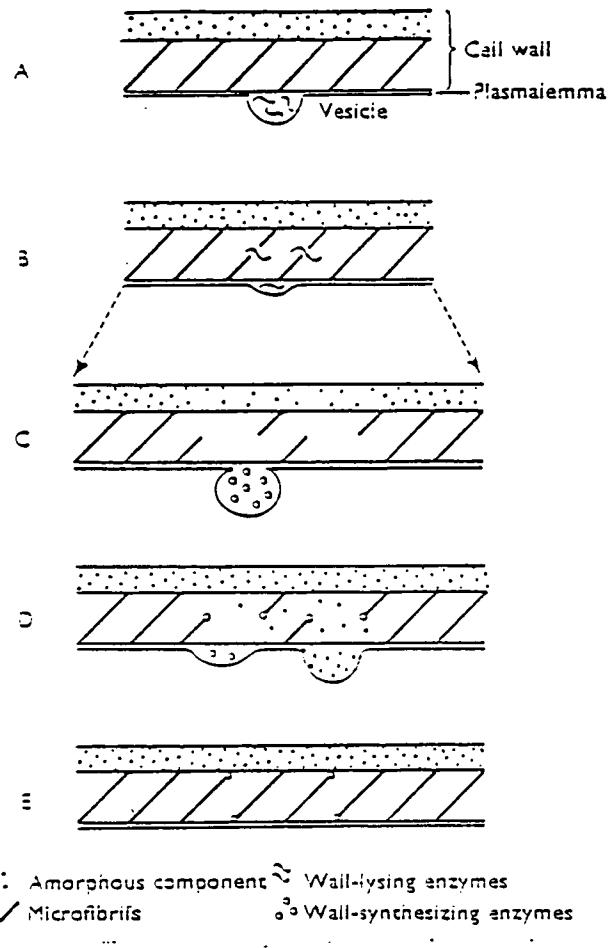


Figure 3.11. Production of a unit of cell wall growth. **A.** Lytic enzymes from a cytoplasmic vesicle are secreted into the wall. **B.** These enzymes attack the microfibrillar skeleton by splitting either inter- or intra-molecular bonds. **C.** The dissociated (broken or thinned out) microfibrils can no longer withstand the high turgor pressure and become stretched out or separated from one another with the consequent increase in surface area of the wall. (No attack on the outer amorphous components is shown but this might prove necessary). **D.** Microfibril synthesising enzymes on the outer surface of the new plasmalemma (formed by exocytosis) rebuild the microfibrils by producing new chains or by extending old ones (or broken ones). In this scheme, the synthetases are assumed to be transported across the plasmalemma (via lipid intermediates ?). Vesicles containing amorphous wall material, in a largely or entirely preformed state, deposit their contents against the wall. Given the high turgor pressure of the cell, the vesicular contents would be forced through the microfibrillar fabric, and most of it would reach the outer surface of the wall where it would somehow be firmly anchored. **E.** In this manner the cell wall has expanded one unit without losing its overall properties, including coaxial arrangement (differential layering) of wall polymers.

hyphae growing at a constant rate, the rate of formation of plastic expandable wall material thus equals the rate of withdrawal of rigidified wall material at the base of the extension zone, hence the reference to a steady state.

The formation solely of only successful anastomosis was observed in the present study. By light microscopy, although cytoplasmic streaming was observed, there was no obvious transfer of organelles, but movement of nuclei and mitochondria could not be detected by this method. Previous studies by Todd and Aylmore (1985) have shown nuclear migration following clamp connection formation, in which participating cells display a donor-recipient relationship. A schematic diagram of this reaction is shown in Figure 3.12. The tetranucleate compartment formed upon fusion of the two hyphae results in the nuclei of the donor cell migrating to the site of anastomosis. Midway between the two hyphae there is an outgrowth which is the beginning of clamp formation, and one of the nuclei remains behind this outgrowth so that upon septum formation it will remain in the original cell. Upon migration of the donor nucleus, the recipient cell nuclei degenerate and are replaced by a conjugate division of the donor pair. In this way two binucleate cells are formed. As the cells are identical it is unknown how the signalling mechanism works which causes dissolution of nuclei which are genetically identical to those which are replacing them.

With multinucleate *R. solani* the fate of the nuclei after fusion is unknown, but as most field isolates are heterokaryons (Anderson, 1984) it is unlikely that nuclear migration will occur following fusion, or segregated colonies would be obtained rather than heterokaryons. Also, unlike binucleate basidiomycetes, there is no septum formation in the fused cell, which remains as a single large unit.

With incompatible fusions a different type of reaction occurs. Anastomosis results in an incompatibility which causes death of the fused cells (Figure 3.1). In *R. solani* the mechanisms for vegetative incompatibility are unknown, and there is no information available on the genetic control of incompatibility. Although post-fusion incompatibility was observed, no video recordings or observations were made as the reaction was happening. Noticeable vegetative incompatibility reactions were localised to certain areas of the hyphae in a similar manner to compatible fusions so

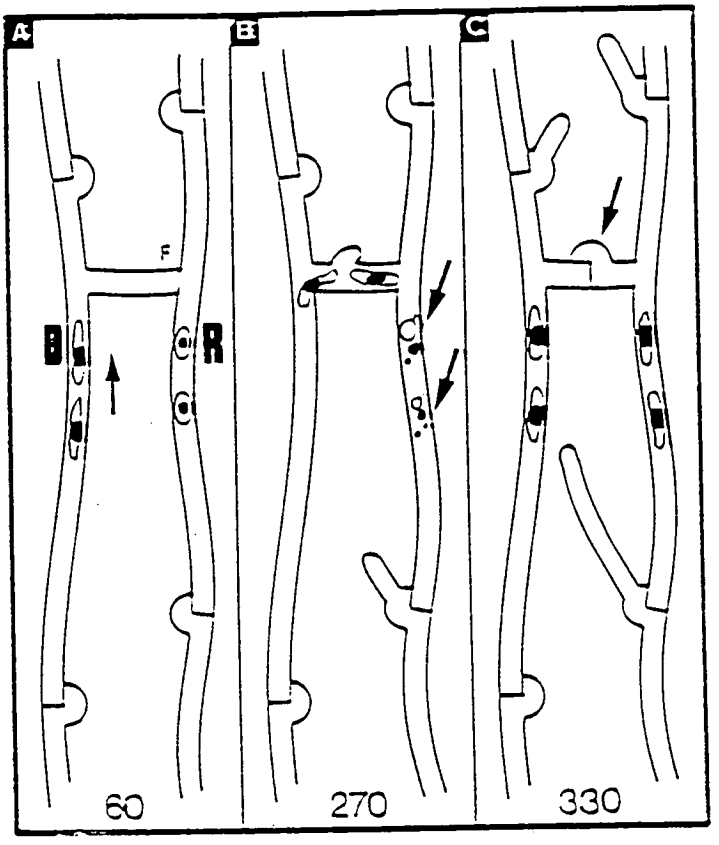
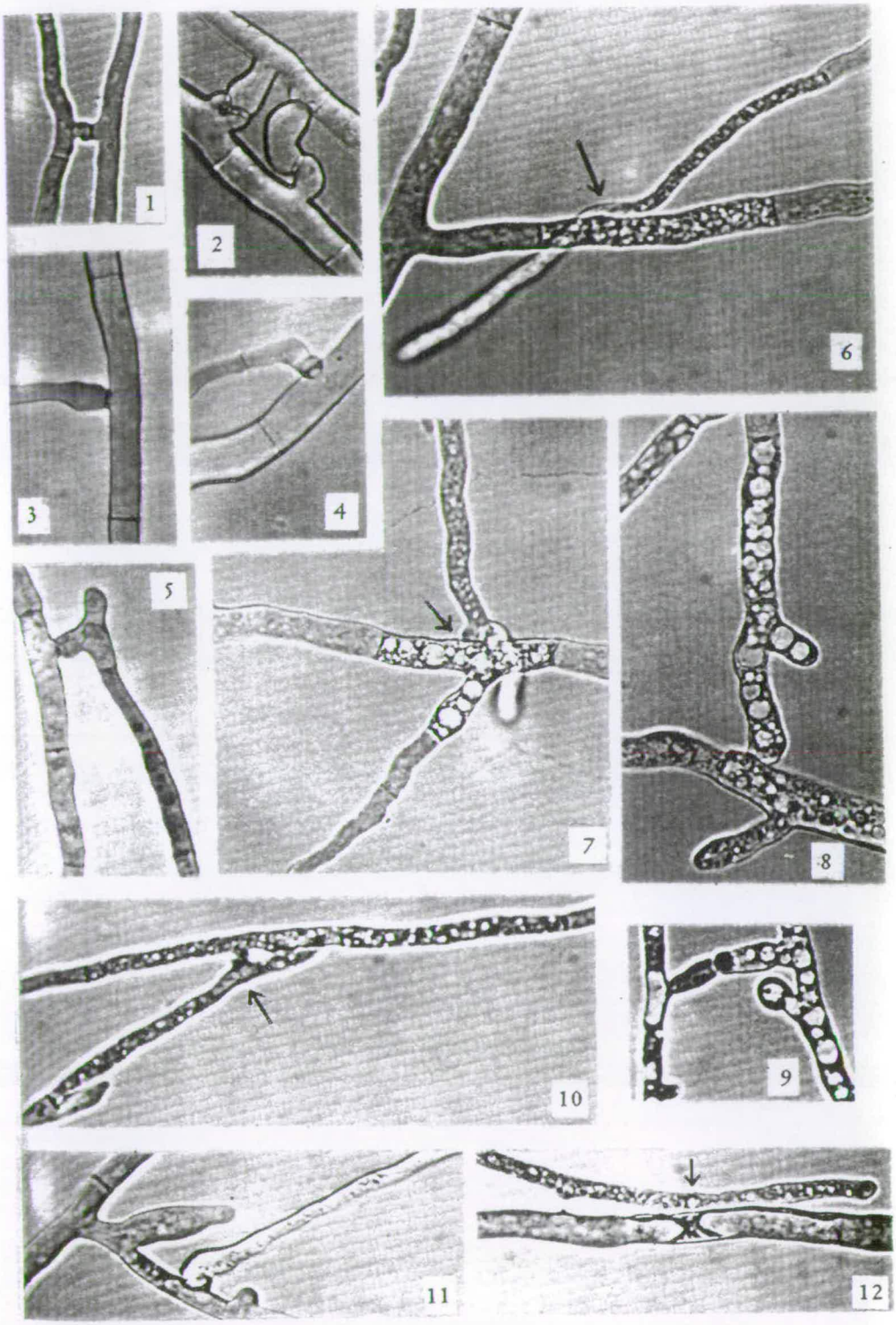


Figure 3.12. The nuclear replacement reaction in *Schizophyllum commune* following fusion of genetically identical dikaryotic hyphae. The numbers represent minutes after fusion. (A) Tip-to-side fusion (F) results in a transient tetranucleate compartment. The nuclei of the donor cell (D) begin migration towards the site of anastomosis while those in the recipient cell (R) round up and remain stationary. (B) Following fragmentation and degeneration of the recipient cell nuclei (arrowed), the donor pair stabilise positions close to the fusion and begin to undergo conjugate mitosis. (C) The fused segment is converted into two binucleate compartments, separated by an intercalary clamp connection (arrowed). Both compartments posses nuclei derived from the original donor cell. (diagram not to scale) (reproduced from Todd & Aylmore, 1985)

the attraction signal must be universal within an anastomosis group no matter whether compatible or incompatible reactions are likely to occur. Vegetative incompatibility has been monitored in *Neurospora crassa*. Garjnobst and Wilson (1956) examined fusion reactions of different mating types of *N. crassa* in which vegetative incompatibility is known to be controlled by two mating types, and, as in *R. solani,* cells could be vacuolated following anastomosis, although in some cases only the fused compartment showed cell death. The vacuolated region was limited by septa (Figure 3.13), each containing a distinct opaque disc or plug in place of the opening. Often two to three consecutive septa were plugged (Garnjobst & Wilson, 1956), and the outermost cells often showed a delayed or graded effect i.e. less granulation and vacuolation distally. The septal pore plugs were most prominent at the height of vacuolation. After a time the vacuoles faded from view, leaving the contents of the affected cells pale and disorganised. Many hours later shrunken protoplasmic remnants could still be seen within the old cell walls. During the late stages of the incompatibility reaction normal cells, immediately behind the outermost plugged septa, often developed new growth points. This observation was also made with *R. solani.* In both cases the regenerated tips discontinued growth or broke out through the wall before reaching the point of union of the parent hyphae. The important result of the fusion and interaction of the two protoplasms appears to be that the affected region of each hyphae is effectively destroyed, a situation analogous to *R. solani.*

One of the questions arising from post-fusion incompatibility in *R. solani* is: do components from the affected region escape into the adjoining region? Pore plugging serves as protection to the rest of the hyphae from an incompatibility reaction. In Ascomycetes the plugging mechanism is well documented (Furtado, 1971) and in Basidiomycetes the dolipore septa are often found to be blocked by electron-dense material (Bracker & Butler, 1964). Such septal sealing is thought to be a two stage process (Todd & Aylmore, 1985). In *S. commune*, upon physical damage to a single cell, pore plugging occurred instantaneously. Blockage of the cell pore channel did not affect the septal swellings or parenthesomes. Pore plugs appear to form *in situ* as if by a process of coagulation. Several minutes after damage there is consolidation of

Figure 3.13. Anastomosis and vegetative incompatibility in *Neurospora crassa*. **1, 2.** Intrastrain fusion. **3, 4.** Interstrain fusions with compatible pairs. **5, 6.** Early stages of the incompatibility reaction. Plugs are visible in septa delimiting affected regions. **7.** Later stage of the reaction showing prominent vacuolation in wild type pair differing in mating type. **8.** Later stages of the reaction showing prominent vacuolation in pair differing in both mating type and heterokaryon phenotype. **9.** Later stages showing prominent vacuolation in pair differing in heterokaryon genotype. **10.** Later stages of reaction in which vacuoles remained small. New growing point at the lower left. **11, 12.** Types of regeneration.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the plug within the channel and eventual degradation of the septal apparatus in the ruptured compartment. At the same time a more permanent seal is formed on the other side of the cross wall. It is unknown how, and to what extent, the stimulus is transmitted through the hyphae, but during regeneration and growth of the damaged hyphae, the plugging of septa not in direct contact with the ruptured cell may be reversible.

Although there is not much biochemical or genetical information of anastomosis in *R. solani*, comparisons with other systems in Basidiomycetes gives an insight into the potential for transfer of genetically important organelles, such as nuclei, between cells. This in turn may lead to information on the limitations to heterokaryon formation in *R. solani*.

Both the results, and the previous discussion section show that light and electron microscopy have given many insights into fungal cytology and the process of anastomosis (Todd & Aylmore, 1985). The problem with light microscopy is the inability to observe subcellular organelles, and with electron microscopy there is always the possibility of artefacts due to the fixation methods used. The use of markers which could follow organeller movement during anastomosis, without artificial manipulation of the hyphae, would give a clearer indication as to the role, and the potential for transfer, of these organelles during and after anastomosis. A wide range of fluorescent probes is available for targeting specific subcellular organelles, and there are reports that these compounds are not cytotoxic in fungi (Darken, 1962; Tsao, 1970; Drummond & Heale, 1985). For the most part, fluorochromes in fungi have been used to examine cell viability and directly visualise organelles, and have been found to be extremely effective in doing so (Butt, 1989). This study confirmed the potential of fluorochromes as a valuable tool for monitoring subcellular components and as a simple method of examining cellular differences such as nuclear number eg. ethidium bromide (Singh & Kumar, 1991), vacuolar size eg. FITC (Butt, *et al.* 1989) and viability eg. FDA (Soderstrom, 1977).

In other cases the use of fluorochromes has been extended to specifically identify fungi in natural materials. The use of the cell wall stain, Calcofluor White MR2, which is analogous to Cellufluor, has been used to identify and count fungal

propagules in soil, or on plant and insect surfaces (Butt, 1989; Darken, 1962; Tsao, 1970). It has often been noted that fluorescence exhibited by labelled fungal propagules in soil is not as strong as similarly labelled propagules on agar plates or glass slides, probably due to quenching by soil particles or some background autofluorescence (A. Stewart, pers. comm.). Nevertheless, pre-labelling of fungal propagules with fluorochromes before the propagules are placed in soil has enabled the propagules subsequently to be located microscopically in soil smears.

Nuclear staining has proved useful in many systems as often there is variation in nuclear number which can be correlated with a change in culture conditions, fungal virulence or stage in the life cycle (Gantotti & Woodske, 1991). In many multinucleate species it is extremely difficult to detect nuclear numbers without a direct staining mechanism and to this end ethidium bromide has been used successfully on a number of species such as *Rhizoctonia solani, Fusarium oxysporum* Schlecht and *Phytophthora infestans* (Mont.) DeBarry (Singh & Kumar, 1991), as has DAPI with *Colletotrichum gloeosporioides* Simmonds (Gantotti & Woodske, 1991). Therefore, vital fluorochromes have been shown in a number of cases to give important information on fungal physiology without the need for fixing of the cells.

Initial investigations of mycelial growth on fluorochrome containing media also confirmed the lack of toxicity of many fluorochromes used (Table 3.4), so they seemed to have potential for examination of anastomosis. Unfortunately, however, the needs in the present study differed from those in others where it was only necessary to detect fungi at the end of an incubation period. Thus, Stewart & Deacon (pers. comm.) could assess the growth made through soil from pre-labelled fungal propagules. Cellufluor, CFDA, CMFDA and Nile red all had the potential to be used as tracers of fungal growth in soil. These flours could be detected after up to 5 days incubation of pre-loaded propagules in soil, and differential staining was used to distinguish between two fungi in soil even when they are morphologically identical (A. Stewart pers. comm.). But for the present purposes it was required that video microscopy be complexed with fluorescence microscopy to follow the movement of fluorochrome tagged organelles during anastomosis, and two insuperable problems

were encountered. First, even brief exposure of the most useful fluorochromes to UV light, those that labelled nuclei or cytoplasmic organelles, led to substantial photo-inactivation of the fluorochromes. Second, relatively brief, periodic exposure of growing hyphae to UV irradiation caused cytoplasmic coagulation and stoppage of hyphal tip growth. Nuclear stains were most toxic in this respect, causing the cells to die rapidly after exposure to UV irradiation. Perhaps because these dyes were so effective in localising to organelles they intercepted much of the ultra-violet irradiation and caused its energy to be localised in the cells. In contrast to this, the cell wall stain Cellufluor had little effect on sensitivity of hyphae to irradiation, perhaps, because the general tagging of the wall did not focus the energy of the UV beam onto intracellular organelles.

It had been hoped that use of vital fluorochromes might label extrachromosomal genetic elements, such as double-stranded RNA in *R. solani.* But comparisons of fluorochrome staining patterns (eg. with acridine orange) of strains of *R. solani* that did and did not contain dsRNA (see Chapter 4) showed no obvious difference. At least partly this could be because dsRNA and dsRNA containing fungal viruses are more plentiful in older than younger regions of hyphae (Buck, 1986a). With *R. solani* only the youngest (tip) regions stained readily with fluorochromes, presumably because of the difficulties of stain penetration or quenching associated with pigmented hyphal walls. So fluorescence staining would not be expected to be of use for detecting differences in mycovirus content in such regions. Alternativly, this may reflect on the sensitivity of detection and a limited abundance of cellular dsRNA.

CHAPTER 4

ANALYSIS OF DOUBLE-STRANDED RNA IN *RHIZOCTONIA SOLANI*

4.1. INTRODUCTION

Rhizoctonia solani is a plant pathogenic fungus of broad host range. In several instances, studies on the genetic basis of plant pathogenicity by fungi have implicated mycoviruses as virulence factors. Mycoviruses are intracellular viruses found in both unicellular and filamentous fungi, and usually consist of RNA, often double stranded, encapsidated in a protein coat. Unlike classical viruses, they do not kill their host, rather host and virus usually exist in harmony in which often the virus is no more noticeable or intrusive than a vacuole.

Many fungi exhibit fascinating biological phenomena as a consequence of double stranded RNA particles, and *R. solani* is no exception. A variety of phenotypic traits have been reported in association with dsRNA. These include (1) Rhizoctonia decline, a degenerative disease of the fungus (Castano & Butler 1978a); (2) increased virulence (Finkler *et al.,* 1985); (3) other observable changes of phenotype related to the element (Zanzinger *et al.,* 1984; Kousik *et al.,* 1994). Reports have been made correlating the presence of dsRNA with reduced virulence in other plant pathogenic fungi such as *Cryphonectria parasitica* and *Ophiostoma ulmi* (Anagnostakis, 1982; Brasier, 1983). With *C. parasitica* direct evidence of the effect of dsRNA was obtained following development of a transformation system allowing direct introduction of purified dsRNA into cells and monitoring of the resulting phenotype (Shapira *et al.*, 1991b). In *R. solani* there is no such direct transformation system is available, so studies on the transfer of dsRNA is limited to the natural method, anastomosis. Anastomosis is a mechanism of cell fusion resulting in transfer of cytoplasm and organelles and is dependent on complex compatibility systems, so successful transfer is limited to specific subsets of the population.

In order to elucidate a role for dsRNA in *R. solani* one must fulfil the equivalent of Koch's postulates and show a phenotypic effect of the dsRNA which transfers with the elements. This chapter investigates the role of double-stranded RNA in *Rhizoctonia solani* by curing of strains and examining phenotypic parameters that might be related to the presence or absence of dsRNA.

4.2. RESULTS

4.2.1. Isolation of double-stranded RNA from *R. solani*

In order to investigate the roles of double stranded RNA (dsRNA) in *Rhizoctonia solani*, initial studies were carried out to investigate the occurrence of dsRNA in *R. solani*, and its distribution within anastomosis groups. Strains were tested for the presence of dsRNA by exploiting the fact that it binds to cellulose in the presence of 15% ethanol. Using a modification of the methods of Morris & Dodds (1979), as described in Chapter 2, total nucleic acid was extracted from mycelia by homogenisation in the presence of phenol and chloroform, then the cell debris was removed by centrifugation. The supernatant was mixed with CF-11 cellulose after the addition of ethanol to 15%, and the slurry was used to make a column. Sequential elution of DNA and single stranded RNA from the column occurred with the addition of buffer containing 15% ethanol. Subsequent elution of dsRNA was achieved upon the addition of buffer only to the column.

Strains from eight anastomosis groups were tested for the presence of dsRNA. Isolated nucleic acid was shown to be dsRNA by nuclease sensitivity tests. The dsRNA was resistant to DNase but sensitive to RNase at low salt concentrations. It is known that dsRNA is resistant to RNase A at high salt but sensitive at low salt levels (Kim *et el.*, 1990). In addition to this it was hydrolysed by 0.3M KOH at 37°C for 16 hours which confirmed the alkali lability of the material (Ralph & Wokick, 1969). Low concentrations of alkali successively remove nucleotides from the ends of RNA molecules. The double stranded nature of the elements was confirmed by resistance to RNase at high salt concentrations. Table 4.1 shows the distribution of dsRNA within anastomosis groups. There appeared to be some anastomosis group specificity to the distribution of dsRNA as none of the anastomosis group 5 strains contained any, but most anastomosis group 4 strains tested had two fragments. However, even though AG4 strains contained dsRNA these were of different sizes, so it is unlikely that the elements were identical. Of 15 strains tested, 3 contained dsRNA elements and in all cases more than one fragment was observed. Figure 4.1a shows dsRNA extracted from a variety of fungal strains using the cellulose CF-11

Strain	Anastomosis Group	Extrachromosomal Element	Double-stranded RNA
SC220	1	No	No
SC121	2	No	No
HV1	2-1	No	No
PS4	2-2	No	No
C127	2-2	2.5kb	No
SC222	3	No	No
R41	3	No	No
PA1	4	No	2.1kb, 2.3kb
I13	4	2.5kb	2.0kb, 2.2kb
C233	4	2.5kb	No
RH5/T	4	2.7kb	No
GM1	5	No	No
RH6/T	5	No	No
R470	5	No	No
FIX1	6	No	No
S3BS1	8	No	2.1kb, 2.3kb

Table 4.1. Distribution of extrachromosomal DNA and double-stranded RNA in *Rhizoctonia solani* All dsRNA sizes are estimated from their electrophoretic mobility in comparison to rotavirus dsRNA. DNA sizes are obtained by electrophoretic mobility relative to λ DNA cut with *Hind III*.

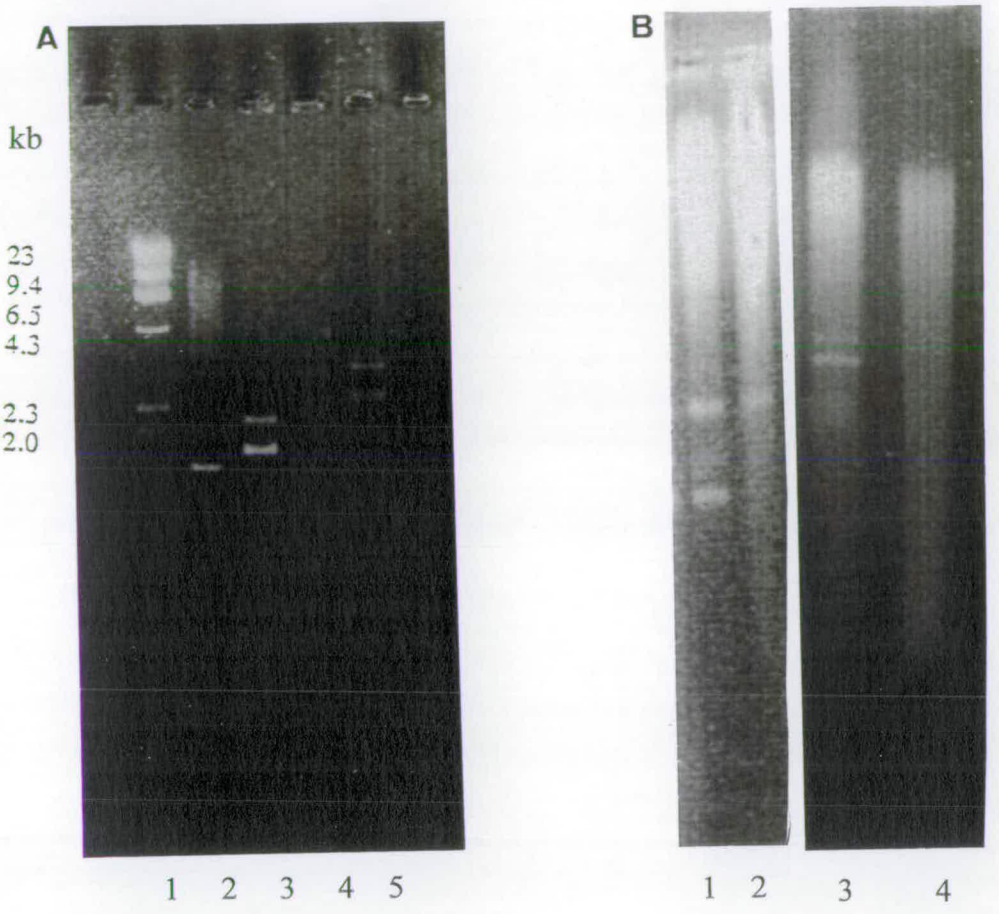


Figure 4.1. A. Agarose gel showing banding patterns of dsRNA from different sources. Double-stranded RNA was isolated using the cellulose CF-11 column of Morris & Dodds (1979) and electrophoresed on 1% agarose (w/v). Lane 1 shows λ DNA cut with *Hind III,* sizes are shown on the left of the photograph. Lanes 2 and 3 show dsRNAs of *Rhizoctonia solani* anastomosis group 4 strains I13 and PA1 dsRNA respectively. Lane 4 shows *Ophiostoma ulmi* log 1/3 d^2 dsRNA and lane 5 shows Rotavirus dsRNA. **B.** Agarose gel (1% w/v) showing total nucleic acid extracted from *R. solani* AG4 strains I13 and PA1 by phenol:chloroform extraction and ethanol precipitation as described in Chapter 2. The photograph shows I13 total nucleic acid (lane 1), I13 after successive hyphal tip subculturing resulting in loss of dsRNA (lane 2), PA1 total nucleic acids (Lane 3) and PA1 after successive hyphal tip subculturing resulting in loss of dsRNA (Lane 4).

column and electrophoresed on 1% agarose. *R. solani* AG4 strains I13 and PA1 are both shown to contain 2 segments of dsRNA of sizes 2.0 and 2.2kb, and 2.1 and 2.3kb respectively. Also included in the figure are *O. ulmi* dsRNA, which is known to confer a 'disease' phenotype on the fungus, and Rotavirus dsRNA, from bovine faeces, both of which were used as positive controls for the cellulose CF-11 column.

4.2.2. Isolation of extrachromosomal DNA

In order to test the efficiency of recovery of dsRNA from the CF-11 column, an aliquot of each sample was removed before addition of cellulose to the supernatant, and electrophoresed on a 1% agarose gel. Examination of total nucleic acid revealed that a few strains contained other extrachromosomal elements not recovered from the dsRNA fraction of the column. Nuclease digestion and lack of alkali hydrolysis showed these elements to be DNA. In the case of AG4 strain I13, the DNA was present in conjunction with dsRNA, but some strains such as AG2-2 C127 were shown to contain DNA only. Further work carried out on the DNA element of AG4 I13 will be described in Chapter 6.

4.2.3. Curing of *Rhizoctonia solani* of double-stranded RNA.

As the dsRNA in *R. solani* is known to be located in older regions of hyphae, and is not present in tip cells (Castano & Butler, 1978a) a simple and effective way of curing cells of dsRNA is by hyphal tip subculture. Two anastomosis group 4 strains, I13 and PA1 were used for curing as both contained two segments of dsRNA but I13 also contained an extrachromosomal DNA element lacking from strain PA1.

Curing of dsRNA was carried out over a 30 day period where sequential hyphal tip cells were removed from the margin of agar colonies and transferred to fresh PDA plates. Subculturing was carried out at 3 day intervals thereby allowing regrowth of the hyphal tip cell before the next subculture. A total of ten consecutive subcultures were carried out on each strain and isolation of total nucleic acid was used to test for the presence of dsRNA (Figure 4.1b). In the strains subjected to repeated hyphal tip subculture there were no detectable levels of dsRNA following this protocol. These strains are referred to as 'AG4 I13 cured' and 'AG4 PA1 cured'.

Double-stranded RNA was consistently isolated from the parent strains and the cured strains were tested repeatedly for the presence of dsRNA over a two year period of normal sub-culturing and were always found to be free of dsRNA.

4.2.4. Investigation of phenotypic effects associated with curing of double-stranded RNA

Pigmentation

Upon curing of dsRNA from the strains AG4 PA1 and AG4 I13, initial observations suggested noticeable effects on pigmentation and sclerotium formation. These effects were restricted to strain PA1 in which curing was associated with increased pigmentation when the mycelia were grown on PDA (Figure 4.2a). The differences in pigmentation were only noticeable in cultures over 5 days old, as initially the mycelia were hyaline in both cured and uncured colonies. Only when the Petri dish was completely covered did the pigmentation of the PA1 hyphae occur. The dsRNA-containing strain remained unpigmented. These pigmentation differences were only observable on PDA, as when cultures were grown on malt extract agar or water agar, no pigmentation was observed in either colony. Therefore the effect was medium dependent. In addition to the reduced pigmentation there was also a noticeable reduction in sclerotium production by the cured strain on both PDA and cellulose. This is illustrated in Figure 4.2b for 21 day old colonies growing from marginally placed inocula on stacks of filter paper moistened with minimal nutrient solution.

With strain AG4 I13 when examined under the same conditions as those above the phenotypes of cured and non-cured forms were identical. There was no difference in pigmentation or sclerotium production.

Linear extension on solid medium

In order to examine the effect of dsRNA on hyphal extension linear extension rates of cured and non-cured strains of AG4 I13 and AG4 PA1 were recorded on PDA, water agar and water agar containing 3% pectin. Water agar containing 3% pectin was included as pectin is an important structural component of

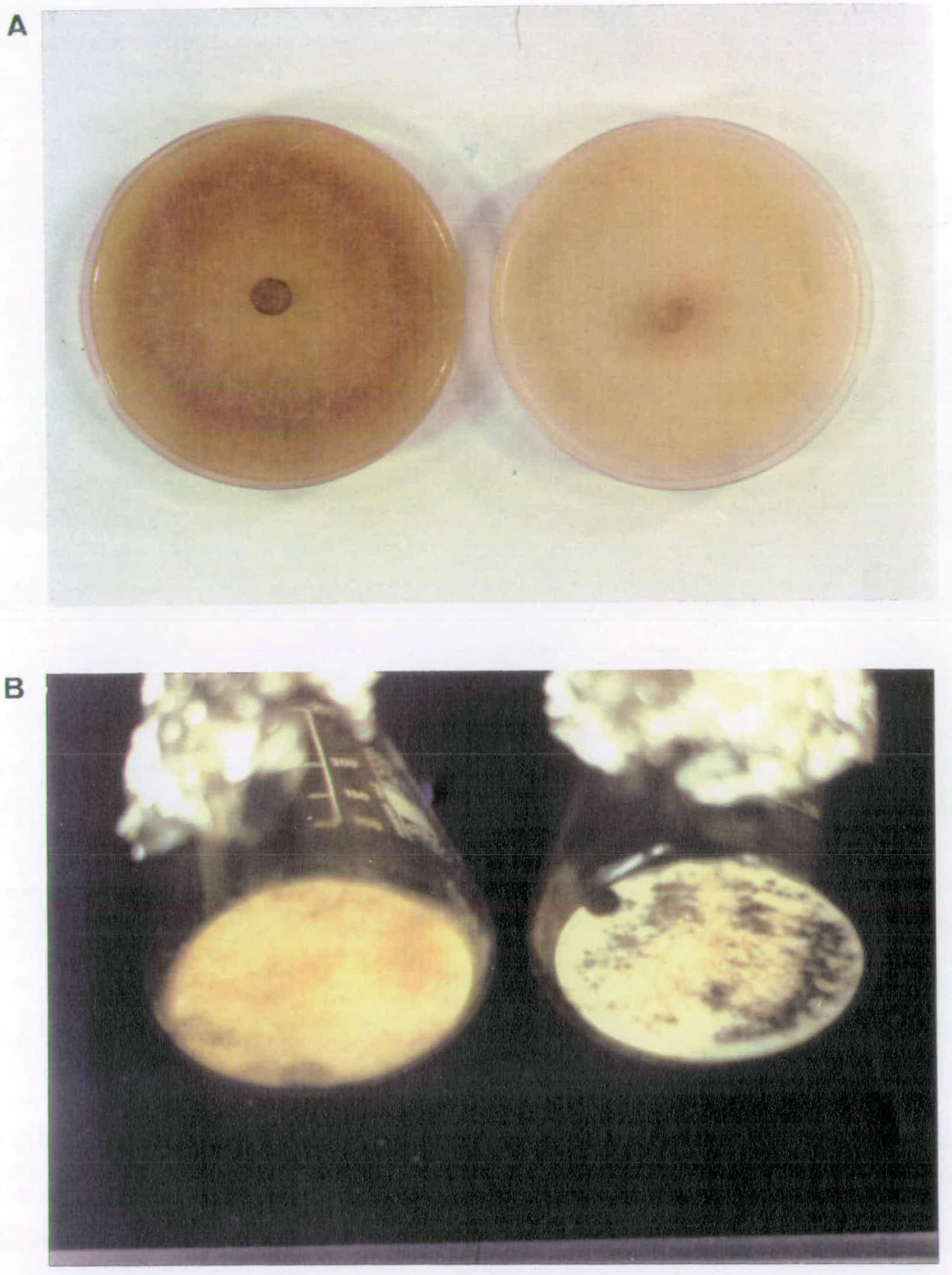


Figure 4.2. Phenotypic differences between *Rhizoctonia solani* AG4 PA1 and its dsRNA-cured derivative. **A.** Petri plates of AG4 PA1 (right) and its dsRNA cured derivative (left) after inoculation of a mycelial covered agar block onto PDA and incubation for 10 days at 25°C. The darker pigmentation of AG4 PA1 cured can be seen. **B.** Strains of AG4 PA1 (right) and AG4 PA1 cured (left) were inoculated into 250ml flasks containing minimal medium and filter paper and incubated at 25°C for 21 days. High levels of sclerotium production of PA1 can be seen in comparison with its dsRNA-cured derivative.

plants and its breakdown is important in pathogenicity. As shown in Table 4.2, the growth rates of dsRNA-cured strains of both I13 anb PA1 were similar to those of the non-cured parents on all types of medium tested. So there was no evidence that presence or absence of dsRNA affected growth rate. However, there were differences, for any one strain, in growth rate across different media. Initially, for example, I13 (cured or non-cured) grew at a similar rate across the three media, but by 48h and especially by 72h, the extension rate of I13 strains had slowed significantly on water agar or water agar plus pectin, relative to the growth rate on PDA. Strain PA1 (cured or non-cured) showed even larger differences in growth rates across the different media and after 24h these differences had already become more pronounced with time. Overall, pectin agar supported little more growth than did water agar, perhaps because the pectin could not be utilised effectively in the absence of supplementary nitrogen or other mineral nutrients. Visual observation showed that colonies on water agar or pectin agar were sparsely branched whereas PDA supported densely branched colonies.

Growth rate of cured and non-cured strains in liquid medium

Changes in mycelial mass upon curing strains of dsRNA were examined by growth in liquid medium. Potato sucrose broth was inoculated with mycelial discs and incubated statically at 25°C for 10 or 25 days. Then the mycelia were collected on filter papers which were washed, dried to constant weight at 80°C and reweighed to obtain biomass yields. Uninoculated control flasks, treated similarly, gave a correction factor which was applied to the data; it represented the difference between the original (recorded) air dry weight and the final oven-dry weight of the filter papers used to collect the mycelia in the experiment.

As can be seen in Table 4.3, the cured form of AG4 PA1 gave a significantly higher biomass after 10 and 25 days than did the non-cured strain but there was no significant difference in biomass between the cured and non-cured forms of AG4 I13.

Strain	Hours	Potato Dextrose Agar	Water agar	Water agar + pectin
I13	24	23 +/- 0.24	22.67 +/- 0.33	23 +/- 0.57
	48	51.6 +/- 0.33	45.67 +/- 0.33	42.67 +/- 0.88
	72	80*	67.67 +/- 0.33	72.67 +/- 0.33
I13 cured	24	23 +/- 0.57	20.6 +/- 0.33	20 +/- 0.57
	48	49.7 +/- 0.88	39 +/- 1.15	36.66 +/- 0.33
	72	80*	62.67 +/- 1.45	53 +/- 1.15
PA1	24	21.66 +/- 0.33	12.33 +/- 0.88	14.33 +/- 1.76
	48	50 +/- 0.12	30.33 +/- 0.88	29 +/- 0.57
	72	80*	49 +/- 0.57	48.66 =/- 1.76
PA1 cured	24	22.66 +/- 0.88	15.66 +/- 0.88	19 +/- 1.15
	48	55 +/- 0.57	25 +/- 0.1	33.33 +/- 2.96
	72	80*	42.33 +/- 0.88	55.33 +/- 0.88

Table 4.2 Linear extension of AG4 strains, I13 and PA1, and their dsRNA-cured derivatives on agar media, recorded as mean colony diameter (mm, mean +/- S.E.M. for 3 replicates) after 24, 48 and 72 h incubation at 25°C. *-maximum.

Time (days)	Strain					
	AG4 PA1		AG4 PA1 cured	AG4 I13		AG4 I13 cured
10	51.4 +/- 0.53	-a-	106.3 +/- 2.20	131.33 +/- 8.07	-nsd-	142.3 +/- 5.61
25	101.03.+/- 6.82	-b-	155.13+/-3.66	192.3 +/- 1.34	-nsd-	209.1 +/- 0.52

Table 4.3. Mycelial dry weights (mg) of dsRNA-cured and non-cured AG4 strains in static cultures of potato-sucrose broth after 10 and 25 days at 25°C. Mean +/- S.E.M. for 4 replicates; values linked by a or b differ significantly from one another ($p<0.05$); other values show no significant differences (nsd).

4.2.5. Plant pathogenicity

Previous reports have correlated the presence of dsRNA in *R. solani* with both increased and decreased virulence (Castano *et al.*, 1978; Finkler *et al.*, 1985). In the present studies, plant pathogenicity tests with cured and non-cured strains were carried out on the following plant hosts: *Daucus carota* var. *sativa* (carrot, Sutton Seeds), *Brassica oeracea* var. *capitata* (cabbage, Sutton Seeds), *Nasturtium officinale* (cress, Sutton Seeds) and *Raphanus sativus* (radish, Sutton Seeds). The method was described in Chapter 2, section 2.2.1. Treatments were incubated at ~22°C using a randomised block design such that pots containing inoculated and uninoculated soil and those containing different host plants were mixed. This meant that any temperature or humidity gradient in the incubation area should not markedly affect the results. At 7-10 days after sowing, seedlings were scored for emergence and seedling health. Pre-emergence damping-off was assessed as failure of seedlings to emerge above ground, and post-emergence damping-off was recorded as seedling that emerged but then collapsed due to *R. solani* infection.

All four strains, cured and non-cured, showed high virulence on all host plants tested (Table 4.4).

4.2.6. Production of extracellular enzymes by cured and non-cured strains

Strains of AG4 PA1 and AG4 I13, with or without dsRNA, were assessed for *in vitro* production of pectic enzymes which are likely to be involved in pathogenicity to plants (Bateman & Miller, 1966), and of cellulase activity, likely to be involved in growth by *R. solani* on cellulosic substrates in soil (Garrett, 1970).

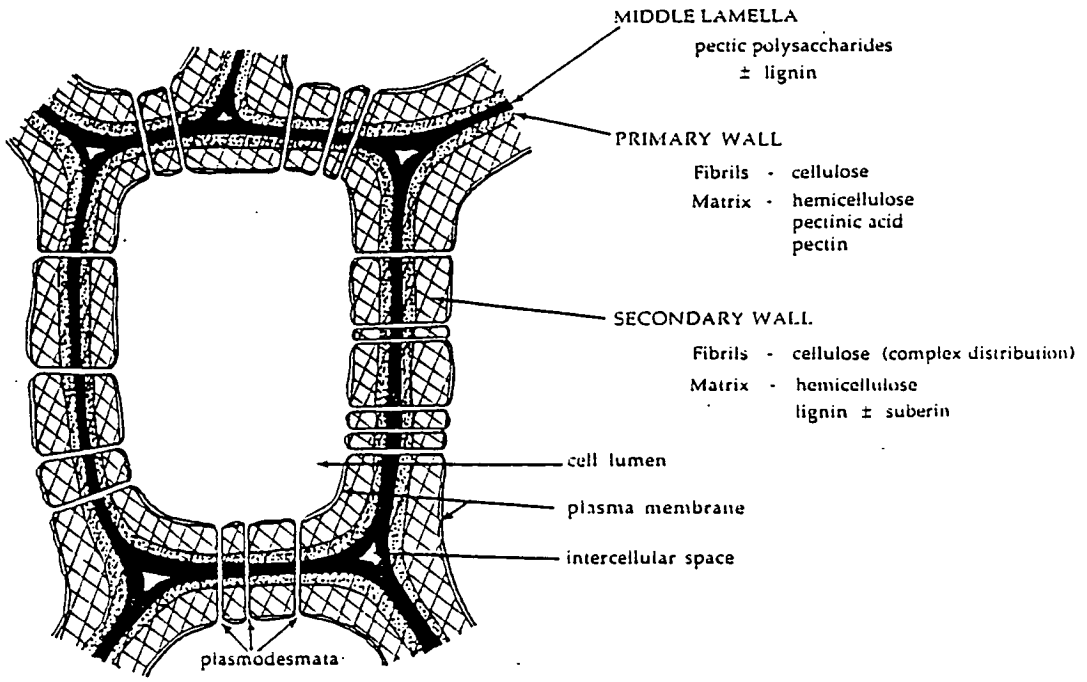
Pectic enzymes

Pectic polymers are chains of predominately α-1,4 linked D galacturonic acid and its methylated derivatives (Bateman & Millar, 1966), important as structural components of the plant cell wall (Figure 4.3). Pectic enzymes are liberated by many plant pathogenic fungi. The battery of pectic enzymes produced differ in substrate preference and reaction mechanism. However, the enzymes all act on the structural components of pectin (Figure 4.3). Due to the structural importance and enzymatic

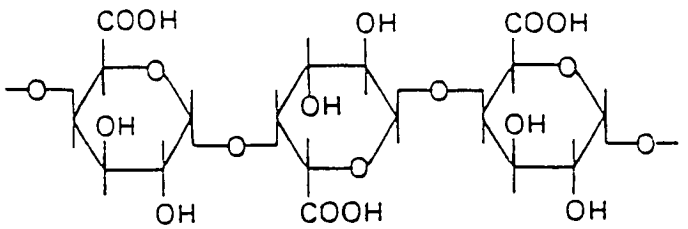
Plant	Control			AG4 PA1			AG4 PA1 cured			AG4 I13			AG4 I13 cured		
	H	Pre	Post	H	Pre	Post	H	Pre	Post	H	Pre	Post	H	Pre	Post
Radish	50	0	0	0	32	14	0	41	3	0	41	4	0	44	6
Cress	49	1	0	0	21	24	0	38	9	0	34	16	0	31	19
Carrot	49	1	0	0	45	5	0	50	0	0	50	0	0	50	0
Cabbage	50	0	0	0	50	0	0	50	0	0	50	0	0	50	0

Table 4.4. Numbers of plant seedlings that were healthy, or showed pre-emergence or post-emergence damping-off 7-10 days after sowing seeds in perlite infested or uninfested with inoculum of different *R. solani* strains. H, healthy; Pre, pre-emergence damping-off; Post, post-emergence damping-off. 50 seeds were used in each treatment.

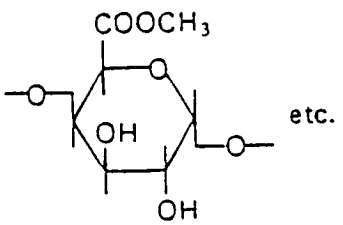
Figure 4.3.



Diagrammatic representation of the plant cell wall (Isaac, 1992)



Pectic acids. Straight chain polymers of galacturonic acid, α 1-4 linked.



Pectinic acids. As above, but with some of the carboxyl groups esterified with methanol.

vulnerability of these polymers, destruction by pectic enzymes can cause plant tissue maceration, cell lysis and also modifications in cell wall structure, allowing other depolymerases to act on their respective substrates. Pectin is found in the middle lamellae of plant cells (Bateman & Millar, 1966).

The production of pectic enzymes is inducible and can be measured very simply by pectic zymograms (Section 2.2.1) This method allows detection of 3 pectic enzymes, polygalacturonase, pectin lyase and pectin estrase, using differential staining in polyacrylamide with ruthenium red, after allowing digestion of the pectin in the polyacrylamide. Cultures of *R. solani* were incubated for 7 days at 25°C in nutrient broth containing pectin, to induce the enzymes, and the culture filtrates were loaded onto a zymogram. Polygalacturonase was detected in all the strains tested, but only the PA1 cured strain produced detectable levels of pectin lyase. Due to poor photographic quality a representative diagram is shown in Figure 4.4. As dsRNA may be affecting the production of pectin lyase a variety of other methods were then used to test the difference in pectic enzyme production by the AG4 PA1 strains with and without dsRNA.

Polygalacturonase and pectin lyase, the two enzymes which were investigated, can be distinguished by their pH optima and substrate specificity. Polygalacturonase has a pH optimum less than 6 and attacks pectate (polygalacturonate), and pectin lyase has a pH optimum of 8.5 and attacks pectin (polymethoxygalacturonate). The enzymes can also act on other pectin substrates but are not efficient on them (Sherwood, 1966). Polygalacturonase is a hydrolase which cleaves the glycosidic bonds of polygalacturonic acid to produce oligogalacturonic acid and galacturonic acid. Pectin lyase is a trans-eliminase which cleaves the α-1,4 glycosidic linkage of pectin to give Δ 4,5, unsaturated bonds (Sherwood, 1966; Marcus *et al.*, 1986).

Blocks of *R. solani* were incubated in the appropriate pectin-containing broth for 5 days at 25°C to allow induction of the pectic enzymes. Controls for the reaction were cultures grown in glucose broth as glucose is known to repress pectin activity (Weinhold & Bowman, 1974). After 5 days, 100µl of culture filtrate was removed and tested for the production of polygalacturonase or pectin lyase by the

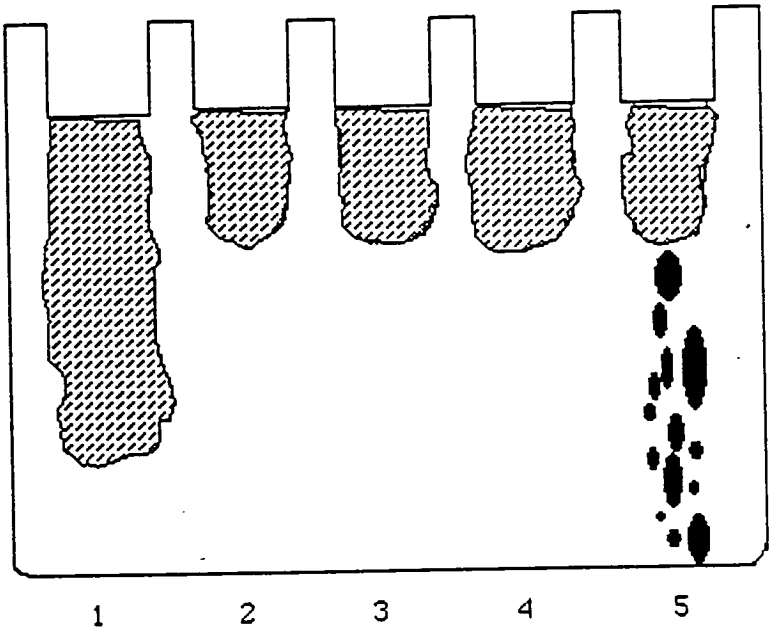


Figure 4.4. Schematic diagram of a pectic zymogram loaded with culture supernatant of *R. solani* grown in pectin containing broth. A 1cm diameter block of mycelium was incubated at 25°C in pectin broth for 7 days. 1ml of supernatant was concentrated to 100µl by TCA precipitation. 50µl of concentrated sample was added to each well. After running, the gel was stained in ruthenium red (0.1%) for 2 hours then destained in water for 2-12 hours. Lanes are as follows: lane 1 polygalacturonase control, lane 2 AG4 I13, lane 3 AG4 I13 cured of dsRNA, lane 4 AG4 PA1, lane 5 AG4 PA1 cured of dsRNA. Upon staining polygalacturonase was observed as a zone of clearing (shown as hatching) and pectin lyase was observed as yellow staining of the gel (shown as black).

thiobarbituric acid assay, whereby unsaturated di- and oligo-saccharides react with thiobarbituric acid to produce red chrymogens with an absorption of 545-550nm. This assay was repeated after incubation of the fungus for 6 to 9 days at 25°C. The control was treated identically, except no fungus was present, only broth. As shown in Table 4.5, both the cured and non-cured strains produced polygalacturonase and pectin lyase, but there was significantly more production of both the enzymes by the cured than non-cured strain after 6 or more days.

As a further test of enzyme production, the above experiment was repeated but the fungus was removed, by filtration, from the enzyme-inducing growth medium after 5, 6, 7, 8, or 9 days. At each of these times the filtrate was incubated with the reaction mixture for 1-5 days to assess enzyme activity. The reason for this is that *R. solani* can metabolise pectin and utilise it as a substrate for growth (Table 4.2). As shown in Tables 4.6 and 4.7, there was again significantly more polygalacturonase and pectin lyase activity in culture broths of the strain which was cured of dsRNA compared with the non-cured strain.

It can be seen from the tables that production of both enzymes by both fungal strains tended to increase with time of incubation of the fungus in the inducing culture broth, but the levels of production by the non-cured strain never reached those by the cured strain. It is also seen that the enzymes were relatively stable because they continued to degrade the substrates (up to what is presumed to be the maximum limit of the assay in the case of the cured strain) during the 5 days of incubation in the enzyme reaction mixtures.

Cellulolytic activity

Rhizoctonia solani is known to utilise cellulose as a growth substrate by producing cellulolytic enzymes (Figure 4.5). Cellulose is a polymer of β (1-4) linked glucan, and is a component of the plant cell wall, so the production of inducible cellulase enzymes may be important in pathogenicity. Levels of cellulolysis were measured using the method of Garrett (1966), as described in Chapter 2. Flasks containing inoculated filter paper wads were incubated for 28 days at 25°C, and the weight loss of the filter paper was measured as an indication of cellulolytic activity.

Incubation Time of Culture (days)	Polygalacturonase (pH 5)[a]			Pectin Lyase (pH 8.3)[b]		
	PA1	PA1 cured	Significance of difference by T-test	PA1	PA1 cured	Significance of difference by T-test
5	0	0	nsd	2.11 +/- 0.19	3.24 +/- 0.88	nsd
6	0	2.05 +/- 0.15	0.05	3.01 +/- 0.07	6.25 +/- 0.23	0.05
7	0.795 +/- 0.04	3.39 +/- 0.56	nsd	3.88 +/- 0.13	8.07 +/- 0.65	0.1
8	1 +/- 0.06	4.68 +/- 0.035	0.02	5.04 +/- 0.19	9.29 +/- 0.83	0.1
9	1.57 +/- 0.05	6 +/- 0.2	0.05	5.65 +/- 0.53	12.55 +/- 1.31	0.1

Table 4.5. Degradation of pectin and polygalacturonate by AG4 PA1 and a derivative cured of dsRNA. Hyphae were incubated in pectin or polypectate containing broth for the appropriate time period then the culture supernatant tested for degradation of substrate by the thiobarbituric acid assay. Data are shown as units of enzyme activity identified as giving a change in absorbance of 0.01 under the conditions of the assay; mean +/- S.E.M. for 3 replicates. a. Polygalacturonase is measured by the degradation of pectin at pH 5. b. Pectin lyase is measured by the degradation of sodium polypectate at pH 8.5. nsd-no significant difference. Values represent readings following substitution of control readings where samples were boiled for 10 min before assay. Control values were always less then 0.03 +/- 0.01.

Culture Incubation Time(days) [a]	Buffer Incubation Time (days)[b]	Enzyme Activity[c] AG4 PA1	AG4 PA1 cured	Significance (of difference between cured and non-cured strains)
5	1	3.3 +/- 0.5	5.50 +/- 1.05	nsd
	2	4.38 +/- 0.28	11 +/- 2.6	nsd
	3	4.92 +/- 0.36	18.9 +/- 1.41	0.1
	4	6.48 +/- 0.74	22.45 +/- 1.41	0.05
	5	6.32 +/- 0.3	28.1 +/- 3.6	nsd
6	1	1.97+/- 0.34	9.7 +/- 1.35	0.1
	2	4.9 +/- 0.7	18.15 +/- 3.55	nsd
	3	7.63 +/- 0.5	20.85 +/- 2.95	nsd
	4	8.01 +/- 0.98	21.5 +/- 9.9	nsd
	5	8.35 +/- 0.25	26.75 +/- 7.05	nsd
7	1	5.25 +/- 1.41	8.7 +/- 1.55	nsd
	2	6.83 +/- 0.96	11.1 +/- 4.5	nsd
	3	8.4 +/- 0.5	20.4 +/- 1	0.1
	4	9.27 +/- 0.32	24.75 +/- 4.95	nsd
	5	11.7 +/- 1.9	27.35 +/- 2.55	nsd
8	1	6.35 +/- 0.55	10.1 +/- 0.47	nsd
	2	6.85 +/- 0.15	20.7 +/- 0.9	0.05
	3	8.32 +/- 0.45	21.2 +/- 4.6	nsd
	4	11.04 +/- 0.94	25.7 +/- 5.9	nsd
	5	10.27+/- 1.2	40.7 +/- 8.9	nsd
9	1	6.95 +/- 0.76	12.9 +/- 0.75	0.01
	2	7.24 +/- 0.42	24.1 +/- 2.3	0.1
	3	11.37 +/- 0.39	27.2 +/- 2.3	0.1
	4	8.42 +/- 1.47	35.9 +/- 4.3	0.1
	5	12.25 +/- 1.65	35.1 +/- 3.4	nsd

Table 4.6. Pectin lyase activity in culture broths of cured and non-cured strains of AG4 PA1. Degradation of substrate was measured by incubation of the fungus in pectin containing broth for induction of enzymes followed by removal of fungus and reincubation of culture supernatant in pectin lyase buffer (pH 8.5). After the required time period substrate degradation was measured by the thiobarbituric acid assay. a -incubation time of substrate and fungus, for induction of enzyme. b - incubation time of substrate and enzyme only. Removal of the fungus is followed by incubation in fresh substrate. c - activity is given in enzyme units. One unit causes a change in absorbence of 0.01 at 549nm; data are mean +/- S.E.M for 3 replicate samples taken from different culture broths. nsd-no significant difference. Values represent readings following substitution of control readings where samples were boiled for 10 min before assay. Control values were always less then 0.03 +/- 0.01.

Incubation Time (days) [a]	Incubation Time (days) [b]	Enzyme Units[c] AG4 PA1	AG4 PA1 cured	Significance (of difference between cured and non-cured strains)
5	1	0	0	nsd
	2	0. 21+/- 0.1	0.7 +/- 0.36	nsd
	3	0.5+/- 0.15	1.23 +/- 0.37	nsd
	4	0.76 +/- 0.25	1.56 +/- 0.4	nsd
	5	0.82 +/- 0.29	2.8 +/- 0.51	nsd
6	1	0.54 +/- 0.03	3.2 +/- 0.93	nsd
	2	0.92 +/- 0.24	6.2 +/- 0.6	0.05
	3	1.04 +/- 0.32	9.15 +/- 1.25	nsd
	4	1.96 +/- 0.43	12.35 +/- 0.05	0.01
	5	2.03 +/- 0.48	20.2 +/- 0.2	nsd
7	1	1.57 +/- 0.036	2.4 +/- 0.4	nsd
	2	1.93 +/- 0.65	4.3 +/- 0.95	0.1
	3	2.23 +/- 0.33	8.26 +/- 1.33	nsd
	4	3.52 +/- 0.52	12.25 +/- 1.65	0.1
	5	4.02 +/- 0.44	15.1 +/- 1.7	nsd
8	1	1.04 +/- 0.026	8.6 +/- 0.75	0.1
	2	1.66 +/- 0.43	12.2 +/- 0.6	0.01
	3	2.4 +/- 0.7	15.1 +/- 0.72	0.01
	4	4.13 +/- 0.83	18 +/- 0.56	0.02
	5	5.5 +/- 0.47	19.2 +/- 0.56	0.05
9	1	0.46 +/- 0.23	9.3 +/- 0.31	0.01
	2	1.36 +/- 0.29	15.7 +/- 1.8	0.1
	3	2.3 +/- 0.45	17.5 +/- 1.73	0.05
	4	3.9 +/- 0.69	18 +/- 1.58	0.02
	5	5.7 +/- 1.04	19.0 +/- 1.04	0.1

Table 4.7. Polygalacturonase activity in culture broths of cured and non-cured strains of AG4 PA1. Degradation of substrate was measured by incubation of the fungus in pectin containing broth for induction of enzymes followed by removal of fungus and reincubation of culture supernatant in polygalacturonase buffer (pH 5). After the required time period substrate degradation was measured by the thiobarbituric acid assay. a - Incubation time of substrate and fungus, for induction of enzyme. b - Incubation time of substrate and enzyme only. Removal of the fungus is followed by incubation in fresh substrate. c- activity is given in enzyme units. 1 unit causes a change in absorbance of 0.01 at 549nm; data are mean +/- S.E.M. for 3 replicate samples taken from different pectin culture broths. nsd-no significant difference. Values represent readings following subtraction of control readings where samples were boiled for 10 min before assay. Control values were always less than 0.03 +/- 0.01.

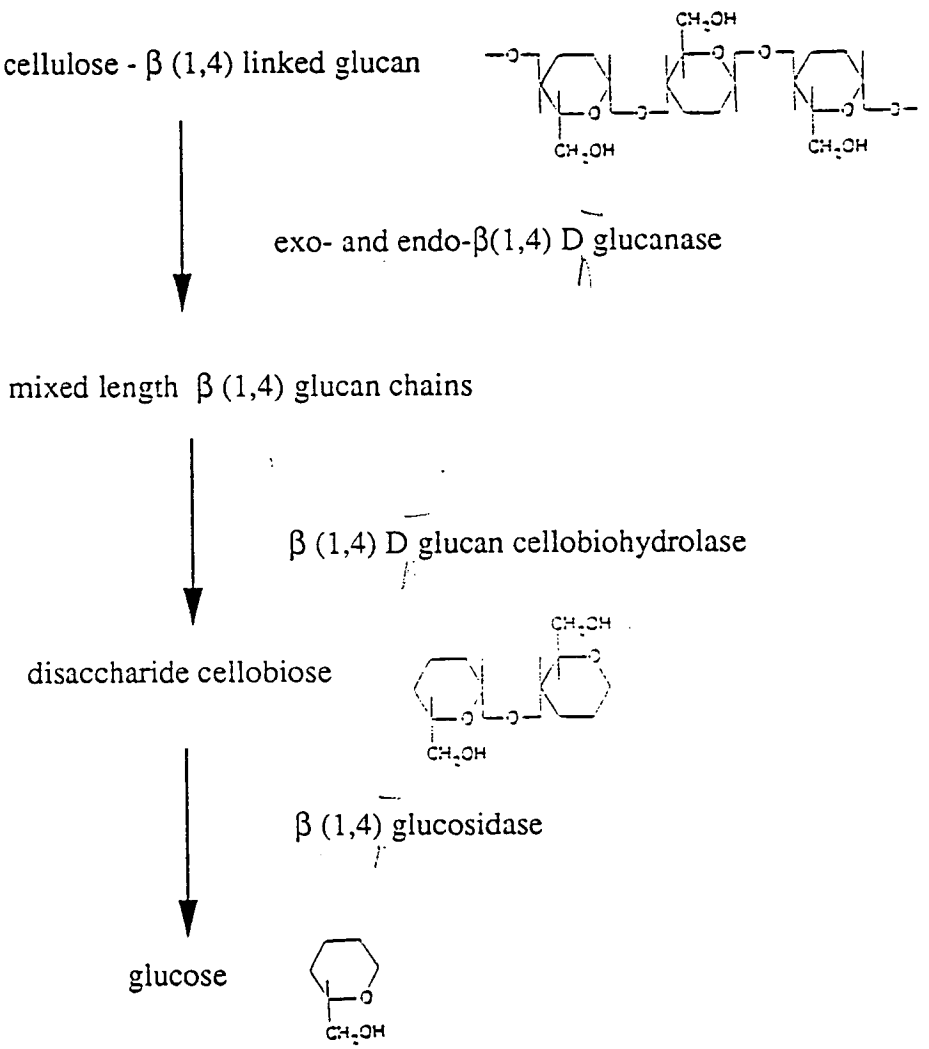


Figure 4.5. Cellulose degradation by fungi.

In this case three cured strains of PA1 were used, each originating from seperate hyphal tip subcultures Table 4.8 shows that the cured strains of both PA1 and I13 had significantly more cellulolytic activity than did the non-cured parents, therefore the two strains appear to metabolise cellulose at different rates (Figure 4.2b).

4.2.7. Anastomosis

Double-stranded RNA-cured and non-cured strains of PA1 and I13 were paired in PDA plates as described in Chapter 2. In all cases the cured strains were found to be vegetatively incompatible with the parents (non-cured) because vegetative cell death followed anastomosis in the fusion zones between colonies (Figure 4.6) At 7 days, after the colonies had formed, a 1cm agar block was removed from the colony of the cured strain at distances of 0.5cm, 2cm and 5cm behind its colony margin. These inoculum blocks were subcultured onto fresh PDA and the resulting colonies were inoculated into potato sucrose broth. After growth, the mycelia were extracted to test for the presence of dsRNA. None of the cured strains was found to contain dsRNA, suggesting lack of successful transfer of dsRNA.

It seems that the incompatibility reaction successfully prevented the transfer of dsRNA into the cured strains from the parental strains. Unambiguous confirmation of this would require repeating the above studies with cured and uncured strains which retained compatibility. This feature will be discussed in more detail in Chapter 5.

4.3. DISCUSSION

The results presented in this chapter show that dsRNA was present in only three of the fifteen tested strains of *R. solani*. It was found in 2 of the 4 strains of AG4 and one strain of AG8. Castano and co-workers (1978) found dsRNA in only 2 of 12 strains tested, from 4 anastomosis groups and Kousik and colleagues (1994) found dsRNA in 12 of 30 *R. solani* strains. In contrast, Zanzinger and colleagues (1984) found dsRNA to be ubiquitous, with 49 out of 50 tested strains of AG1 to 5

Strain	Weight loss of filter paper (mg)*
PA1 with ds RNA	310.9 +/- 26.57
PA1 cured strain 1	1114.2+/- 161.22[a]
PA1 cured strain 2	917.6 +/- 52.4[a]
PA1 cured strain 3	994.3 +/- 25.35[a]
I13 with dsRNA	382.1 +/- 20.02
I13 cured	1153.1 +/- 166.5[b]

Table 4.8. Cellulolysis of cured and non-cured strains of *R. solani* AG4. *weight loss = mean +/- S.E.M for 4 replicates after 4 weeks incubation at 25°C. PA1cured strains 1, 2 and 3 are from separate hyphal tip subcultures. a - significantly different from non cured strain PA1; $P< 0.01$. b - significantly different from non cured strain I13; $P< 0.01$.

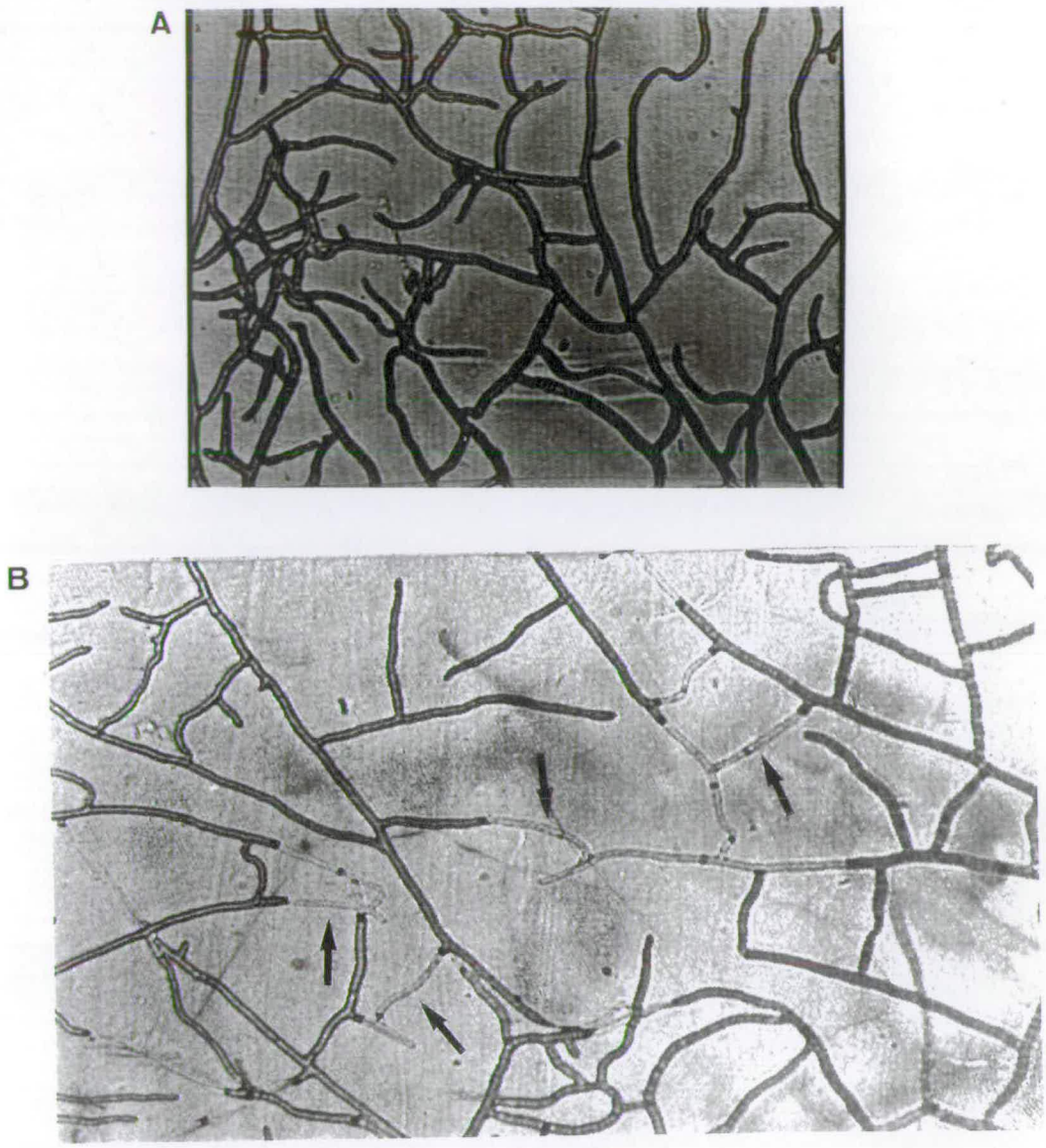


Figure 4.6. Anastomosis and vegetative incompatibility in *Rhizoctonia solani* strain AG4 PA1. Two mycelial covered PDA blocks were placed 2cm apart on a water agar covered glass slide and incubated at 25°C for 36h. Hyphae were observed with a microscope attached to a video camera. Photographs were produced from video copy processor prints. **A.** The interaction between two colonies of AG4 PA1 results in complete anastomosis allowing the formation of a fused cell. **B.** The interaction between PA1 and a hyphal tip derivative cured of dsRNA results in anastomosis followed by vegetative incompatibility and localised cell death indicated by hyphal transparency (arrows).

containing dsRNA. They found at least 2 segments of dsRNA in each of the 49 strains and these segments ranged in size from 0.6kb to over 8kb; several strains contained up to 8 segments. In the present work only 2 segments were obtained from each of the three positive strains. Double-stranded RNA has been found in several strains of other plant pathogenic fungi including *Gaeumannomyces graminis* var *tritici, G. graminis* var *avenae* (E.M. Turner) Dennis (Rawlinson *et al.,* 1973) and *Agaricus bisporus* (Lang.) Imbach (Tavantzis & Smith, 1979) even when the cultures were apparently healthy.

One of the main differences between the study of Zanzinger *et al.* (1984) and the present work is that all the strains used by Zanzinger *et al.* (1984) were obtained directly from the field, categorised into anastomosis groups and then tested for dsRNA, whereas in the present study the strains were mainly from culture collections. As dsRNA is known to be lost on hyphal tip subculture (Castano & Butler 1978) there is a chance that it had been lost during continued subculture of some of the strains used here. However, Kousik and co-workers (1994) showed stable maintainance of dsRNA upon serial subculturing for periods of over one year. Indeed dsRNA was isolated in the present work from strains that had been routinely subcultured on PDA in the laboratory for over two years.

It is important to identify the extrachromosomal element as dsRNA. This was done here by nuclease treatments; digestion by ribonuclease and resistance to deoxyribonuclease, by specific binding to a cellulose CF-11 column in the presence of buffer containing 15% ethanol and by alkali hydrolysis of the fragments at low alkali concentrations (Castano *et al.,* 1978; Zanzinger *et al.,* 1984; Finkler *et al.,* 1985).

The dsRNA extracted in the present study had an identical banding pattern on repeated extractions over a three year period. These results correlate with those of Kousik and colleagues (1994) who consistently obtained dsRNA elements of the same size from extractions over a one year period. This result is in direct contrast to that of Finkler and workers (1985) who found that over a period of one year repeated extractions resulted in different banding patterns where smaller fragments of dsRNA appeared with culturing. Such variations are also found in *Cryphonectria parasitica,*

where small dsRNAs appear during repeated subculturing and are presumed to be the result of internal deletions in a larger dsRNA (Shapira *et al.*, 1991a; Rae *et al.*, 1989). This has also been found in *Gaeumannomyces graminis* (Buck *et al.*, 1981). The large dsRNA of *C. parisitaca* is approximately 12kb and that of *G. graminis* is approximately 6kb. When smaller dsRNAs have been isolated from other species, it is always in the presence of other larger dsRNA segments (Shapira *et al.*, 1991a; Stanway & Buck, 1984). It is possible that the small (1.8-2.2kb) dsRNAs of *R. solani* represent the minimum size that can replicate autonomously, so they remain stable in size during subculturing.

The presence of dsRNA has been associated with phenotypic changes in many plant pathogenic fungi. One of the most striking cases concerns the hypovirulence phenotype of *C. parasitica.* In this case strains of the fungus containing the large (12kb) dsRNA appeared debilitated, with altered colony morphology, slow growth and reduced conidiation and pigmentation (Anagnostakis, 1984a), and with much reduced virulence which has been attributed to specific genes encoded by dsRNA (Shapira *et al.*, 1991a). The dsRNA of other fungi such as *Helminthosporium victoriae* and *Ustilago maydis* affect production of toxins which act against other strains of the same species. *H. victoriae* produces the toxin victorin which is active against all strains which express the victoria-type resistance to the rust fungi. The presence of dsRNA in this fungus was associated with reduced levels of toxin production in addition to slow growth rate and lysis of aerial mycelia (Lindberg, 1960). In *U. maydis* dsRNA elements have been shown to encode toxins which act to inhibit other strains of the fungus (Koltin, 1988). In *S. cerevisiae* dsRNA acts in a similar manner, where killer strains of *S. cerevisiae* kill other *S. cerevisiae* strains (Bostain *et al.*, 1984). Double-stranded RNA in *Ophiostoma ulmi* results in slow-growing 'diseased' isolates which have reduced viability of conidia and impairment of sexual reproduction (Brasier, 1983).

Phenotypic effects of the presence of dsRNA in *R. solani* have been investigated mainly by examination of fitness of the strains and pathogenicity. When dsRNA was first detected in *R. solani* it was associated with a condition called Rhizoctonia decline ie. failure of strains to survive on agar or in soil (Castano &

Butler, 1978b). Initially it was not realised dsRNA was involved in this disease. The disease reported was a debilitation of an anastomosis group 1 strain which resulted from routine subculture. The debilitated strain was phenotypically different from healthy strains. It had decreased pigmentation and a reduction in sclerotium formation. These features resemble those of the PA1 strain, cured of dsRNA, as described in this chapter.

In the present study, the attempted curing of dsRNA in AG4 strain PA1 often led to failure of hyphal tip subcultures to regenerate. This might have been because the hyphal tip subcultures were taken too close to the edge of the colony resulting in damage of tip cells. Alternatively, it may be that many of the tip subcultures could have been unable to regenerate due to loss of fitness. One of the original cured strains did not survive more than three subcultures, but as the other cured strains were easily maintained in culture it may be assumed that debilitation in this case was not due to loss of dsRNA.

Further characterisation of the 'disease' symptoms in the strain isolated by Castano & Butler (1978a) included a 10-fold decrease in linear extension rate and decreased virulence. In the present study, the linear extension rates of AG4 strains I13 and PA1 on agar showed no difference after curing of dsRNA, so the findings differ from those of Castano & Butler (1978a) in that dsRNA in I13 and PA1 evidently is not associated with a decline phenomenon. The findings also differ from those of Zanzinger *et al* (1984) in that the only strain isolated by these workers which was devoid of dsRNA had an increased growth rate but no other phenotypic differences. As the phenotype of increased pigmentation and reduced sclerotia formation found in PA1 was not repeated with strain I13 it is impossible to say whether the phenotype is definitely attributable to removal of specific dsRNA elements (as I13 and PA1 have segments with different electrophoretic mobility) or due to a secondary factor removed upon hyphal tip subculturing. Unfortunately, attempts to transmit dsRNA between parent strains and cured strains were unsuccesful owing to the lack of compatibility between strains, so it was not possible to correlate the presence of dsRNA and phenotypic changes more precisely. However, growth of cured and dsRNA containing strains in liquid medium revealed

that the mycelial mass produced by the cured strain of PA1 was significantly higher than that for the parent (dsRNA containing) strain (Table 4.3) even though there was no effect on linear extension rate on agar. This is in contrast to dsRNA containing strains of *Phytophthora infestans* which were shown to have mycelial weights significantly higher than those without dsRNA (Tooley *et al.*, 1989).

As a result of the change in phenotype on curing strains PA1 of dsRNA, further studies were carried out on pathogenicity, and the production of enzymes important for invasion of the fungus. As mentioned earlier, the association of dsRNA with reduction of pathogenicity is of extreme interest due to the use of hypovirulent strains in biological control of chestnut blight caused by *C. parasitica* in Europe (Grente, 1965) and elucidation of the role of specific dsRNA segments in affecting virulence of *C. parisitica* in the USA (Choi & Nuss, 1992a,b). This study indicated the presence of an open reading frame of dsRNA which encodes proteins responsible for hypovirulence, and a second open reading frame which encodes a protein for hypovirulence-associated traits such as slow growth and reduced conidiation. Transformation of *C. parasitica* with a plasmid containing a cDNA copy of the open reading frame for hypovirulence resulted in integration of the cDNA into the fungal chromosome and transcription of the fragment to form a dsRNA virus which was packaged in vesicles and could be transferred, via anastomosis, to similar strains reducing their virulence. The integrated form should be transferred during sexual reproduction (Chen *et al.*, 1993) thereby ensuring its stable inheritance in recombinant environmentally fit hypovirulent strains which it is hoped will provide a means of control for chestnut blight in the USA.

As dsRNA may be important in pathogenicity work was carried out on the role of dsRNA in pathogenicity of *R. solani*. Plant pathogenicity tests on four plant hosts showed no significant difference in the virulence of either I13, a known virulent strain (Finkler *et al.*, 1985), or PA1, whose virulence status was unknown, and their cured derivatives. All four strains were highly pathogenic to all four plant hosts tested (Table 4.4). These results correlate with those of Zanzinger and colleagues (1984) who state that the presence of dsRNA is not related to pathogenicity as dsRNA was found in 98% of strains which ranged from avirulent to

highly pathogenic. The work of Kousik and co-workers (1994) also agrees with this, stating that pathogenicity is independent of dsRNA in *R. solani*. Lack of effect of dsRNA on pathogenicity has also been observed in other fungi including *P. infestans* (Tooley *et al.*, 1989) and *Diaporthe phaseolum* (Cooke & Ell.) Sacc. (Lee *et al.*, 1990). In both these reports pathogenic and non-pathogenic strains were found which were associated with dsRNA.

The latter such work conflicts with that of Finkler and colleagues (1985) and also with that of Castano *et al.* (1978) and Zanzinger *et al.* (1984), but for different reasons. Finkler *et al.* (1985) found that 32 isolates from107 tested, from Israel were, non-pathogenic on eleven plant hosts tested, and all these non-pathogenic strains lacked dsRNA. In contrast, the pathogenic strains contained dsRNA so dsRNA was directly correlated with virulence. Further studies on four virulent and four non-virulent AG4 strains showed that hyphal tip subculturing of the virulent strains resulted in hypovirulence in 1% of cases with corresponding loss of dsRNA, but virulent strains were never obtained from hypovirulent ones. One of the strains used in the present study, I13, was supplied by A. Finkler and was one of the virulent strains used in the aforementioned paper. The sizes and number of dsRNA segments isolated in the present study were the same as reported by Finkler (1985). Hyphal tip subculture resulted in removal of dsRNA but the cells remained virulent (Table 4.4), suggesting that dsRNA was not directly related to the pathogenicity of *R. solani*. This is in agreement with the work of Zanzinger (1985) and Kousik and colleagues (1994).

Other work on pathogenicity of *R. solani* suggests that a natural population of non-pathogenic strains are present in the environment and that they are as fit as the pathogens. Studies by Ichielivich-Auster *et al* (1985) indicated that prior inoculation of seedlings with these naturally non-pathogenic strains caused a decrease in disease (79% & 94%) when the plants were subsequently inoculated with pathogenic strains. This protection appeared to be limited to the genus *Rhizoctonia* (there was also significant protection with *R. zeae*), because there was no protection against *Fusarium oxysporum* or *Pythium aphanadermatum* (Edson) Fitzpatrick on tomatoes or cucumbers, respectively. As there was protection with *R. zeae,* it suggests that

cytoplasmic compatibility is not needed for protection, so the avirulent strain may be acting to induce host defence mechanisms or merely by competition for resources. Other workers suggested that protection of this type in other pathosystems was due to the elicitation of phytoalexin production by the plant (Cardoso & Echandi, 1987) or competition for leaf exudates between pathogenic and non-pathogenic strains of *R. solani* (Burpee & Goulty, 1984). The mechanism for seedling protection by *R. solani* was investigated by Sneh and colleagues (1989) and it was shown that protection of the seedlings did not involve the production of inhibitory substances by seedlings. However, a 3 to 4-fold increase in calcium ions in the cortex of the seedlings was recorded which may help to reduce their degradation by pectolytic enzymes of plant pathogens (Bateman, 1963; McGuire & Kelman, 1986). It had previously been reported that as Ca^{2+} content increased with the age of the plant so did resistance to *R. solani* (Bateman & Lumsden, 1965). Although the increase in Ca^{2+} may have an effect, the authors concluded that this was not the main mechanism of protection, as protection was lost on removal of the fungus. They concluded that the presence of the hyphae of the hypovirulent isolate provides a barrier which blocks recognition of the plant by virulent isolates (Sneh *et al.*, 1989) and that the reason it did not protect from other strains such as *Pythium* spp. and *Fusarium* spp. was that they have different recognition sites for infection to those already occupied.

A major role in symptom development and pathogenicity of *R. solani* is played by pectolytic enzymes (Bateman, 1963; Bateman & Lumsden, 1965; Van Etten *et al.*, 1967), without which the fungus would not be able to invade efficiently. In fact, pectic enzymes are now implicated as a feature of many host-pathogen interactions, and their involvement in the degradation of the constituents of host cell walls is very important (Bateman & Miller, 1966). Pectic enzymes have been shown to be involved in the primary infection of plants by removal of ruthenium red positive substances, presumably pectic materials, from plant cell walls, at approximately 18 hours after infection of cotton, which is then followed by damage to cellulose, another important cell wall component (Weinhold & Motta, 1973). As loss of pectic substances occurred before penetration of the fungus the enzymes were thought to be important in cell wall breakdown to allow ease of infection, and both

polygalacturonase and cellulase activities have been detected in lesions caused by *R. solani* at all stages of infection. Much work has been carried out on pectic enzymes in *R. solani* and their importance in pathogenicity, although as pectic enzymes are ubiquitous in nature the presence of these enzymes does not necessarily indicate pathogenicity (Bateman & Miller, 1966).

In the present study, although dsRNA did not affect pathogenicity of strains on the plant hosts tested, it was of interest to examine whether there were any change in cell wall digesting enzymes upon curing of dsRNA. Initial studies indicated a significant increase in both polygalacturonase and pectin lyase produced by the cured strain of isolate PA1 compared with the original PA1 strain, but no equivalent difference was found for strain I13.

The culture medium and length of culture time has been shown to greatly influence the production of pectic enzyme (Sherwood, 1966) which was why further experiments here included removal of the fungus from the culture filtrate and the addition of enzyme-containing culture supernatant to fresh substrate at the appropriate pH. The importance of substrate and pH in specific enzyme activity has also been shown for *Fusarium oxysporum* f. sp. *lycopersici* (Sacc.) Snyder & Hansen, *Fusarium solani* f. sp. *phaseoli* Kendrick & Snider, *Colletotrichum trifoli* and *Botrytis cinerea* Pers. (Sherwood, 1966; Bateman, 1966; Hancock, 1966) all of which produced pectic enzymes in a similar manner to *R. solani. Sclerotium rolfsii* Sacc. and *Rhizopus stolonifer* (Ehrenb.) Lind have been shown to lower the pH of the growth medium to such an extent that only polygalacturonase is produced (Sherwood, 1966). Polygalacturonase is thought to be the most important enzyme initially in infection, so lowering of the pH of plant tissues by fungi may be a very valuble aid to infection. In the current study incubation of fungus in pectin broth for up to 9 days did not result in a drop in pectin lyase levels suggesting that no substrate limitation had occurred. Although there was no observable difference in plant pathogenicity, removal of dsRNA did appear to increase pectic enzyme production.

Previous studies on pectic enzymes have shown that the enzymes are produced by both highly virulent and avirulent strains of *R. solani* (Sherwood, 1966, Marcus *et al.,* 1986), but studies on pectin lyase by Marcus and colleagues (1986)

showed it to be produced only by virulent strains while polygalacturonase was produced by both virulent and hypovirulent strains. These results are in agreement with the present work, as both pectic enzyme types were found in the dsRNA-containing PA1 strain and its cured derivative (Tables 4.6 & 4.7), both of which are pathogenic (Table 4.4) and produce pectin lyase. In the present study, however, clear differences in the levels of polygalacturonase and pectin lyase produced were observed for PA1 and PA1 cured.

Further studies carried out by Marcus *et al.* (1986) purified the enzymes to homogeneity and found two different types of polygalacturonase present which differed in size and pH specificities. Endopolygalacturonase I had a molecular weight of 34,000Da and a pH optimum of 4.8, whereas endopolygalacturonase II had a molecular weight of 37,000Da and acted optimally at pH5.4. Breakdown of substrate resulted in the production of octa-, hepta-, penta-, tetra-, tri-, di- and monogalacturonic acids by both polygalacturonases and pectin lyase suggesting a random cleavage mechanism by these enzymes. The assay used in the present work presumably measured the cumulative action of polgalacturonase I and II. The studies carried out by Marcus *et al.* (1986) showed that the production of both polygalacturonase II and pectin lyase was much higher in the virulent strain than in the hypovirulent strain tested, both of which were from anastomosis group 4, but levels of polygalacturonase I were the same in both strains. In the present work, both strains were virulent but levels of pectic enzymes were higher in the dsRNA cured strain.

Pectic enzymes are only one of the means by which fungi degrade plant tissues. But, *R. solani* is able to degrade pectic material under a variety of conditions, which makes the enzymes important in the complex of factors involved in pathogenicity. In this thesis cellulose breakdown was also investigated, as cellulose is a structural component of plant cell walls (Isaac, 1992) and reports have shown that cellulose breakdown, and therefore cellulase activity, occurred in the lesions of hypocotyls infected with *R. solani* at all stages of lesion maturation (Van Etten *et al.*, 1967). In the present study cellulose breakdown was markedly increased in the cured PA1 strain when compared with the dsRNA-containing strain, suggesting once more

that there was an increase in fitness of the dsRNA-free strain. Increased cellulolytic activity may have asisen partly as a result of increased growth of the PA1 cured strain whose mycelial dry weight was found to be over double that of PA1. There were also many more sclerotia produced by the parent PA1 strain (Figure 4.4b), suggesting that the strain had gone into a resting stage whereas the cured strain did not produce many sclerotia so it perhaps remained active in cellulolysis for longer.

The results of the pectic and cellulolytic activities of AG4 strain PA1 and its cured derivatives, although indicative of an increased fitness of the dsRNA cured strains, must be viewed with caution. Subsequent anastomosis experiments carried out in attempts to fulfil Kochs postulates by reintroduction of the dsRNA into the cured strains failed due to lack of compatibility between the cured and non-cured strains. When paired on PDA or water agar, attempted anastomosis resulted in complete fusion followed by a localised areas of cell death (Figure 4.6). So hyphal tip subculturing of both strains resulted not only in loss of dsRNA, but also in loss of a vegetative compatibility factor which presumably was under nuclear control. The strains used in the study were originally field isolates so were presumed to be heterokaryotic and, as cells of *R. solani* are multinucleate, it is unknown how many different nuclear types are present in each cell. Unlike binucleate basidiomycetes (Todd & Aylmore, 1985), nuclear number is not regulated by clamp connections in *R. solani*. It is assumed that each nucleus contains specific vegetative compatibility genes and loss of a nuclear type will result in loss of these genes leading to vegetative incompatibility with the parent strain which contains all nuclear types. It may also be assumed that pectic and cellulolytic enzymes are encoded by nuclear genes, in which case the regulation of these enzymes would also be nuclear encoded. However, the involvement of dsRNA in this regulation cannot be ruled out because Powell & Van Alfen (1987) showed that part of the dsRNA genome of *Cryphonecria parasitica* is involved in down-regulation of several nuclear genes and a similar penomenon may also occur in *R. solani*.

For further work on *R. solani* it will be essential to develop an efficient transformation system so that extracted and purified dsRNA can be introduced into

cells with a uniform genetic background, to test specifically the effects of dsRNA on the fungal phenotype.

CHAPTER 5

VEGETATIVE COMPATIBILITY IN *RHIZOCTONIA SOLANI* ANASTOMOSIS GROUP 4

5.1. INTRODUCTION

In many fungi vegetative compatibility systems are present and act to restrict the transfer of nuclear and cytoplasmic elements between species. The genetic systems governing vegetative incompatibility have been reported in many genera, especially Ascomycetes such as *Fusarium* (Cullen *et al.*, 1983), *Cryphonectria* (Anagnostakis, 1977) and *Neurospora* (Garbjnost & Wilson, 1956), and the simplest of these incompatibility systems are those in which strains which are identical at a particular set of loci are capable of forming a stable heterokaryon, while those which differ at any of these loci are incapable of forming a vegetatively stable heterokaryon.

Vegetative incompatibility systems that have been studied in fungi are governed by polygenic and heterogenic somatic recognition systems (Mylyk, 1975). A heterogenic system is one in which a compatible reaction occurs when alleles are identical at all governing loci. In a homogenic system the alleles must be identical for a compatible pairing (Adams, 1988).

Multinucleate basidiomycetes such as *Rhizoctonia solani* have a vegetative incompatibility system which acts as a secondary barrier following anastomosis. In incompatible strains anastomosis occurs by hyphal fusion and is followed by a killing reaction as a result of formation of heterokaryotic cells which are composed of incompatible nuclei (Molnar *et al.*, 1990). Most field isolates of *R. solani* are heterokaryotic but in most anastomosis groups heterokaryosis is difficult to assess because the multinucleate cells lack clamp connections which are often indicative of heterokaryosis in basidiomycetes. In anastomosis group 4, mating compatibility has been reported to be controlled by a single complex mating locus with multiple alleles (Anderson, 1982; Adams & Butler, 1982).

Previous work in this thesis (Chapter 4) has shown that hyphal tip subculture of the presumed heterokaryotic AG4 strains I13 and PA1 has generated strains that are vegetatively incompatible with the parent I13 and PA1 strains. This chapter aims to investigate the phenomenon in AG4 by isolation of single hyphal tip subcultures and examination of their ability for compatible fusion. In addition to this it examines the role of dsRNA from strains formed by hyphal tip subculture.

5.2 RESULTS

5.2.1 Analysis of single hyphal tip subcultures of AG4 PA1

Initially ten single hyphal tip subcultures of strain PA1 were isolated by the method described in Chapter 2. The tip cells were allowed to regenerate to form a colony and tested for vegetative compatibility with the parent and for the presence of dsRNA.

Microscopic analysis of vegetative compatibility

Fusion studies were first carried out microscopically using the method described in Chapter 3 for analysis of anastomosis. 1cm diameter blocks of mycelia were inoculated 2cm apart on a water agar coated microscope slide. Slides were inoculated at 25°C for 24-36hrs and examined microscopically at a magnification of x 700 and the interactions produced were equivalent to Figures 3.1 B and C.

Microscopic examination of all ten hyphal tip cultures (T1-T10) paired against PA1 and against each other was carried out in duplicate, so a total of 110 observations were made, and cells examined for compatibility following fusion. Unfortunately, clear compatible and incompatible distinctions could not be made using this method due to the following complications. (1) As the cells were growing on water agar, nutrient availability was very low, so retraction of the cytoplasmic contents of many hyphal branches had occurred due to nutrient depletion. These ghost cells closely resembled cells in which a vegetative incompatibility reaction had occurred. (2) Due to ghosting, often a self anastomosis would appear to be incompatible. Therefore unless each hyphal source could be clearly identified, there was no clear distinction between vegetative incompatibility and ghosting.

Due to inconclusive results by microscopic examination a second method for identification of vegetative incompatibility was examined.

Macroscopic analysis of vegetative incompatibility

Previous work on heterokaryon formation in *R. solani* AG4 strains used Petri plate tests. Pairing of strains on PDA resulted in two types of interaction. (1).

Heterogenic incompatibility in AG4 caused barrage formation, a killing reaction which occurs between different isolates in the same anastomosis group, but does not occur when an isolate is paired with itself. (2). Homogenic incompatibility forms tufts at the boundaries between two homokaryons (Anderson, 1984).

In the present work barrage formation was used to assess heterokaryon breakdown. A barrage may result when colony margins of incompatible strains grow into each other and react in an antagonistic manner. Barrage formation requires hyphal fusion, so is only seen when testing strains from the same anastomosis group. In the barrage region numerous lethal fusions will occur so the area of contact and cell death may be sharply delimited (Adams, 1988). Barrage formation with AG4 PA1 and its hyphal tip derivatives (T1-T10) was investigated by inoculating two 1cm diameter mycelial covered agar blocks 2cm apart on a Petri dish containing PDA. Plates were incubated at 25°C for 7 days and examined for growth and interaction.

Pairing of the original dsRNA cured strain of PA1 with the parent PA1 strain resulted in the formation of a distinct zone of death at the colony interaction site (Figure 5.1). This death zone was visible without microscopy and examination of the barrage area microscopically revealed only dead cells in the area of interaction. The barrage appeared to be a simple method to identify incompatible hyphal tip subcultures, so tip subcultures T1-T10 were paired against PA1.

By plate analysis only strains T2 and T5 were incompatible with the parent (PA1) (Figure 5.1), but they were compatible with each other. In order to further characterise the vegetative compatibility groups present within PA1 more extensively, a further 68 hyphal tip subcultures were taken from PA1 and paired against the parental culture PA1. From these tips only four were found to be incompatible with the parent strain. These tip cultures were designated T12, T13, T14 and T16 (Figure 5.2). Initially T12 and T14 were only weakly incompatible with PA1 but routine subculture of these strains resulted in stronger incompatibility when subsequently tested against the parent PA1.

Compatibility of all the subcultures incompatible with PA1 was tested by barrage formation and the results are shown in Table 5.1. From the data it appeared that there were two compatibility groups among the strains that were incompatible

Figure 5.1. Anastomosis and vegetative incompatibility between AG4 PA1 and various tip subcultures (labelled T with subscript). The tip-subcultures were obtained by removal of hyphal tips from PA1 and incubation for 3 days at 25°C to allow regeneration. Upon regeneration and new colony formation pairings of the tip subcultures with both PA1 and all other tip subcultures were carried out. 1cm diameter agar blocks, from the colony margin, were placed 2cm apart on PDA and the plates incubated at 25°C for 7 days. The photographs show examples of the reaction types which occurred. **A.** Pairing of PA1 and T_2 resulted in barrage formation at the colony interface due to vegetative incompatibility. **B.** A similar incompatibility reaction can be seen with PA1 and T_5. **C.** Pairing of both PA1 incompatible strains, T_2 and T_5 with each other results in complete compatibility. There is no barrage formation or zone of death. **D. E. F. G.** Pairing of T_5 with four other tip cultures which were incompatible with PA1. In all cases T_5 forms a barrage indicating vegetative incompatibility. A similar reaction was observed with T_2 (not shown). Pairing of all of the cultures, T_{12}, T_{13}, T_{14} and T_{16}, with each other resulted in compatibility in all cases (see Figure 5.2 and Table 5.1). All ~~compatibility~~ compatibility reactions were also confirmed by light microscopy.

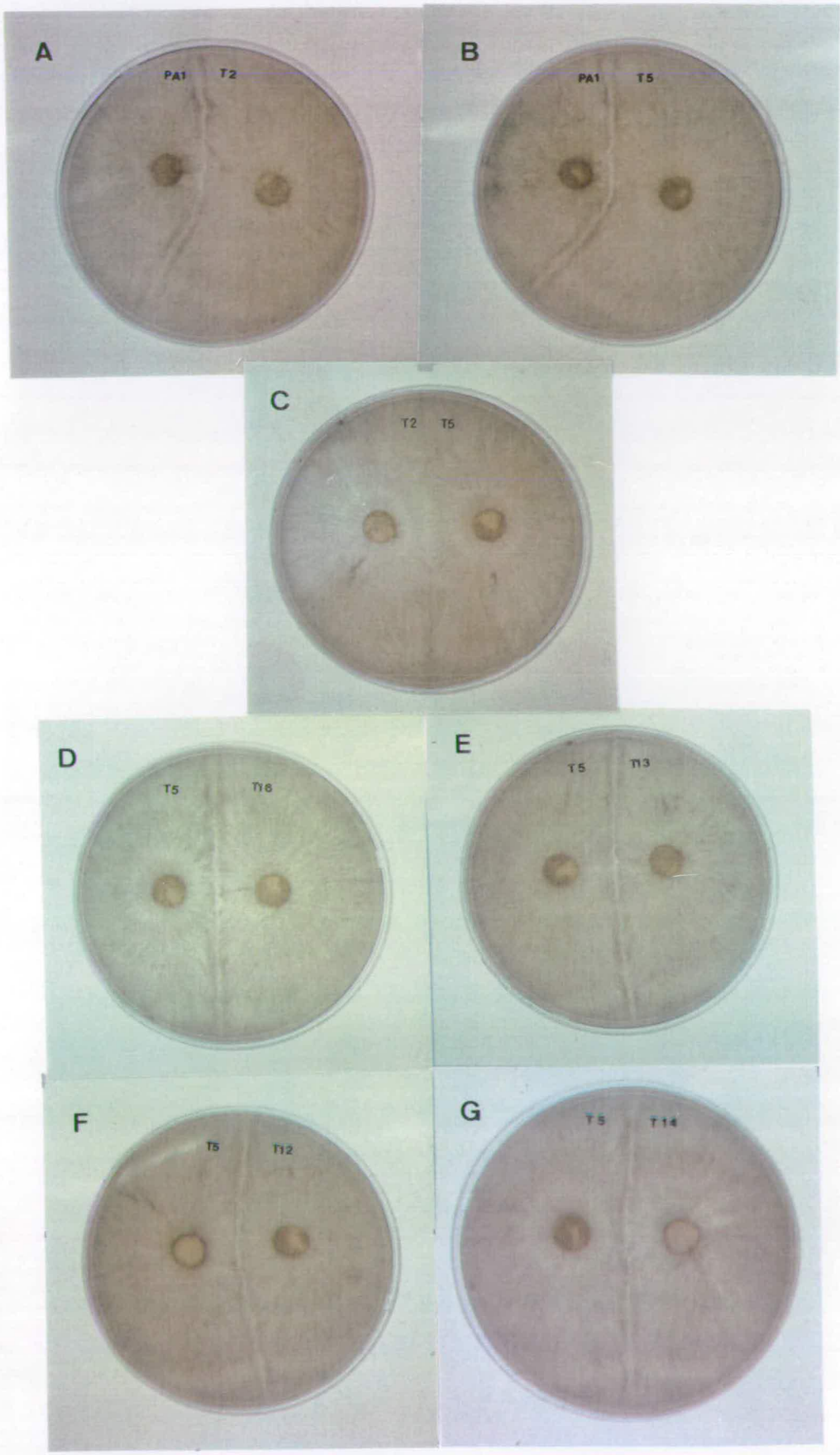
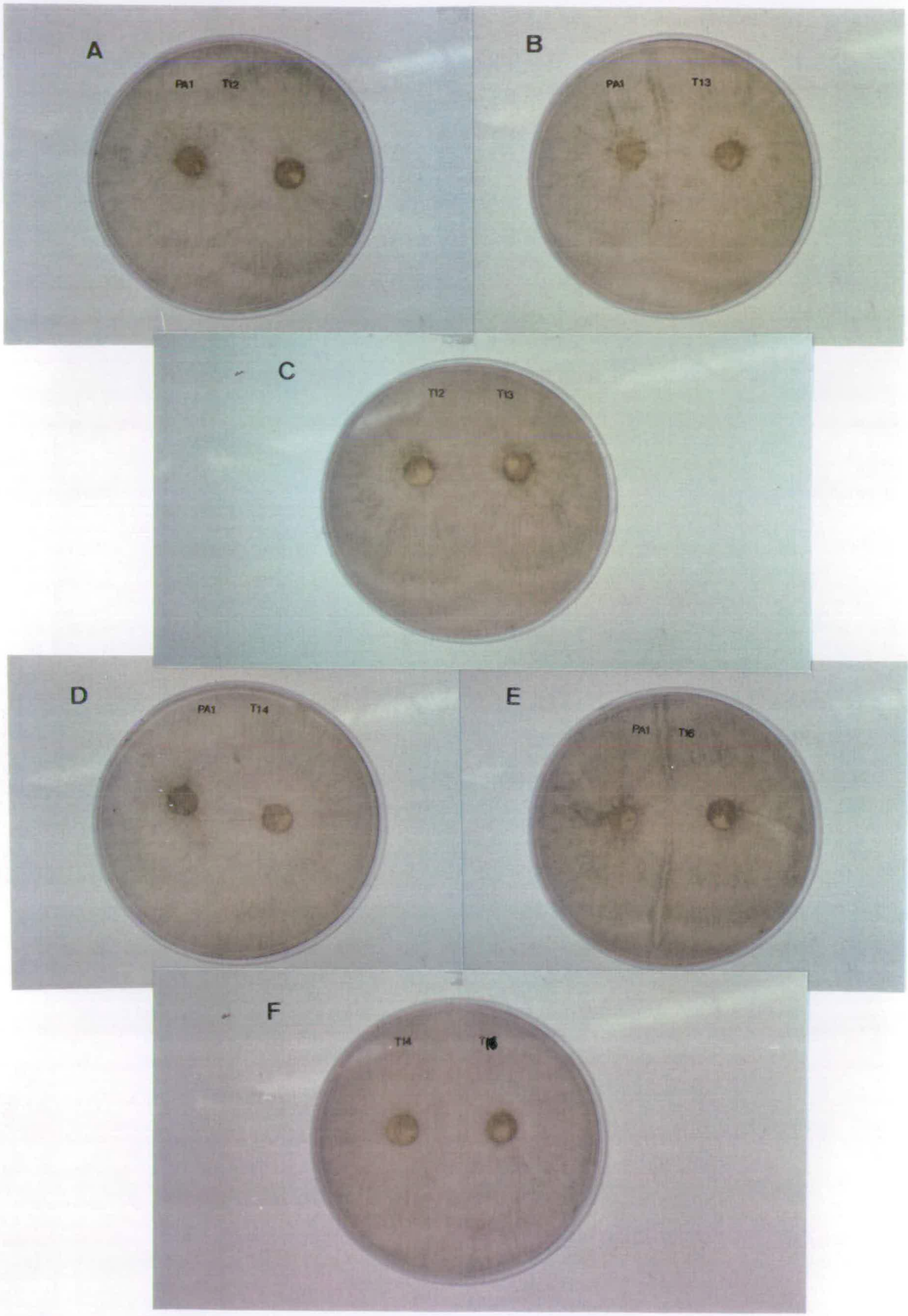

A
PA1
T2
B
PA1
T5
C
T2
T5
D
T5
T16
E
T5
T13
F
T5
T12
G
T5
T14

Figure 5.2. Anastomosis and vegetative incompatibility in AG4 PA1 and tip subcultures T12, T13, T14 and T16. Tip subcultures, designated Tx, were isolated by removal of hyphal tips from PA1 and incubation for 3 days at 25°C to allow regeneration. Upon regeneration and new colony formation pairings of the tip subcultures with both PA1 and all other tip subcultures were carried out. 1cm diameter agar blocks, from the colony margin, were placed 2cm apart on PDA and the plates incubated at 25°C for 7 days. The data shown are examples of the reaction types which occurred. **A.** Barrage formation at the colony interface of PA1 and T12 due to vegetative incompatibility. The incompatibility reaction is very weak and can only be observed in the centre of the interaction zone. **B.** Vegetative incompatibility between PA1 and T13. **C.** T12 and T13 were mutually compatibile. **D.** Very weak incompatibility indicated by barrage formation between PA1 and T14. **E.** Barrage formation between PA1 and T16. **F.** Compatible reaction upon pairing of T14 and T16. All compatibility reactions were also confirmed by light microscopy.


A
PA1
T12
B
PA1
T13
C
T12
T13
D
PA1
T14
E
PA1
T16
F
T14

	Strain						
	PA1	T2	T5	T12	T13	T14	T16
PA1	+	-	-	-	-	-	-
T2	-	+	+	-	-	-	-
T5	-	+	+	-	-	-	-
T12	-	-	-	+	+	+	+
T13	-	-	-	+	+	+	+
T14	-	-	-	+	+	+	+
T16	-	-	-	+	+	+	+

Table 5.1 Anastomosis between parent incompatible hyphal tip subcultures of AG4 PA1. Two 1cm diameter mycelial covered agar blocks were placed 2cm apart on PDA, and incubated at 25°C for 7 days. Examination of the plates for anastomosis and vegetative compatibility was carried out. + denotes successful anastomosis and - denotes attempted anastomosis resulting in barrage formation due to localised cell death. All compatibility/incompatibility reactions were verified by light microscopy.

with the parent PA1. All strains within a group were mutually compatible, while those in the opposite group always formed a barrage. Figures 5.1 and 5.2 also show this. Strains T2 and T5 are compatible with one another, as are T12, T13, T14 and T16, but T2 and T5 were incompatible with any of the other incompatible tips (Figure 5.1). In all cases these results were supported by microscopic analysis showing a zone of death where there was barrage formation. Also, as PDA is a nutrient-rich medium there was very little cellular death outside the barrage region as a result of nutrient depletion.

5.2.2. Parental reformation

From the data presented in Table 5.1 there appears to be 2 vegetative incompatibility groups present in the hyphal tip subcultures each of which is distinct from PA1. Cells within each group are mutually compatible, but those in different groups are always incompatible. Previous work by Anderson (1984) has shown barrage formation in *R. solani* AG4 to be a result of heterogenic incompatibility reaction, a vegetative incompatibility mechanism which prevents cellular integration between non-like individuals. It was considered that occasional fusion of strains of each of the groups might result in regeneration of a strain compatible with the parent PA1.

To test this hypothesis all incompatible interactions were paired on PDA for 7 days, then 6 mycelial blocks were removed from the colony interaction area down the length of the zone of junction. The mycelial blocks from the interaction zones were placed on fresh PDA plates and the resulting colonies were then paired with PA1, as described in section 5.2.1, and examined for compatibility. It was hoped that although there was cell death, a limited amount of cytoplasmic and nuclear transfer may have occurred between the pairings to regenerate a parental type strain. However all the colonies derived from the subcultures were incompatible with PA1 and no evidence for parental reformation could be found.

5.2.3. The role of dsRNA in vegetative incompatibility

All of the single hyphal tip subcultures, T1-T10 and T12, T13, T14 and T16, were tested for the presence of dsRNA using both the cellulose CF-11 column method and the method for isolation of total fungal nucleic acid described in Chapter 2. In all cases a single hyphal tip subculture had resulted in loss of dsRNA, whereas it was still present in strain PA1. Figure 5.3 shows total nucleic acid preparations from PA1 and strains T1 to T5, showing this loss of dsRNA. Of the fourteen hyphal tip strains, six were incompatible, and eight compatible with PA1 (see Figure 5.3). So this suggests that dsRNA had no role in the observed vegetative compatibility reactions.

Further evidence was obtained from strain T1. Initial hyphal tip subculture showed T1 to be compatible with PA1 (Figure 5.4A) and it was shown to have lost dsRNA (Figure 5.3). A further single tip subculture (strain T1A) resulted in incompatibility with PA1 (Figure 5.4B) and T_1 but the strain T1A was still compatible with T12, T13, T14 and T16. Therefore in this case vegetative incompatibility was obtained by subculture of an originally dsRNA-free strain that was compatible with the dsRNA containing parent.

5.2.4. Transfer of double-stranded RNA

Hyphal tip subculture T1 was shown to be compatible with PA1 (Figure 5.4A), but had lost dsRNA. Pairing of PA1 and T1 on PDA was carried out and the plate incubated for 10 days at 25°C to allow anastomosis and transfer of dsRNA. 1cm diameter blocks of mycelium-covered PDA were removed from the T1 colony at 0.5cm, 2cm and 4cm behind the region of overlap with PA1. Extraction of both total nucleic acid and dsRNA from this strain showed no transfer of dsRNA from PA1 to T1, at least over the distances tested.

5.2.5. The role of nuclear number in vegetative compatibility

Previous reports on vegetative compatibility have stated that cell death between fused strains is the result of the presence of two incompatible nuclei in the heterokaryon (Molnar *et al.,* 1990). If the compatibility status of a heterokaryon is

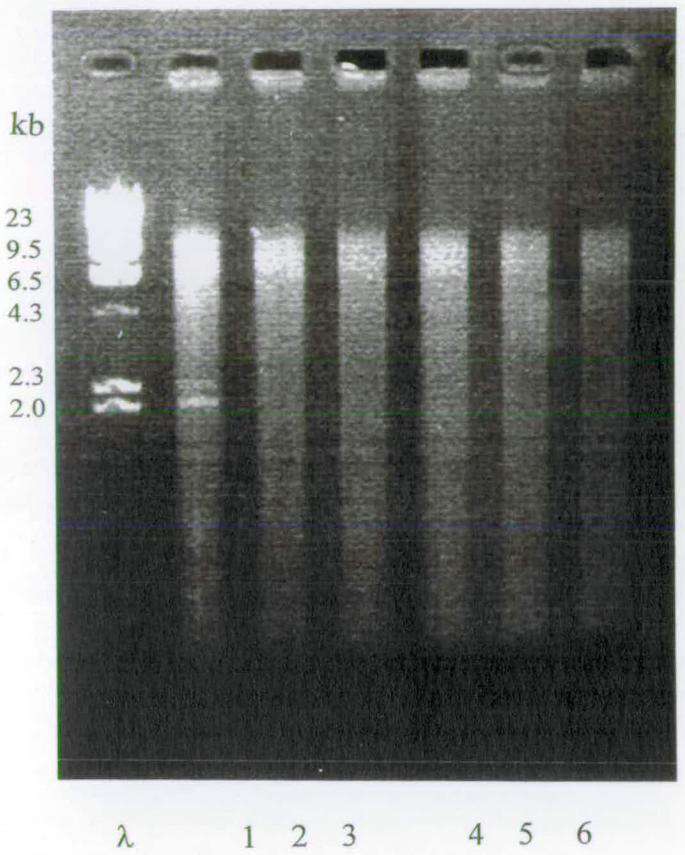


Figure 5.3. The dsRNA content of AG4 PA1 and single hyphal tip derivatives. AG4 PA1 and 5 single hyphal tip subcultures (T1-T5), irrespective of their compatibility groupings (which are shown in Table 5.1), were incubated, with shaking, for 10 days at 25°C in PSB. The culture media was filtered off and total nucleic acid was extracted from each of the strains using phenol and chloroform as described in Chapter 2. Following ethanol precipitation samples were electrophoresed on 1% agarose (w/v). λ DNA digested with *HindIII* to give size markers shown at the left of the photograph. Lane 1 shows AG4 PA1 total nucleic acid. Lanes 2-6 show total nucleic acid recovered from single hyphal tip subcultures T1 to T5.

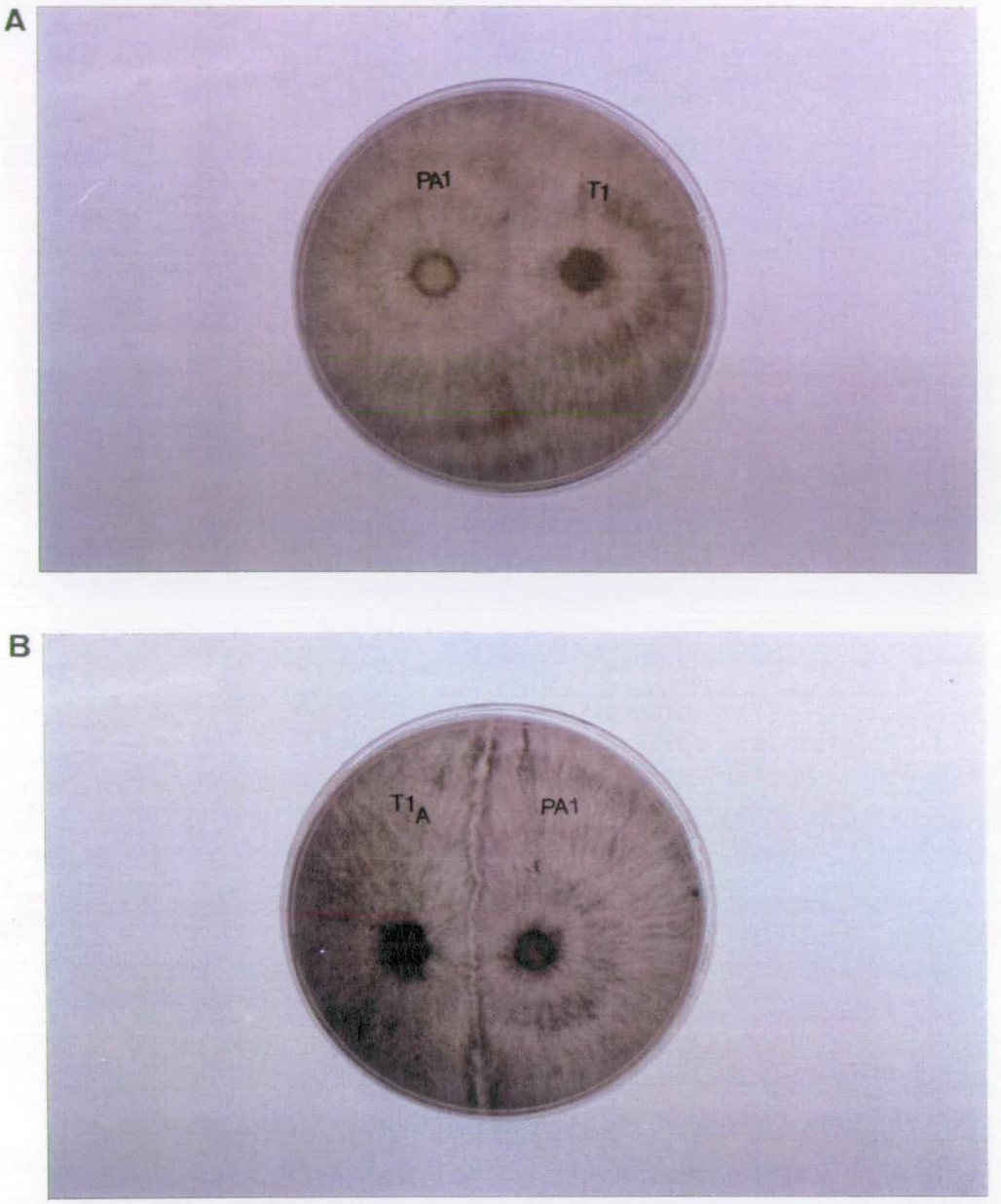


Figure 5.4. Anastomosis of AG4 PA1 and two successive hyphal tip subcultures T1 and T1A. Tip subcultures, designated Tx, were isolated by removal of hyphal tips from PA1 and incubation for 3 days at 25°C to allow regeneration. Upon regeneration and new colony formation pairings of the tip subcultures with both PA1 and all other tip subcultures were carried out. 1cm diameter agar blocks, from the colony margin, were placed 2cm apart on PDA and the plates incubated at 25°C for 7 days. **A.** Single hyphal tip subculture resulted in a compatible strain T1, which had lost dsRNA (see Figure 5.3). **B.** Tip subculturing of T1 resulted in an incompatible strain T1A, which formed a barrage upon pairing with the parent and T1 (data not shown), indicating that dsRNA plays no role in vegetative compatibility.

conferred by all the compatibility loci in each nucleus, then the loss of any nuclear type would possibly change the vegetative compatibility status. In order to explain the possibility that nuclear number (and thus the probability of there being different nuclear types) per hyphal cell is related to vegetative compatibility, nuclear counts of the hyphal tip cells of all fourteen tip subcultures were carried out by DAPI staining of the nuclei. The data are presented in Table 5.2 and show quite a variation in nuclear number between strains. PA1 is a multinucleate strain with nuclear number varying from 2 to 6 in individual cells.

Analysis of variance of the nuclear numbers showed there to be a significant difference in the mean number of nuclei per tip cell of the 15 tested strains. Separate analysis of each of the compatibility groups showed there was no significant difference in the number of nuclei in tips of strains T2 and T5 (mutually compatible) and no difference in nuclear number between strains T12, T13, T14 and T16 (mutually compatible). So cells of strains in the same compatibility group seemed to have a similar mean number of nuclei per hyphal tip. However, inspection of Table 5.2 shows that there was no absolute relationship between nuclear number per tip and compatibility grouping. For example, strains T5 and T16 had similar nuclear number per tip although they were mutually incompatible.

5.2.6. Nuclear segregation in PA1

Although within a colony a variety of nuclear numbers were observed, upon closer examination of the colony, cells which branched from a main hypha often appeared to have a similar nuclear number to the main hyphal cell from which they had arisen. This clustering of branch tips with similar nuclear number may be important in the partial incompatibility reactions observed upon fusion with the parent (Figure 5.2) so individual colonies on PDA were examined for distribution of nuclear number per tip cell. Unfortunately, the analysis could not be as comprehensive as was hoped, because DAPI did not give uniform 'staining' of nuclei in all regions of the fungal colonies which were assessed. This was partly due to failure of stain penetration (or fluorescence) of some hyphae, especially those that were pigmented. So the analysis was done by scanning DAPI-stained colonies under

Strain	Tip Cell		Sub-apical Cell	
	Sample Size	Mean Nuclear Number +/- S.E.M.	Sample Size	Mean Nuclear Number +/- S.E.M.
PA1[c]	20	4.2 +/- 0.27	10	3.5 +/- 0.31
T1c	20	2.9 +/- 0.14	10	2.7 +/- 0.26
T2[a]	10	3.7 +/- 0.11	10	3.5 +/- 0.31
T3[c]	10	4.0 +/- 0.10	nt	nt
T4[c]	20	3.3 +/- 0.23	10	3.0 +/- 0.21
T5[a]	16	3.1 +/- 0.21	10	4.0 +/- 0.26
T6[c]	10	4.0 +/- 0.15	nt	nt
T7	10	4.1 +/- 0.16	nt	nt
T8	10	4.0 +/- 0.10	nt	nt
T9	10	4.1 +/- 0.13	nt	nt
T10	10	4.5 +/- 0.26	nt	nt
T12[b]	10	3.4 +/- 0.22	10	3.1 +/- 0.26
T13b	10	3.6 +/- 0.19	10	3.3 +/- 0.21
T14[b]	14	3.7 +/- 0.17	10	3.7 +/- 0.29
T16[b]	10	3.2 +/- 0.27	10	3.2 +/- 0.29
5% LSD		0.739		0.54

Table 5.2. Nuclear numbers in hyphal tip cells shown and subapical cells of *R. solani* strain AG4 PA1 or strains derived from it by hyphal tip subculture. Nuclear numbers were obtained by staining mycelia with DAPI and counting nuclei under fluorescence microscopy at a magnification of x 700. nt - not tested. Strains followed by the same letter are in the same vegetative compatibility group.

a fluorescence microscope and making hyphal counts in any regions where a group (cluster) of adjacent hyphal tips were clearly seen to be part of a branching system from a common 'source' hyphal cell (Figure 5.5). Table 5.3 shows results for variable numbers of such clusters (4-7) in the different strains. Each of these clusters had variable numbers of tip cells that showed fluorescence.

Statistical analysis was carried out using binomial expansion. For this analysis initially the frequency distribution of nuclear number per tip was calculated for all the tips in clusters of each strain. For example, for strain PA1 in Table 5.3, there were 28 tip cells counted, of which 1 cell had 2 nuclei, 9 cells had 3 nuclei, 15 cells had 4 nuclei and 3 had 5 nuclei. So the probability (p) of any single tip having 3 nuclei was 9/28 or 0.321, and the probability of any single tip not having 3 nuclei (q) was 1-p or 0.697. Then, for cluster 1 the probability of finding 3 tips out of the total 3 that all contain 3 nuclei is obtained by binomial expansion. The general form of this is $(p + q)^n$. For a total of 3 hyphal tips it is:

$$(p + q)^3 = p^3 + 3p^2q^2 + 3pq^2 + q^2.$$

In the case of the cluster 1 of strain PA1, all 3 tips had 3 nuclei so the probability of this occurring by chance was given by p^3 $(= 0.321^3)$ $= 0.033$. This level of probability indicates significant tendency for hyphal tips within the cluster to have similar numbers of nuclei.

Analysis of this sort were done for each cluster of each strain, selecting only the most common nuclear number in each cluster (eg. 3 nuclei per tip in cluster 3 of PA1, but 4 nuclei per tip in cluster 6 of PA1, etc.). As a further illustration of the method, consider cluster 2 of PA1. In this case there were six tip cells, four of which had 3 nuclei per tip and two of which did not have 3 nuclei per tip. The probability of this occurring by chance is given by calculating $p^6 + 6p^5q + 15p^4q^2$, this being part of the full binomial equation which is:

$$(p + q)^6 = p^6 + 6p^5q + 15p^4q^2 + 10p^3q^3 + 15p^2q^4 + 6pq^5 + q^6.$$

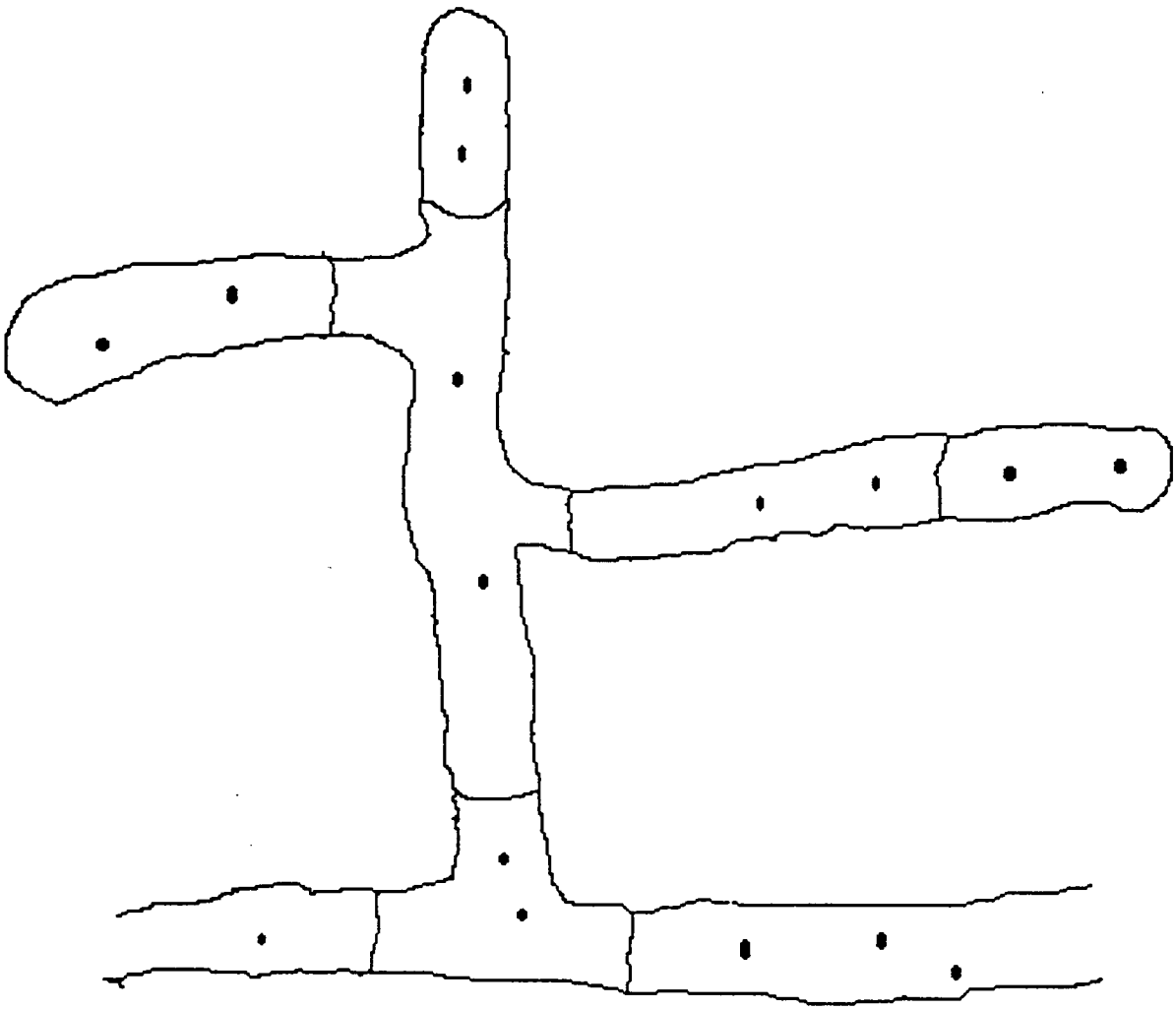


Figure 5.5. An impression of clustering in a side branch from a main hyphae. ● -nucleus

Table 5.3. Counts of numbers of nuclei (stained with Dapi) in tip cells within clusters of tips, a cluster being defined as a group of tip cells that originated from a simple branch of the fungus (see Figure 5.5). Binomial expansion was used to calculate the probability of finding the observed distributions of nuclear numbers per tip by chance (see text for details). For example, for strains PA1 the probability of finding 3 nuclei in all 3 tips (cluster 1) is 0.033 by chance (because only 6 of the total 28 tips counted for strain PA1 had 3 nuclei in them). So this indicates a significant departure from expectation of randomness and suggests some local control over nuclear number within a cluster. *significant deviation from randomness.

Strain	Cluster number	No. of tips in cluster	Nuclear number in each tip	Probability Value
PA1	1	3	3 3 3	0.033*
	2	6	2 3 3 4 3 3	0.088
	3	4	4 4 4 3	0.367
	4	5	4 4 5 4 3	0.565
	5	4	4 4 4 4*	0.080
	6	6	4 5 4 5 4 4	0.410
T1	1	4	4 4 4 4*	0.017*
	2	8	3 3 3 3 3 3 3 3	0.017*
	3	9	3 3 4 3 3 3 2 3 3	0.413
	4	4	4 4 4 4	0.017*
T2	1	4	4 5 3 4	0.227
	2	4	4 3 5 2	0.6985
	3	6	4 5 6 5 5 5	0.024
	4	4	4 4 4 2	0.057
	5	4	2 2 2 2	0.002*
	6	5	3 3 3 3	0.001*
T4	1	4	4 4 4 3	0.0012*
	2	7	3 3 3 3 3 2 2	0.880
	3	4	3 3 3 3*	0.251
	4	4	3 3 3 2	0.665
	5	5	3 3 3 3 2	0.544

Strain	Cluster Number	No. of tips in cluster	Nuclear number in each tip	Probability Value
T5	1	8	3 3 3 3 4 4 5 5	0.314
	2	4	4 3 6 3	0.800
	3	9	3 3 4 3 4 5 4 4	0.140
	4	7	3 3 3 3 3 3 3	0.024*
	5	5	4 4 4 3	0.061
	6	9	3 3 3 2 3 3 4 3 3	0.145
T12	1	4	4 3 3 4	0.180
	2	4	4 4 4 3	0.650
	3	5	4 4 4 4 4*	0.168
	4	3	4 3 4	0.780
	5	4	5 5 4 4	0.480
T13	1	5	3 3 4 3 2	0.346
	2	9	4 4 4 3 3 3 3 3 3	0.349
	3	4	3 3 3 3	0.11
	4	8	2 3 3 2 4 4 4 5	0.367
T14	1	4	3 3 3 3*	0.037
	2	5	3 3 4 3 4	0.509
	3	5	4 4 4 5 3	0.509
	4	4	4 5 4 4	0.262
T16	1	4	2 2 2 2	0.004*
	2	3	3 3 3	0.095
	3	4	3 3 3 4	0.022*
	4	7	3 3 3 3 3 4 2	0.017*
	5	4	2 2 3 2	0.051
	6	7	3 3 3 3 4 3 2	0.178
	7	6	5 5 5 4 4 3	0.01*

In the case of cluster 2 the probability of finding 4 of the 6 tips to contain 3 nuclei (given that the frequency of 3 tips per cell is 0.321 overall) is 0.088, which is not significant (a probability of 0.05 being chosen by convention to be the least significant difference).

As can be seen in Table 5.3, for all 47 clusters that were recorded, 11 showed significant evidence of similar nuclear number per tip within the cluster. In 6 further cases there was a highly constant nuclear count in the cluster but it was insignificant because those particular nuclear numbers had a relatively high frequency of occurrence in those strains as a whole.

The evidence thus points to a strong tendency for the hyphal tips derived locally from a single parent hyphal cell to have a similar number of nuclei to one another. Put in other terms, the hyphal tips in cluster 1 of strain PA1, for example, were similar to one another but different from, say, the tips in cluster 5 of the strain. Evidently, the colony of strain PA1 consisted of a mosaic of clusters of tips that differed from one another in nuclear number.

5.2.7. Phenotypic effects of vegetative incompatibility

Upon hyphal tip subculturing, the cultures were losing dsRNA or both dsRNA and specific nuclear genes/type. Therefore cultures were examined for phenotypic changes due to loss of these factors. Initially examination of colony morphology was carried out, but, there was no change in pigmentation or sclerotium formation upon single hyphal tip subculture whether cells had lost only dsRNA, or also showed a change in vegetative compatibility. As this differed from the results with a previously cured PA1 strain, described in Chapter 4, characterisation of pectic and cellulolytic enzyme production of the new cured strains was carried out. Five of the hyphal tip strains were used in these assays, in addition to PA1. These subcultures were chosen as they fall into three main groups. Two cultures had lost dsRNA but remained compatible with PA1, the other three cultures came from the two parental incompatible groups shown in Table 5.1 which no longer displayed parental compatibility.

Pectic enzymes

The role of pectic enzymes in the pathogenicity of *R. solani* is described in Chapters 1 and 4. Pectic enzyme production was measured essentially as described in Chapter 2. Inoculum blocks of the 6 strains to be tested were incubated in pectin-containing broth (see Chapter 2) for 7 days at 25°C to induce production of enzymes. The fungus was filtered off and the supernatant containing enzymes was added to an equal volume of medium to assess polygalacturonase or pectin lyase (described in Chapter 2). Samples were taken, in duplicate, on days 1 to 5 after incubation in the appropriate buffer and levels of substrate degradation measured by the thiobarbituric acid assay (see Chapter 2). A boiled sample was used as a control for the assay.

Tables 5.4 and 5.5 show pectic enzyme activity for the 6 strains tested. Analysis of variance of the data showed that there were significant differences in the levels of both enzymes produced by the strains on each day, but there was little evidence of consistency of strain differences at all assessment times.

The most notable finding was the very high level of polygalacturonase activity of strain T13 relative to all other strains. This difference first became evident on day 2 and was very marked on days 4 and 5 of the assay. Strain T13 was the only hyphal tip transfer that showed a marked increase in enzyme relative to the parent strain, similar to the increase on polygalacturonase activity of the dsRNA-cured strains described in Chapter 4.

Cellulase activity

The activity of cellulase in each of the above six strains was measured as described in Chapter 2. Four replicates of each strain were incubated for 4 weeks at 25°C and loss of weight of filter paper was measured with respect to uninoculated controls. Table 5.6 shows that there was no significant difference in any of the strains.

5.2.8. Analysis of single hyphal tip subcultures of I13

In order to confirm the vegetative incompatibility found in hyphal tip subcultures of AG4 PA1, single hyphal tip subcultures of another AG4 strain I13

Strain	Day				
	1	2	3	4	5
PA1	8.18	9.29	10.37	8.32	10.30
T1	10.5	10.67	11.3	9.95	11.85
T2	7.36	10.48	9.45	9.64	10.06
T4	7.34	11.02	11.39	10.23	11.47
T12	6.0	11.63	10.78	10.72	10.91
T13	8.68	8.97	8.75	9.27	10.27
5% LSD	0.582	0.098	0.47	0.114	0.214

Table 5.4. Pectin lyase activity of strain PA1 and individual hyphal tip subcultures derived from it. Hyphae were incubated for the appropriate time period at 25°C and the culture supernatant tested for degradation of substrate by the thiobarbituric acid assay. One enzyme unit causes a change in absorbance (595nm) of 0.01 under the conditions of the assay. Pectin lyase is measured by the degradation of sodium polypectate at pH 8.5. Data are the means of two replicates used in each assay.

Strain	Day				
	1	2	3	4	5
PA1	1.77	1.86	2.34	2.80	3.16
T1	2.37	2.65	3.26	4.22	4.97
T2	2.36	2.91	3.92	3.71	4.01
T4	3.46	2.90	2.92	3.14	2.94
T12	1.55	1.73	2.55	2.74	2.94
T13	2.66	4.96	4.91	8.01	23.48
5% LSD	0.121	0.0924	0.2	0.097	0.097

Table 5.5. Polygalacturonase activity of strain PA1 and individual hyphal tip subcultures derived from it. Hyphae were incubated for the appropriate time period at 25°C and the culture supernatant tested for degradation of substrate by the thiobarbituric acid assay. One enzyme unit causes a change in absorbance (595nm) of 0.01 under the conditions of the assay. Polygalacturonase is measured by the degradation of pectin at pH 5. Data are the means of two replicates used in each assay.

Strain	Weight loss of filter paper (mg)
PA1	388.3 +/- 13.45
PA1 T1	350.7 +/- 21.12
PA1 T2	408.1.+/- 7.01
PA1 T4	387.1 +/- 26.09
PA1 T12	384.6 +/- 25.56
PA1 T13	399.1 +/- 16.82

Table 5.6. Cellulolysis of hyphal tip subcultures of *R. solani* AG4 PA1. Subcultures were obtained by removal of cells at the colony margin allowing regeneration of the new tip colony. Cultures derived from tip cells have been assigned the prefix T. Cellulolytic activity of AG4 PA1 and both compatible and incompatible hyphal tip subcultures was assessed by the method of Garret (1966). Filter paper inoculated cultures were incubated at 25°C for 4 weeks and cellulose breakdown (as weight loss of filter paper) was calculated with respect to uninoculated controls. Weight loss is shown as the mean +/- S.E.M. for 4 replicates. The weight loss recorded represents respiration of the cellulose by *R. solani*.

were carried out in a similar manner to that of PA1. In total, 124 hyphal tip derivatives were tested for compatibility with the parent strain and only 6 were found to be incompatible by plate analysis (Figure 5.6). Anastomosis of each of the parent-incompatible strains with each other was carried out and the results are shown in Table 5.7. A similar barrage formation to that of PA1 was observed and microscopic examination showed cell death in that region. Partial incompatibility was also observed (Figure 5.6), among some of these strains when they were first subcultured and it led to complete incompatibility after further subculturing of the strains. The results in Table 5.7 suggest the presence of two mutually incompatible groups of strains, one comprising of strains S2 and S4, the other comprising of strains S3, S5 and S11. Both of these groups were incompatible with the parent strain I13.

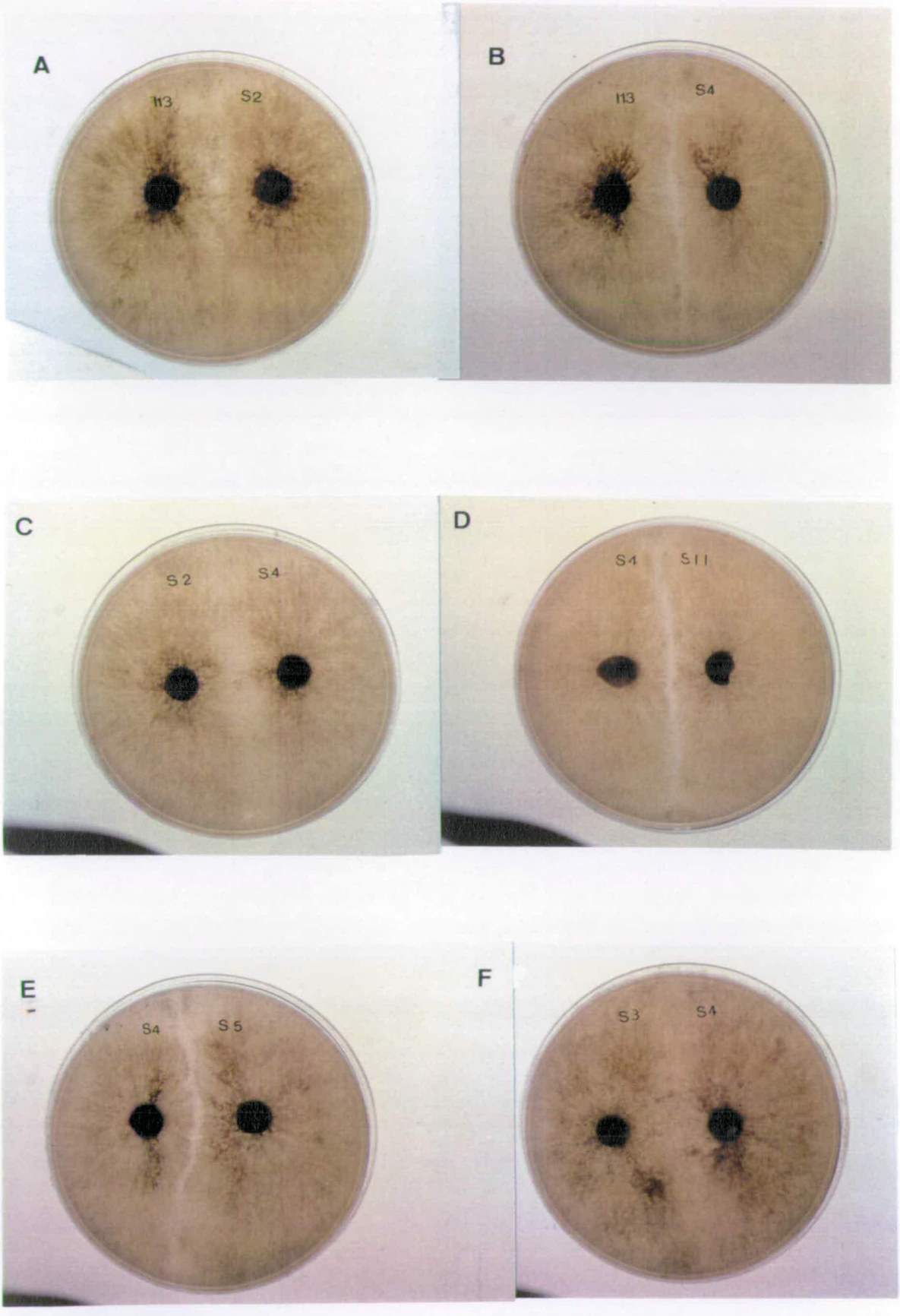
As with PA1, nuclear counts of hyphal tip cells were carried out in an attempt to correlate nuclear number with vegetative incompatibility. As can be seen in Table 5.8 the mean number of nuclei per tip cell of strain 113 and its derivatives was greater than for PA1; between 7.3 and 9.5 for I13 with between 2.9 and 4.5 for strain PA1. This may help to explain the lower number of incompatible-tip cultures isolated for I13. Analysis of variance of nuclear number showed a significant difference overall and suggested that strain T2 had significantly fewer nuclei per cell than did the parent (I13) or strain T4. However, the difference was small in absolute terms so may not be meaningful.

5.3. DISCUSSION

Vegetative incompatibility represents a means of discriminating self from non-self, and therefore reflects a phenomenon that is carried throughout most of the biological world.

Previous studies on both vegetative and sexual compatibility in *Rhizoctonia solani* have used single basidiospores to ensure isolation of a homokaryotic colony, followed by pairing of isolates to test for heterokaryosis (Whitney & Parmeter, 1963; Adams , 1988; Cubeta *et al.,* 1993). In contrast to previous work, the work described

Figure 5.6. Anastomosis and vegetative incompatibility in AG4 I13 and tip subcultures. Tip subcultures, designated Sx, were isolated by removal of hyphal tips from PA1 and incubation for 3 days at 25°C to allow regeneration. Upon regeneration and new colony formation pairings of the tip subcultures with both I13 and all other tip subcultures were carried out. 1cm diameter agar blocks, from the colony margin, were placed 2cm apart on PDA and the plates incubated at 25°C for 7 days. The data shown are examples of the reaction types which occurred. **A.** Weak incompatibility can be observed with I13 and S2. **B.** Stronger incompatibility is observed upon the interaction of I13 and S4. **C.** Compatibility upon pairing of S2 and S4. **D. E. F.** Pairing of S4 with three other tip cultures which were incompatible with I13. In all cases S4 forms a barrage indicating vegetative incompatibility. A similar reaction was observed with S2 (not shown). Pairing of all of the cultures, S3, S4 and S5, with each other resulted in compatibility in all cases (see Table 5.7).


A
I13
S2
B
I13
S4
C
S2
S4
D
S4
S11
E
S4
S5
F
S3
S4

	Strain					
	I13	S2	S4	S3	S5	S11
I13	+	-	-	-	-	-
S2	-	+	+	-	-	-
S4	-	+	+	-	-	-
S3	-	-	-	+	+	+
S5	-	-	-	+	+	+
S11	-	-	-	+	+	+

Table 5.7. Anastomosis between strain I13 and parent-incompatible hyphal tip subcultures. Two 1cm diameter mycelial covered agar blocks were placed 2cm apart on PDA and incubated at 25°C for 7 days. Examination of the plates for anastomosis and vegetative compatibility was carried out. + denotes successful fusion and - denotes attempted fusion resulting in barrage formation due to localised cell death. All compatibility/incompatibility reactions were confirmed by light microscopy.

Strain	No of Cells Counted	Mean Nuclear Number +/- S.E.M.
I13	10	9.5 +/- 0.5
T2[a]	12	7.3 +/- 0.58
T3[b]	10	8.2 +/- 0.36
T4[a]	10	8.8 +/- 0.25
T5[b]	10	8.4 +/- 0.58
T11[b]	10	8.6 +/- 0.45
5% LSD		1.34

Table 5.8 Nuclear numbers in hyphal tip cells of strains I13 or strains derived from it by hyphal tip subculture. Nuclear numbers were obtained by staining mycelia with DAPI and counting nuclei by fluorescence microscopy at a magnification of x 700. Strains followed by the same letter are in the same vegetative compatibility group.

in this chapter has focused on heterokaryon breakdown as a means of analysing the vegetative compatibility types present. This stemmed from chance observation that hyphal tip subculturing of AG4 strain PA1, to cure the strain of dsRNA, led to colonies that were no longer compatible with the parent (presumably heterokaryotic) strain. To my knowledge, this has never been reported before. It is of potential interest for several reasons. First, it might provide a means for breakdown of parental heterokaryotic genotypes in natural, field conditions eg. by variation in nuclear number in branch or tip cells, thereby creating a number of mutually incompatible strains from a parent mycelium. It is normally assumed that 'field' isolates of *R. solani* are heterokaryons and exist as extensive mycelial networks in soil, growing from one discrete source of substrate (eg. cellulose) to another, through soil. Second, if such breakdown of parental genotypes occurs in nature (or at least if parent-incompatible strains arise periodically perhaps by branching or from detached hyphal tips), then this could serve to limit the frequency of transmission of dsRNA or other extrachromosomal elements between mycelia. Third, it is still unknown how important the sexual stage of *R. solani* is in nature. The inconspicuous sexual stage is seldom seen (Parmeter, 1970), but can sometimes be induced to form on the surface of soil in culture. The segregation of genes by breakdown of heterokaryons (if this is how incompatible strains arise) could serve to generate variation in nature even if the sexual stage is uncommon. Lastly, the generation of parent-incompatible strains by hyphal tip transfer could provide an experimental tool for understanding the genetics of *R. solani*; if it is caused by the random assortment of nuclear genotypes in hyphal tips of a heterokaryon then it could suggest that the control of heterokaryosis in *R. solani* is much looser than the tightly regulated control of dikaryones in other basidiomycetes such as *S. commune* (Todd & Aylmore, 1985) and *C. versicolour* (Aylmore & Todd, 1984). For all these reasons, the phenomenon whereby parent-incompatible strains could be generated by hyphal tip subculture of parental strains was deemed to merit detailed study.

Pairings of parent-incompatible hyphal tip-derived cultures from the AG4 strains, I13 and PA1, suggested that these strains fell into two major groups that were incompatible with one another (Tables 5.2 & 5.7). But within each group most of the

strains were compatible with one another. These data could suggest the segregation of two nuclear types each with its own set of vegetative compatibility genes. The result would be (as observed) that pairing of strains of the two different groups would lead to anastomosis followed by barrage formation, then localised cell death (Figures 5.1, 5.2 and 5.5). Work by Anderson (1984) has shown barrage formation in *R. solani* to be the result of a heterogenic incompatibility mechanism in which strains of the same anastomosis group anastomose, then upon fusion a vegetative incompatibility reaction occurs. The vegetative incompatibility reaction results in cell death in both of the fused cells and in 5 or 6 cells on either side of the fused cell. When two colonies interact on agar there is a large number of attempted anastomoses, so where the colonies meet there will be mass cell death in a restricted area. This cell death is visible to the naked eye and, as it resembles a barrier preventing interaction between the two colonies, is termed a barrage. Barrage formation is solely a vegetative mechanism and heterogenic incompatibility serves to prevent somatic integration between genetically dissimilar individuals (Rayner *et al.*, 1984). No studies have been carried out into the genetic control of heterogenic incompatibility in *R. solani,* but the intensity of the reaction varies in different pairings (see Figure 5.2) indicating it is probably controlled by several genes.

Barrage formation in AG4 has been observed upon pairing of different heterokaryons, pairings between heterokaryons and homokaryons and pairings between different homokaryons (Anderson, 1984; Cubeta *et al.*, 1993).

Barrage formation has been studied in more detail in ascomycetes such as *Fusarium* spp. (Leslie, 1990), *Neurospora crassa* (Esser & Bliach, 1973), *Podospora anserina* (Griffin *et al.*, 1992) and *Cryphonectria parasitica* (Anagnostakis, 1977); in these fungi vegetative incompatibility is thought to restrict the transfer of nuclear and cytoplasmic elements during growth. In these strains incompatibility systems are all allelic, such that strains which are identical at a particular set of loci are capable of forming a stable vegetative heterokaryon, and those which differ at any of these loci are incapable of doing so. There are 7-15 heterocompatibility (*het*) loci per genome in *Neurospora* (Beadle & Coonradt, 1944), *Aspergillus* (Caten & Jinks, 1966), *Cochliobolus* (Leach & Yoder, 1983), *Ophiostoma* (Brasier, 1983), *Cryphonectria*

(Anagnostakis, 1982) and *Fusarium* (Correll *et al.,* 1988), and strains of the same species which differ at any of these loci are unable to form a heterokaryon. There are thought to be analogous loci in basidiomycetes. Unfortunately the molecular mechanisms involved in heterogenic incompatibility are unknown, although in *N. crassa* ten different incompatibility loci have been identified and mapped to 5 of the 7 chromosomes. Also, preliminary evidence is available for multiple alleles at 2 of these loci (Leslie, 1993). Wilson and colleagues (1961), have shown that the agent responsible for incompatibility is proteinaceous, and further work by Williams & Wilson (1966) showed there to be an RNA component associated with it. These workers suggested that a intracellular membrane receptor for the protein is a prerequisite for killing.

Work carried out in *R. solani* on heterokaryon formation from two homokaryons shows many interesting features, some of which are quite different to the findings of the present study. Early studies into compatibility in *R. solani* were carried out as most field isolates are heterokaryotic (Whitney & Parmeter, 1963; Garza-Chapa & Anderson, 1966), and a knowledge of heterokaryosis is important in understanding and interpreting the results of studies on the biology and control of *Rhizoctonia* diseases. Heterokaryons of AG4 were formed from homokaryons with mutations in genes affecting either two different steps in the infection cycle (Anderson, 1984): growth, but no attachment to the plant or formation of infection cushions but no penetration. This experiment indicated that heterokaryons could complement homokaryotic mutations to make the heterokaryons highly pathogenic, so heterokaryosis appears to ensure pathogenicity.

Work on compatibility in the sexual stage (*Thanatephorus cucumeris*) of *R. solani,* has shown the presence of two closely linked nuclear genes, named H-factors, which are thought to be responsible for compatibility. Field isolates have been shown to consist of two H-factors, HX and HY, and heterokaryosis will only occur between strains with different H-factors (Anderson *et al.,* 1972). The H-factor is thought to control heterokaryosis and promote outbreeding. It does not control hyphal fusion but controls sexual compatibility, nuclear pairing and the stability of the heterokaryon (Puhalla & Carter, 1976). Such a system has also been reported to influence

compatibility in the sexual stages of *R. solani* (Anderson, 1984). The presence of these H-factors promoting outbreeding in anastomosis group 4 might explain the wide host range of this group, and although heterokaryosis is not needed for pathogenicity it has been reported to increase virulence (Garza-Chapa & Anderson, 1966). This may be one explanation for the heterokaryotic nature of field isolates, including I13 and PA1 both of which are highly pathogenic. In pairings of vegetative cells identical heterokaryons are needed for compatibility, whereas in the rare sexual stage opposite H-factors are needed for compatibility. Thus, in *R. solani,* there appear to be two opposing systems in operation: one which promotes diversity and one which restricts it.

In ascomycetes vegetative compatibility groups serve as a natural means to subdivide a fungal population and the loci and alleles are thought to be selectively neutral with respect to traits such as pathogenicity and vegetative viability. In basidiomycetes, work by Whitney & Parmeter (1963) showed that pairing of single basidiospore isolates on agar gave rise to new cultural types readily distinguishable from either contributing strain. These new strains had the cultural characteristics of both 'parent' strains and showed predictable inheritance of physiological characters of the two contributing strains, including pigmentation and colony morphology. Later studies showed that pairing of two homokaryons obtained, via basidiospores, from an individual parent strain resulted in tuft formation at the interaction zone; removal and plating of mycelia from this tuft resulted in cultures with the characteristics of both parent strains (Garza-Chapa & Anderson, 1966). Other multinucleate basidiomycetes such as *Heterobasidiun annosum* (Fr.) Bref. (Stenlid & Rayner, 1991), *Coriolus versicolor* (Leslie, 1993) and *Stereum hirsutum* (Willd. ex Fr.) S.F. Gray (Coates *et al.,* 1981) have been shown to form tufts upon pairing, and for many years heterokaryosis was scored on the basis of tuft formation.

More recently work by Cubeta and colleagues (1993) with *R. solani* AG4 showed that although tufts often will form upon heterokaryosis, the process can occur without tuft formation. In their study phenotypic differences such as pigmentation suggested heterokaryosis in the absence of tufts. In other pairings a bow-tie reaction was observed where the putative heterokaryon expanded outwards towards one or

both ends of the Petri dish. These studies conclude that although tuft formation is a good indicator of heterokaryon formation it is not a prerequisite for cell type mixing.

Cubeta and colleagues (1993) suggested that a test of heterokaryosis was pairing the putative heterokaryon with the parent homokaryons from which it was derived. This resulted in a zone of clearing, which is indicative of an antagonistic response, or formation of a white line at the interaction zone. The zone of clearing is the incompatibility reaction observed in the present study. In the present study barrage formation was consistently seen; there were no other types of incompatibility reaction such as tuft formation. Pairing of two different AG4 strains, PA1 and I13, also resulted in barrage formation, as would be expected when two unlike heterokaryons are allowed to anastomose, it is notable that pairings of hyphal-tip subcultures of strain PA1 also gave similar barrage formation. But of interest also is the finding, here, that hyphal tip subcultures could give rise to strains that initially showed only weak barrage formation with one another or with the parent, but displayed strong barrage formation when the separate strains were repeatedly subcultured before anastomosis.

One of the major considerations in vegetative incompatibility in *R. solani* is the role of nuclei. Upon anastomosis nuclear migration has been observed (Whitney & Parmeter, 1963), and it has been suggested this may lead to heterokaryosis. Nuclear numbers in tip cells of both PA1 and I13 were assessed in the present study. The tips were shown to be multinucleate and although the nuclear number per tip cell varied this is commonly found in *R. solani* strains (Butler & Bracker, 1970). There was certainly no evidence that the parent-incompatible hyphal tip cultures had consistently reduced nuclear numbers compared with the parent strains from which they were derived. So it might be assumed that these parent-incompatible daughter strains were homokaryons (or at least less heterogenic) formed by random assortment of nuclear genotypes in the hyphal tip cells. Although due to lack of parental reformation this is not conclusive.

In a study by Cubeta and colleagues heterokaryon formation in *R. solani* nuclear transfer has been shown to be both bi-directional and unidirectional (Cubeta *et al.*, 1993). In the latter study nuclear migration was detected by subculturing from

either side of the hyphal interaction zone following pairing of homokaryons, and the mycelia were then paired with both parents (homokaryones) and putative heterokaryons. Using this method, 12 from 30 heterokaryons showed bi-directional migration, and 3 of 30 showed unilateral migration. Migration of nuclei throughout the colony was obvious in strains which were phenotypically different, such as in pigmentation, as transfer of pigment-forming ability could be observed from the tuft further back into the colony. However, not all putative heterokaryons showed strong incompatibility responses when paired back with their original parents, so in such cases there was no clear evidence of nuclear migration.

From the work by Cubeta and co-workers (1993) on nuclear migration into heterokaryons there appears to be variation in the direction, and occurrence, of migration. Also, previous observations by Flentje and Hawn (1963) did not reveal nuclear migration through the septal pore. Nonetheless, the work by Cubeta *et al.* (1993) suggests a role for nuclear migration in compatibility as mycelial subcultures from either side of the junction gave rise to heterokaryotic colonies which were somatically compatible with tuft isolates; in the absence of nuclear migration heterokaryon formation would be restricted to a relatively narrow region where a tuft forms. Secondly, the bow-tie reaction was observed, where a morphologically distinct heterokaryon expanded from the centre of the interaction zone to one or both sides of the plate. Similar bow-tie formation has been observed in *S. hirsutum* (Coates *et al.*, 1981) as the partial or complete replacement of certain nuclei from one strain to another. Although in this present study no direct evidence was obtained for nuclear loss, there was a lower number of nuclei in some hyphal tip subcultures (see Tables 5.2 & 5.8) though not all of them.

Previous work has suggested that only two nuclear types are present in field isolates of *R. solani* and that equal numbers of each nuclear type are present per cell (Whitney & Parmeter, 1963). The data in the present study do not support this. For example in PA1 the parent strain has a mean of 4.2 nuclei per cell whereas the tips of incompatible strains had means ranging from 3.1 to 3.7. If there were equal numbers of two nuclear types per cell the number would be expected to drop by half upon incompatibility. As only approximately one quarter of nuclei seem to be lost in

incompatible tip cultures of PA1, and even less from I13, it suggests either there are more than two nuclear types present in the parent strain or there are unequal numbers of nuclear types can be tolerated under certain conditions. It may also be, if nuclear number is regulated, that loss of one nucleus results in replacement by another nucleus, not necessarily of the same type.

Heterologous cell fusion can lead to nuclear fusion (Ellingboe, 1965). Although homologous nuclear fusion may take place at a low frequency; consequently a fusion cell may contain nuclei of genetically separate origin, it does not appear to be stimulated by homologous cell fusion in eucaryotes (Lane, 1981). Heterologous cell fusion does, however, lead to karyogamy between sexual gametes in the same species and interspecificity between somatic cells in fungi (Kevei & Peberdy, 1977). Heterogenic somatic fusion within a species can also stimulate karyogamy. In basidiomycetes such fusions have been shown to lead to somatic recombination which is often quite selective. Two types of matings have been made: homokaryon x homokaryon to form a dikaryon or heterokaryon by recombination, or homokaryon x dikaryon to form a new dikaryon due to recombination and subsequent dikaryotisation of the homokaryon (Ellingboe, 1965). As a result of recombination events the new heterokaryons are somatically incompatible with both parental types.

Sectoring of colonies may occur following recombination in some cells but not in others. Sectoring due to recombination may help explain partial compatibility as, if recombination occurred between two cells upon fusion, the recombined cells would differ from either the parent or the homokaryons. Sectoring can spread throughout a colony and pairing of the recombined sector with either homokaryons or heterokaryons would result in barrage formation (Cubeta *et al.*, 1993). Purification of the recombinant sector would result in a new compatibility group which would not pair with existing compatibility groups (Elllingboe, 1965), so formation of the original heterokaryon would never occur.

To show definite loss of a nuclear type, resulting in loss of a vegetative compatibility gene, nuclear markers are needed. There are some auxotrophs of *R. solani* with respect to thiamine, lithium chloride and nitrate reductase (Anderson,

1982; Puhalla & Carter, 1976; Bolken & Butler, 1974) and mating of auxotrophs resulting in complementation would show nuclear complementation. Unfortunately most auxotrophs are unstable and, as they must be modified to be auxotrophic, this technique may not be useful for the study of natural isolates. To show nuclear loss more specific techniques like RAPD (randomly amplified polymorphic DNA) and PCR analysis of the fungal chromosome would be needed to show loss of specific markers.

The main reason for investigation into vegetative incompatibility resulted from the incompatibility between hyphal tip subcultures in I13 and PA1 which coincided with apparent loss of dsRNA and an increase in the production of some cellular enzymes important in pathogenicity in some strains (Chapter 4). Examination of the tip subcultures of PA1 showed the loss of dsRNA from both parent-compatible and parent-incompatible strains (Figure 5.3). As this loss occurred from all strains, some of which were still compatible with the parent it excludes dsRNA as a factor in vegetative compatibility, so compatibility must be encoded by nuclear or mitochondrial genes. Previous studies on *Neurospora* (Beadle & Coonradt, 1944), *Aspergillus* (Croft & Jinks, 1977), *Fusarium* (Correll *et al.*, 1988) and *Cryphonectria* (Anagnostakis, 1982) have shown vegetative compatibility to be nuclear encoded, therefore it is expected that the genes are encoded by the nuclei in *R. solani,* and are perhaps related to the H-factor genes. Indeed, it is thought that heterogenic and homogenic incompatibility mechanisms interact inversely.

Further evidence that loss of dsRNA was not involved in compatibility was obtained by a successive subculture of strain T1, a parent-compatible strain without dsRNA, to produce T1A which was incompatible with PA1. This incompatibility was the same as that of T12, T13, T14 and T16. The original daughter T1, which was compatible with PA1, was paired with the parent to try to transfer dsRNA between strains thereby fulfilling Kochs postulates. However, no transfer of dsRNA was observed by this method suggesting either that dsRNA remained localised in PA1 (the subcellular location of the dsRNA has not been clearly determined although recent studies indicate a cytoplasmic location (Kousik *et al.*, 1994; Lacksman & Tavantziz, 1994) or that it was transferred at undetectable levels. This is in contrast

to *C. parasitica* and *O. ulmi* dsRNA which can be transferred very efficiently between cells by anastomosis (Anagnostakis & Day, 1979; Brasier, 1983). In *C. parasitica* at least, it is thought that even incompatible strains may be able to transfer dsRNA, as the dsRNA is so close to the hyphal apex (B. Hillman, pers. comm.). Unlike the case in the latter fungi, dsRNA in *R. solani* is largely thought to be located further back in the hyphae (Buck, 1986a), hence the ease of curing by hyphal tip subculture. Transfer during anastomosis may therefore be very slow. A very recent study by Lacshsman & Tavantzis (1984) has shown dsRNA to be integrated into the chromosome of *R. solani,* in some cases, so this possibility cannot be eliminated in this study. The possibility that in some 'cured' strains the dsRNA has integrated via a DNA intermediate and others have not may reflect on vegetative incompatibility. Although it is unlikely that dsRNA is involved in vegetative incompatibility it cannot be excluded from the reaction at the present time.

Although Koch's postulates were not fulfilled, ie. it was not possible to demonstrate a relationship between dsRNA and strain compatibility or phenotype, it is still of interest that pectic and cellulolytic enzyme activities varied between strains. For example, both pectin lyase and polygalacturonase activity showed a large degree of variation (Tables 5.4 & 5.4) but only in the case of strain T13 was there any significant increase in enzyme production relative to that of the parent strain. This was seen with polygalacturonase, and the difference was almost as great as that described in Chapter 4 for dsRNA-cured strains when compared with the parent PA1.

The cellulase assay showed no significant differences between strains. Both sets of data differ from those in Chapter 4 which showed a significant increase in both pectic and cellulose enzyme production upon repeated hyphal tip subculture and which also resulted in loss of dsRNA. In the present chapter all strains analysed resulted from single hyphal tip subcultures and, although all had lost dsRNA not all were incompatible with PA1.

These results show two interesting findings. (1). Double-stranded RNA seems to play no identifiable role in vegetative incompatibility or the regulation, or production, of important pathogenic enzymes in *R. solani.* (2) Changes in vegetative incompatibility, presumably resulting from changes in nuclear genotype, did not

affect the production of the aforementioned enzymes in a consistent manner. Unfortunately the data do not explain the results obtained in Chapter 4. It might be postulated that successive hyphal tip subculture has resulted in a mutation in the strain, or that loss of a negative regulator has resulted in increased production of the enzymes.

The data presented in this chapter represent novel findings as to the breakdown of a heterokaryon. Double-stranded RNA does not appear to play a role in vegetative compatibility or specific enzyme production in AG4 strains so further studies are needed to elucidate both its role (if any) and also the genetic basis of vegetative incompatibility.

CHAPTER 6

ISOLATION AND CHARACTERISATION OF AN EXTRACHROMOSOMAL DNA ELEMENT FROM *RHIZOCTONIA SOLANI* ANASTOMOSIS GROUP 4

6. 1. INTRODUCTION

Double-stranded RNA particles have been identified from a variety of plant pathogenic fungi and, in a number of cases the presence of these elements has been correlated with a reduction in fungal virulence. However, it has only recently become apparent that many fungal species contain extrachromosomal DNA elements (for a review see Meinhardt *et al.,* 1990). Unlike bacterial plasmids, which are normally circular elements and often confer a selective advantage to the cells such as antibiotic resistance, eucaryotic plasmids appear to be linear, but their study has been neglected as most appear to be cryptic in function. It was not until plasmids with known functions were characterised, such as the *Kluyveromyces lactis* killer plasmids (Gunge *et al.,* 1981) and *Fusarium oxysporum* virulence plasmids (Kistler & Leong, 1986), that wider interest was stimulated in the distribution and function of eucaryotic linear DNA elements.

With *Rhizoctonia solani,* the characterisation of extrachromosomal dsRNA elements, and their function, has been investigated; but only one group of workers has reported the presence of extrachromosomal DNA elements in this organism (Hashiba *et al.,* 1984; Miyasaka *et al.,* 1990; Miyashita *et al.,* 1990), where they were reported to be involved in the attenuation of virulence. During the extraction of dsRNA from *R. solani* strain I13 (Chapter 4) the presence of an extrachromosomal DNA element was detected. Although Hashiba and colleagues (1984) had correlated extrachromosomal DNA elements with reduced virulence of *R. solani,* in the present study strain I13 retained its DNA element upon curing and also retained its virulence (Table 4.4). This suggested that extrachromosomal DNA is not always associated with decreased pathogenicity. Further study of this extrachromosomal DNA is presented in this chapter.

6.2. RESULTS

6.2.1. Isolation of a DNA Element from *Rhizoctonia solani*

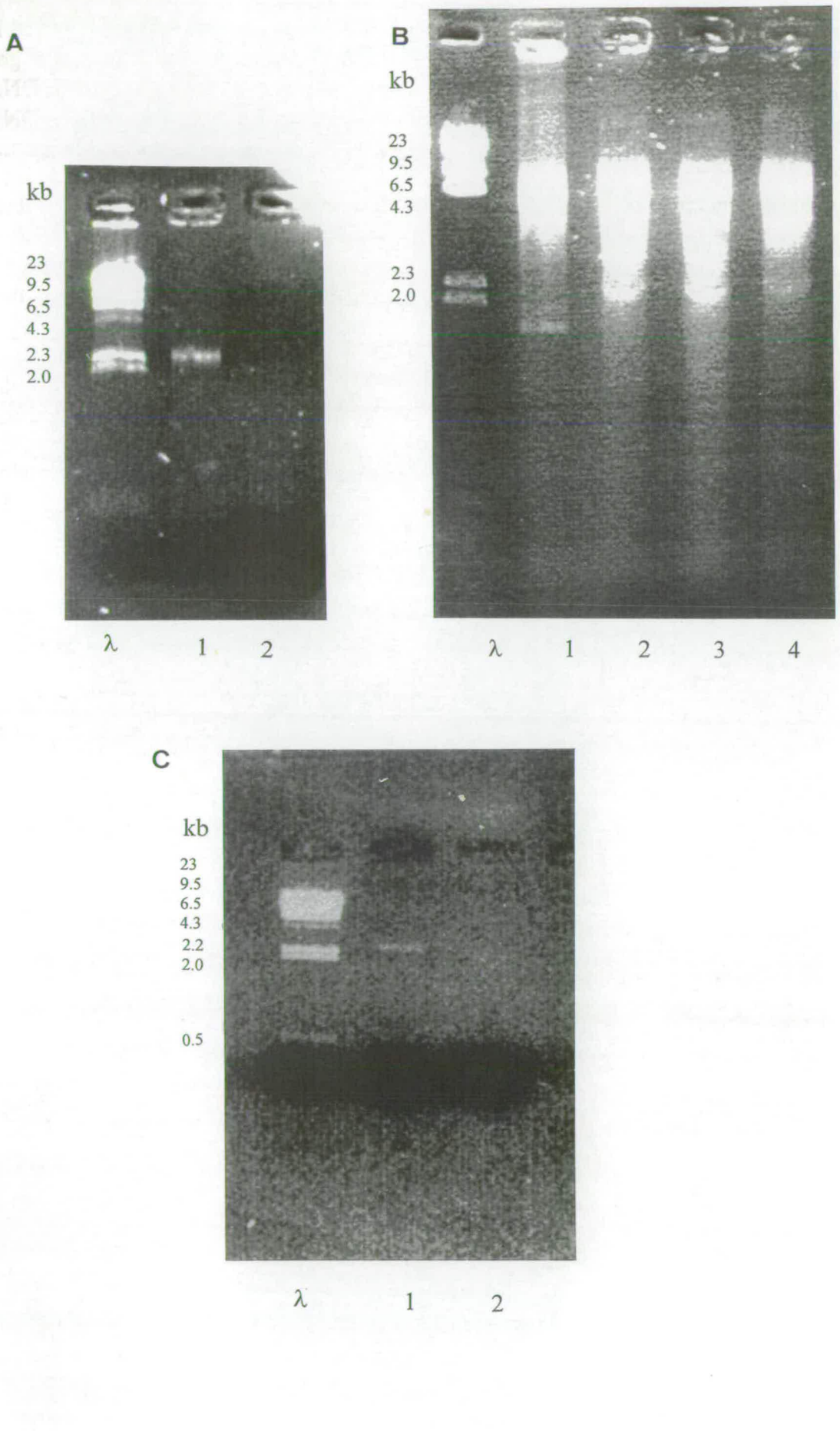
Initially, analysis of extrachromosomal elements of *R. solani* was carried out using a cellulose CF-11 column for the specific isolation of dsRNA following the method of Morris & Dodds (1979). However, a low yield of dsRNA was obtained. To test if much of the dsRNA was being lost during the column procedure total nucleic acid was isolated from *R. solani* by a simple phenol:chloroform extraction procedure followed by ethanol precipitation (see Chapter 2). Isolation of total nucleic acid appeared to result in the presence of an additional genetic element in AG4 I13; it migrated with a size of 2.4-2.5kb, which was larger than the dsRNA elements observed previously (see Figure 4.1). In order to confirm the nature of this element it was incubated with 50 units of either ribonuclease A or deoxyribonuclease I for 30 minutes at 37°C. The element was found to be resistant to the action of ribonuclease but succeptable to that of deoxyribonuclease (Figure 6.1a) and so this band was presumed to be a DNA plasmid. Purification and partial characterisation of the element was carried out.

6.2.2. The effect of DNA on *R. solani* virulence

Even after curing strain I13 of dsRNA by hyphal tip subculture (Chapter 4), the strain still contained extrachromosomal DNA and was found to be highly virulent on a variety of plants (Table 4.4).

As the DNA elements were retained during hyphal tip subculture, attempts were made to cure the strains of plasmids by exposure of the macerated mycelia to ultra-violet light. Mycelia (0.5g) was macerated using a mortar and pestle and the fragments were spread onto a Petri dish. They were irradiated for times ranging from of 2 to 60 minutes at 254nm, using a strip light at a distance of 15cm from the Petri dish, and then were incubated to allow growth from any viable fragments. Irradiation proved unsuccessful in plasmid curing, probably due to the pigmentation of the mycelial walls which may provide protection against UV irradiation. Heat-induced plasmid curing was also attempted by incubating mycelia at 30°C for 10 days to

Figure 6.1. A. I13 extrachromosomal DNA element was isolated by phenol:choroform extraction and ethanol precipitation as described in Chapter 2. After digestion for 30 mins with 50 units RNase A the sample was electrophoresed in 1% agarose (w/v) and the extrachromosomal DNA removed from the gel, genecleaned and electrophoresed on a 1% agarose (w/v) gel. Lane 1 shows λ DNA cut with *HindIII*. Lane 2 shows purified I13 DNA (5μg). Lane 3 shows I13 DNA digested with 50 units DNase I for 30 minutes. **B.** Total I13 nucleic acid before and after hyphal tip subculture. Curing of extrachromosomal dsRNA and DNA were attempted by continuous hyphal tip subculture, every 3 days over a period of 30 days followed by total nucleic acid extraction as described in Chapter 2. λ DNA was digested with *HindIII*. Lane 1 shows total I13 nucleic acid before hyphal tip subculture. Lanes 2, 3 and 4 show total I13 nucleic acid after hyphal tip subculture (lane 2). Further attempts to cure the DNA were carried out by exposure to UV light for 30 mins (lane 3) and heat treatment by incubation at 35°C for 7 days (lane 4). **C.** 1% agarose gel showing alkali hydrolysis of I13 extrachromosomal DNA element. The element (5μg) was purified by phenol:chloroform extraction and RNase digestion as described in Chapter 2. Lane 1 shows λ DNA digested with *HindIII*. Lane 2 shows purified I13 DNA. Lane 3 shows purified I13 DNA following hydrolysis with 0.1N NaOH for 16 hrs at 36°C.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1
2
3
4
C
kb
23
9.5
6.5
4.3
2.2
2.0
0.5
λ
1
2

induce stress related curing, but the DNA could still be isolated from cells (Figure 6.1b). This suggests that, unlike dsRNA, the DNA element is located equally in hyphal tip cells as well as further back in the mycelia.

Removal of the DNA element and therefore comparison of virulence of strain I13 with and without the plasmid could not be achieved. Nevertheless biochemical and molecular characterisation of the plasmid was carried out.

6.2.3. Structural characterisation of the extrachromosomal DNA element from AG4 I13.

The nature of the DNA element was examined using criteria previously applied to *R. solani* plasmid pRS-64 (Miyashita *et al.*, 1990) and the killer toxin plasmids, K1 and K2, from *K. lactis* (Sor *et al.*, 1983; Kikuchi *et al.*, 1984). As the element was present in low quantities, a bulking up procedure was used which consisted of isolation of total nucleic acid from 10g wet mycelia, followed by digestion with 10 units of ribonuclease A for 30 minutes at 37°C. The digested nucleic acid was then electrophoresed on a 1% agarose gel where the extrachromosomal DNA could be clearly identified. Bands of extrachromosomal DNA were cut from the gel and 'Genecleaned' (Chapter 2). The clean DNA was resuspended in 20µl of sdH_2O to give a more concentrated sample which could be further manipulated.

A linear nature of the molecule was suggested as the undigested plasmid appeared largely as a single form, unlike the covalently closed circular, open circular and nicked forms of circular plasmids. Random prime labelling of the whole element could also be carried out following heat denaturation (100°C for 10 min) using α-^{32}P dCTP and the Klenow fragment of *E. coli* DNA polymerase I, which will only act on a single stranded linear or circular DNA template (Sambrook *et al.*, 1989). If the molecule was circular (and did not display significant regions of complementarity), it would label inefficiently. Label incorporated into the DNA was 80% (Table 6.1) as estimated using TCA precipitation and scintillation counting as described in Chapter 2. Hydrolysis of the element by 0.1N NaOH also supported the

	Counts per Minute		
	Before Washing	After Washing	% Incorporation
I13 Element	250,000	200,000	80
Background	25	28	

Table 6.1. Measurement of percentage incorporation of radiolabel into the extrachromosomal DNA element of *R. solani* AG4 I13. Efficiency of incorpoation of radiolabel into the sample was taken by spotting 100µl of labelled reaction mixture onto a GT/C membrane filter which was placed in a scintillation vial and counted. The filter was then washed in 10% trichloroacetic acid to remove the unincorporated radiolabel and counts per minute were reassessed. The percentage incorporation of radiolabel into the DNA fragment was calculated from this.

linearity of the element as there was complete denaturation of the molecule (Figure 6.1c).

As the molecule appeared linear, it must possess specific structural components at its ends which prevent degradation and possibly serve as primers for replication of the element. The previously reported plasmid from *R. solani* AG4, pRS-64 was shown to have hairpin loops at either end of the molecule (Figure 1.4) (Miyashita *et al.* 1990a). This is in contrast to most eucaryotic linear plasmids, of which the *K. lactis* plasmids are typical examples, which contain protein covalently attached to their 5' ends. To distinguish between these two possibilities, the I13 plasmid found in the present study was subjected to exonuclease digestion, radiolabelling and proteinase digestion.

Examination of the 5' end of the molecule

The availability of the 5' end for modification was tested by 5' end labelling of the molecule by polynucleotide kinase and γ-^{32}P dATP. The DNA, enzyme and dNTPs were incubated at 37°C for 1 hour, then the reaction was stopped by heat inactivation of the enzyme for 10 minutes at 70°C. A simultaneous experiment was carried out using pBluescript, linearised with *SmaI* to give a blunt end, to test efficiency of labelling. No detectable label was seen to be added to the 5' end of the I13 element after polyacrylamide gel electrophoresis, and exposure to photographic film. In contrast pBluescript labelled efficiently.

As no labelling of the I13 element occurred there was thought to be a blockage at the 5' end of the element. Exonuclease digestion with λ exonuclease which specifically digests linear DNA in a 5' to 3' direction, was therefore carried out to verify this possibility. 16 units of enzyme were added to 15μg I13 DNA at 37°C and, after time intervals of 0, 2, 5, 10 and 20 minutes, aliquots were removed and the enzyme was inactivated by the addition of EDTA to a final concentration of 50mM, on ice (Chapter 2). The DNA was then subjected to agarose electrophoresis. Figure 6.2a shows that the element of I13 DNA was resistant to λ exonuclease, whereas pBluescript linearised with *SmaI,* was digested within two minutes (Figure 6.2b).

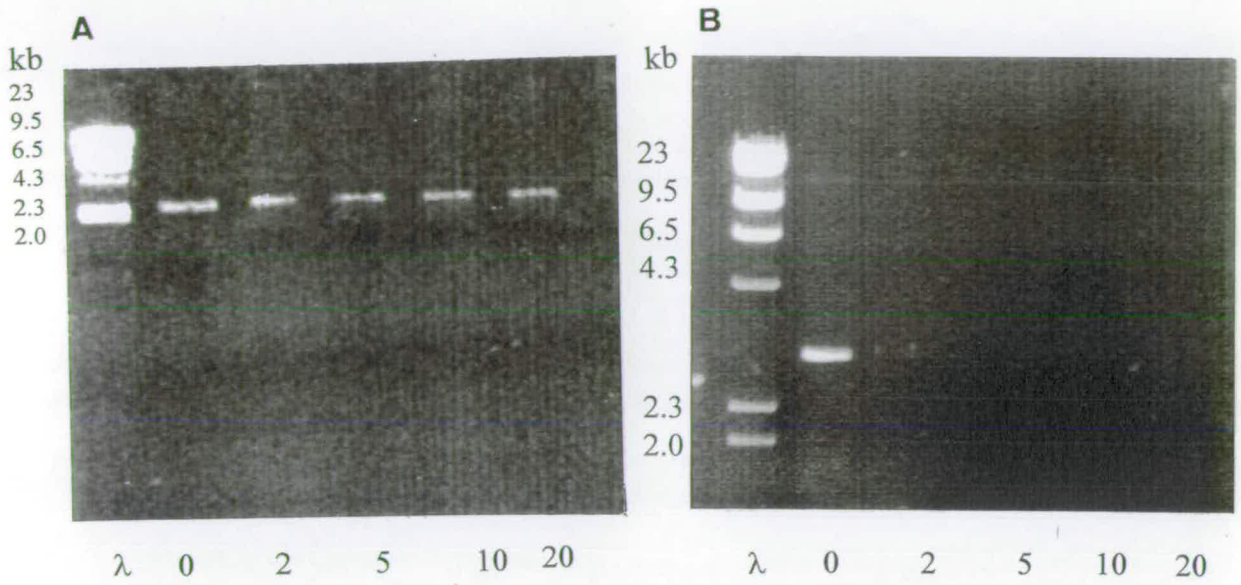


Figure 6.2. λ exonuclease digestion of the 5' end of AG4 I13 extrachromosomal DNA element. **A.** 15 μg of I13 DNA was incubated in the presence of 16 units λ exonuclease (a 5'-3' exonuclease) for 0, 2, 5, 10 or 20 minutes, after which time the reaction was stopped by the addition of EDTA on ice. Samples were electrophoresed on 1% agarose (w/v). λ DNA was digested with *HindIII* to give size markers. The sizes are shown at the side of the photograph. Labels show I13 DNA digested for the undernoted time by λ exonuclease. The DNA was not susceptible to digestion by the nuclease at any of the time periods tested. **B.** Positive control showing λ exonuclease digestion of 15μg linearised pBluescript under the same conditions as in A. As in A, λ DNA was digested with *HindIII* and the sizes of the fragments are at the side of the photograph. The linearised plasmid is digested after less than 2 minutes incubation with the enzyme.

To test whether protein was present at the 5' end of the molecule, the element was incubated with 10μg proteinase K for 30 minutes at 37°C and subjected to electrophoresis. There was no detectable shift in band size after incubation with proteinase K which might have suggested removal of covalently attached proteins. However unmodified migration may be due to any attached proteins being of very small size, or the large size of the DNA fragment making removal of small amounts of protein undetectable under these conditions.

A repetition of the λ exonuclease experiment was carried out after incubation of the I13 element with proteinase K, so that if removal of protein occurred, leaving free ends, then the enzyme might now be able to digest the DNA. No λ exonuclease digestion of the element occurred under these conditions, suggesting either extremely tight protein binding or a structural blockage at the 5' end.

Examination of the 3' ends of the molecule

Access of the 3' end of the element to nuclease digestion was examined in a similar manner to that of the 5' end, except that attempted digestion was carried out using exonuclease III, an enzyme which specifically degrades from the 3' OH end of blunt or recessed 3' DNA, or single-stranded nicks in DNA, in the presence of magnesium ions. 15μg of I13 DNA and 38 units of enzyme were incubated at 37°C, in a water bath, for periods of 0, 2, 5, 10, 20 and 30 minutes, then samples were removed from the mixture and adjusted to a concentration of 50mM EDTA on ice (see Chapter 2). A simultaneous control experiment was carried out, as before, using blunt-ended, linearised, pBluescript as the substrate. Figure 6.3a shows digestion of the 3' ends of the I13 element while 6.3b shows digestion of the plasmid control. In both cases, the DNA has clearly been digested by exonuclease III, although the I13 DNA does not digest as cleanly nor as rapidly as the control plasmid and so gives a smeared effect. The unusual digestion of the 3' end I13 DNA may be due to the blocked 5' end preventing ease of accessibility of the complementary 3' end for the enzyme.

The freedom of the 3' end was also tested by attempting to label this end with 1 unit of T4 DNA polymerase and α-^{32}P dCTP, by the rapid end-labelling method

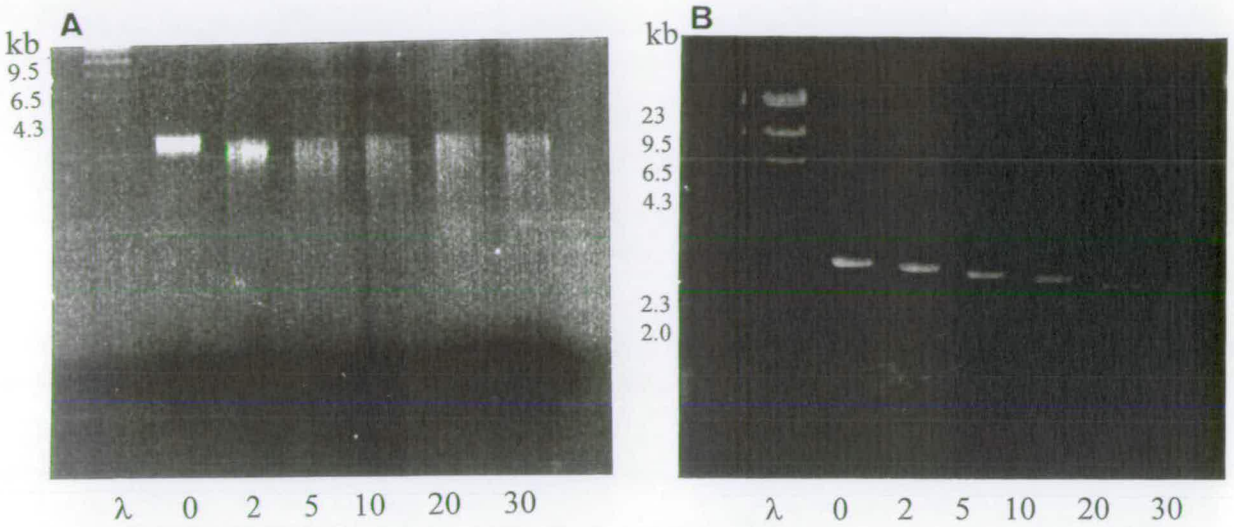


Figure 6.3. Exonuclease III digestion of the 3' end of AG4 I13 extrachromosomal DNA element. **A.** 15μg I13 DNA was incubated, at 37°C, for 0, 2, 5, 10, 20, and 30 minutes, with 38 units of exonuclease III (a 3'-5' exonuclease), then the reaction was stopped by the addition of 2μl of 0.5M EDTA on ice. Samples were electrophoresed on 1% agarose. The numbers at the side of the photographs are λ DNA digested with *HindIII*. Labels show I13 DNA digested for the undernoted time period by exonuclease III. Progressive digestion of I13 extrachromosomal DNA was observed over the time period. **B.** Positive control showing 15μg linearised pBluescript digested with exonuclease III, as in A. Progressive digestion of the plasmid can also be seen here.

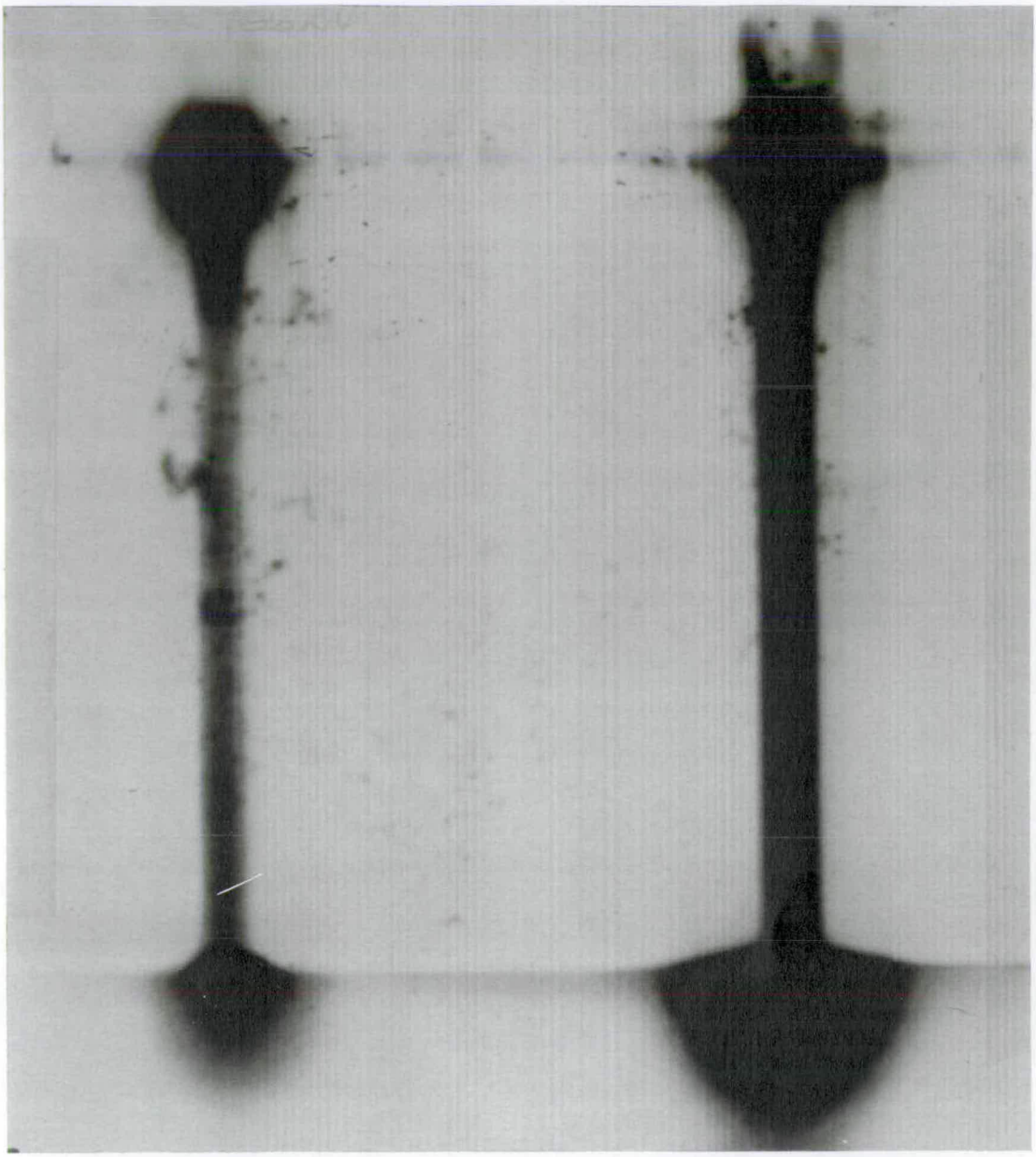


Figure 6.3. C. 3' end labelling of I13 nucleic acid with 32P α-dCTP (3000μCi/ml) and T4 DNA polymerase. I13 extrachromosomal DNA was purified by phenol:chloroform extraction and 'Genecleaned' as described in Chapter 2. Purified DNA (~5μg) was incubated with 2.5 units polymerase for 1 hour at 37°C. pBluescript linearised with *EcoRV* was labelled under the same conditions as a control. Samples were electrophoresed in 5% polyacrylamide. Lane 1 shows labelled pBluescript. Lane 2 shows labelled I13 extrachromosomal DNA.

(Sambrook *et al.*, 1989). The DNA and radiolabel were incubated at 37°C for 1 hour with the enzyme and the reaction was stopped by heat inactivation of the enzyme at 70°C for 10 minutes. Labelled DNA was visualised by polyacrylamide gel electrophoresis and autoradiography (Figure 6.3c). The 3' ends of the element were still available for labelling under the conditions described, further confirming that there was no significant blockage at the 3' end of the molecule.

6.2.4. Partial *SauIIIA* digestion of I13 DNA element

Cloning and sequence analysis were therefore carried out in order to gain further information about the element and to elucidate its function. Direct blunt end cloning of the element was attempted, before and after filling in the ends with DNA polymerase I (Klenow fragment) and dNTPs, but it proved unsuccessful in all cases. This was thought to be due to the blockage of the 5' ends of the element (shown in the above studies to nuclease digestion and radiolabelling). To circumvent the possible blockage at the ends of the element, a partial *SauIIIA* digest of the DNA was carried out to make a *SauIIIA* library in which internal fragments of the I13 element could be cloned and analysed by sequencing. 20µg I13 DNA was digested with 1 unit of *SauIIIA* at 37°C for 10 minutes. The reaction was stopped by the addition of 20mM EDTA on ice. The digested DNA was electrophoresed on 0.7% agarose (w/v) (Figure 6.4a) where a range of fragments from less than 100bp to the full length fragment were observed. The digested DNA was then extracted from the gel and Genecleaned. Purified fragments were cloned into pBluescript, which had been cut with *BamHI*, at relative concentrations of vector:insert of 1:10. Ligation was carried out at 16°C for 12-16 hours and the ligated vector containing insert was transformed into competent *E. coli* JM109 or NM522 cells (see Chapter 2). A blue/white screen was employed, using X-gal, for colonies which were resistant to ampicillin.

White, ampicillin-resistant colonies, presumed to carry pBluescript containing I13 DNA, were patched onto LBamp and minimal media. Small scale plasmid preparations were carried out from the patched colonies by the method of He *et al.* (1991), and the plasmid DNA was digested with *PvuII*. The restriction endonuclease *PvuII* was specifically chosen as the enzyme cuts either side of the polycloning site to

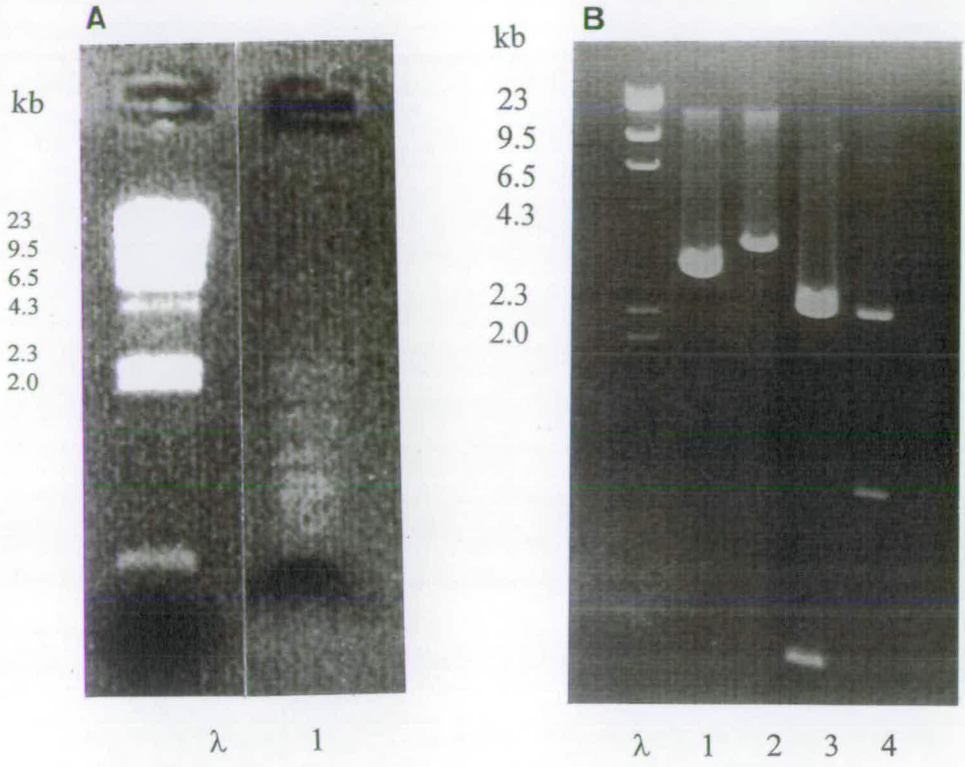


Figure 6.4. A. *SauIIIA* digestion of I13 extrachromosomal DNA was carried out by incubating 20μg purified DNA with 1 unit of enzyme at 37°C for 5 minutes. The reaction was stopped by the addition of 20mM EDTA on ice and the digested DNA was electrophoresed on 0.7% agarose (w/v). Panel A shows λ *HindIII* markers. Lane 1 shows I13 DNA digested by *SauIIIA*. **B.** Plasmid minipreparations of pBluescript and pMC1 digested with *PvuII* and *EcoRI*. Plasmid DNA was extracted from *E. coli* JMI09 by the method of He *et al.* (1991) as described in Chapter 2. In both cases pBluescript was digested as well as pMC1 (pSK containing I13 DNA insert). Lanes 1 and 2 show pBlueskript and pMC1 digested with *EcoRI*. Lanes 3 and 4 show pBluescript and pMC1 digested with *PvuII*. The excised *PvuII* fragment from pBluescript is 445bp.

liberate a 445bp fragment from pBluescript DNA. Linearisation of the plasmid was also obtained by digestion with *EcoRI* (Figure 6.4b). The presence of the flanking plasmid sequence allowed detection of small inserts on a 1.5% agarose gel (w/v).

Southern blotting and hybridisation to each of the inserts with random prime α-^{32}P dCTP-labelled I13 DNA element was carried out to check that the inserts were correct. By southern analysis only 21 clones were obtained, most of which contained very small fragments of <50bp in length. However, only 3 larger clones were obtained containing around 500-600bp, so these fragments were further analysed by sequencing.

6.2.5. Nucleotide sequencing of pMC1

Double-stranded and single-stranded sequencing of pMC1, a plasmid containing approximately 600bp of I13 extrachromosomal DNA (Figure 6.4b), was carried out using pBluescript T_7 and T_7 reverse primers (Table 2.4). The plasmid containing 600bp was used as it was the largest clone obtained.

Single-stranded DNA was obtained using the M13 origin of replication on pBluescript and the helper bacteriophage M13 KO7 (Vieira & Messing, 1987). Single and double-stranded sequencing was carried out using the method described in Chapter 2. 257bp of sequence was obtained using the T7 primer and 319bp was obtained using with the T7 reverse primer. However, as there was no overlap of sequence, an internal primer, P1 (Table 2.4), was used to sequence the intervening region. The combined sequence from all primers gave a DNA fragment of 643bp, as shown in Figure 6.5.

Some of the other clones were also sequenced. All contained at least partial overlap with pMC1, but there appeared to be rearrangements within their sequence as part of the pBluescript polylinker was found to be present within the insert and was flanked by I13 DNA. Due to these problems with recombination, the other clones were not used for further analysis.

```
  1  ccgggGATCA CGCATCAGTT CATAGAACCT AGCGCCTTCA GACTTTTTTG
 51  GCCTCTCGAG GTAGCTTAAA TTTCTCTTAC AGAAATTACA AATAAGTCCC
101  ACTGGGCCCT CGTTATGGCA TCTCAGACCA ATATATGGGG CCTAGCTTTC
151  AAATGCTAGG GCCTACTAGG CTACCTTTGG GGGTTCCGAA AGGTAGACGG
201  TCTTGGCCGC TAAGGGAGTG ACTCCTTAGC ACTATAGCTC ACGCTATAGA
251  GAGGCATGGC AACCGATTGC ACGCCAGCTC AGCCAGCTAC GCCTGAAGCA
301  CTATTGACAC CCGGAAGCAT ATAGGTTGGT GGTTTGTAAA ACCGCCAGCC
351  GCACGACTAA TGTGTACCGG TTGGGGACGA ACGTAATCCT GTTCGATAAA
401  GATAAGTGGA GCATATTGCT CTCTTACCAG ATTGGAGTAG ACCTTCTTAT
451  CTTTTATCGG TTTCTTACGG TGACATCATT GAGTGGTGTC AATAGGCACC
501  CCACAAGACT CGGGGCTCGA GTTTTCACTC GACGCTCGTC TTGTGAGGGT
551  GCTCTCAAGG AGCCTCCGCT CACCCAGCAA AGCTGAATGG GCGGCTTCTT
601  GAGATGATTA GGCCGTAGTA GAACCCTACG GCTTAACGGG AACTAGTCAC
651  Taggtga
```

Figure 6. 5. Nucleotide sequence of the 643bp insert of pMC1 cloned into the *BamH1* site of pBluescript. The sequence is shown as reading from the pBluescript T7 primer, and pBluescript sequence flanking the insert is shown in lower case. The *Bam HI* site at either end is underlined.

6.2.6. Sequence analysis of pMC1

Database analysis of the sequence was carried out using the Wisconsin package Version 7.3, June 1993.

The GC content of the sequenced fragment was 48%. Hypothetical translation of the DNA into protein in all frames showed no continuous open reading frames in either strand which spanned the length of the insert, by common codon usage and mitochondrial codon bias; as previously reported linear DNA plasmids from *R. solani* are thought to be mitochondrially located (Wako *et al.*, 1991; Jabaji-Hare *et al.*, 1994). In mitochondria of filamentous fungi the stop codon UGA is read as tryptophan (Fox, 1987), so often lengthens the open reading frame. Open reading frames were produced by the map program designed by Schroeder & Blattner (1982) and are shown schematically in Figure 6.6, using either valine or methionine as the initiation codon. Only a single open reading frame of over 250bp and three of over 150bp in length were obtained. However, two open reading frames extended to the edges of the cloned fragment and so their full length could not be determined.

Fasta analysis (Pearson & Lipman, 1988) was carried out to try to elucidate the function of I13 by homology to known sequences in the database. In particular, specific checks were carried out for identification of the presence of conserved regions of DNA polymerase, as a polymerase would be expected if the element is autonomous. However, Fasta analysis showed no extensive homology to any other sequences in the database, even at the DNA level. Alignment was also carried out to the partially sequenced *R. solani* AG5 plasmid pRS188 (Jabaji-Hare, 1994), but only 37% identity was obtained over the whole length of pMC1.

6.2.7. Isolation of the termini of I13 DNA

Sequencing of the internal *SauIIIA* fragment of I13 DNA, pMC1, allowed application of a modification of a RACE (rapid amplification of cDNA ends) experiment (Frohman *et al.*, 1987) to try to amplify and analyse the rest of the I13 element. The experiment exploited the fact that the 3' ends of the DNA may be available for labelling. A polyA tail was added to the 3' end of the DNA by incubation with terminal transferase at 37°C for 10 minutes in the presence of

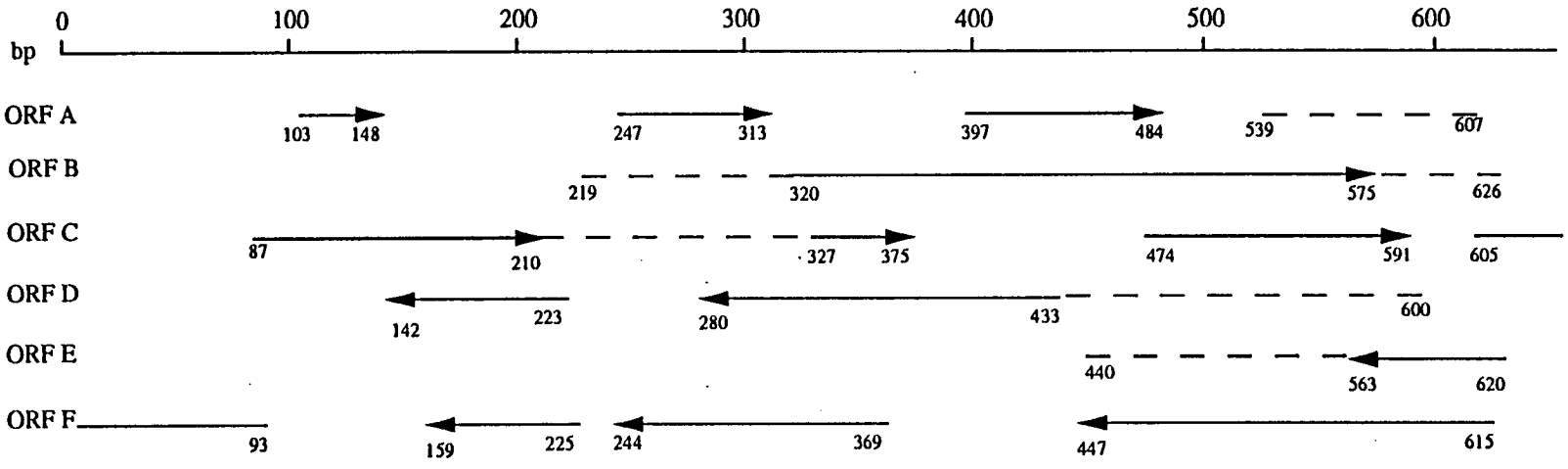


Figure 6.6. Open reading frames of pMC1. Open reading frames of the translated product of sequenced clone pMC1 as shown by MAP analysis (Schroeder & Blattner, 1982). Arrows represent the direction of protein sequence and numbers represent the nucleotide positions, where 1 is the initial nucleotide of pMC1 upon sequencing from the pBluescript T7 primer. Where dashed lines are continuous with filled in lines they represent mitrochondrial codon usage of filamentous fungi where the stop codon UGA is read as tryptophan.

dATP. This enzyme will specifically catalyse the sequential addition of dATP to the 3' end of the molecule, resulting in a tail.

As internal sequence was available, oligonucleotides from the sequence were designed such that priming of DNA synthesis is towards the outside of the cloned fragment. The primers listed in Table 2.4, P2 and P3, were designed in this way. A polymerase chain reaction (PCR) was set up using the polyadenylated I13 DNA, the poly dT adapter primer (Table 2.4) and either P2 or P3. The conditions for PCR were as described in chapter 2. Upon PCR a smear of DNA was obtained (Figure 6.7). This was expected as the length of the poly A tail added to the 3'-OH end of the molecule would vary. As both primers contained sites for restriction enzymes, the PCR product was cut with the appropriate enzymes after phenol:chloroform extraction and electrophoresed. The poly T adapter primer contained sites for *PstI, PvuII, BalI, EcoRI* and *BamHI.* The internal primers both contained *XhoI* sites. The *XhoI* sites were chosen as they are absent from the insert in pMC1 as shown by the sequence. Upon cutting with any of the aforementioned enzymes, the size of the products diminished greatly suggesting the presence of internal sites for those enzymes in the DNA. Cloning of the products gave many very small clones of less than 100bp. This was not unexpected as transformation favours the internalisation of smaller plasmids. In an attempt to increase the insert size, further clonings were carried out after cutting the PCR product with a single enzyme which recognised one of the primers, after filling in the blunt ends. Ligation was carried out into a plasmid which had the appropriate protruding termini at one end of the linear vector DNA and a blunt end at the other. Transformation was efficient, giving a large number of white ampicillin-resistant colonies. However all of these still appeared to have very small inserts.

Southern hybridisation of all the clones showed no homology with the I13 element. Some of the small fragments were sequenced and all contained a polyA tail of 19bp but no sequence which overlapped with pMC1 was detected. The experiment was repeated on a number of occasions but the same result was obtained in each case.

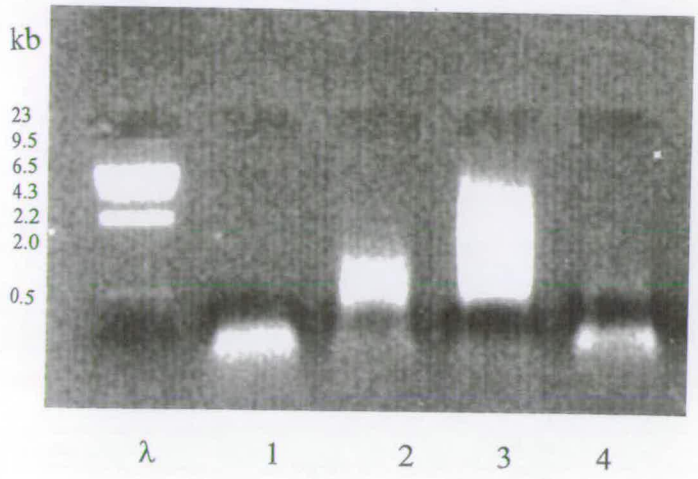


Figure 6.7. Polymerase chain reaction (PCR) amplification of tailed fragments of I13 extrachromosomal DNA. Size markers are given, being λ DNA digested with *Hind III*. Lane 1 shows control for tailed product 1 (primers only). Lane 2 shows PCR product 1. Lane 3 shows PCR product 2. Lane 4 shows control for PCR product 2 (primers only). Both PCR products were obtained using I13 DNA to which a polyA tail has been added using terminal transferase as a template. Product 1 has been primed using a polyT primer (P1), which has a linker on the end containing sites for specific restriction endonucleases and internal I13 primer 2. Product 2 is the result of the same polyT primer used in conjunction with internal I13 primer 3. Using these two primers it was hoped to amplify the I13 extrachromosomal DNA fragment in 2 sections.

6.2.8. Genetic relatedness of DNA elements from *R. solani*

Many fungal species, such as *Podospora anserina* (Esser, 1990) and *Neurospora crassa* (Bertrand *et al.*, 1985), excise mitochondrial plasmids during senescence. To test the uniqueness of the I13 DNA element, cross-hybridisation studies to *R. solani* strains from a range of anastomosis groups and to a variety of other plant pathogenic fungi, were carried out and the subcellular location of the plasmid was investigated.

Total nucleic acid was isolated from 11 species of *R. solani* comprising 7 anastomosis groups. I13 DNA, labelled by random priming, was used as a probe to hybridise to southern blots of *R. solani* total nucleic acid using the formamide protocol described in Chapter 2. The purified element was also included as a positive control. Figure 6.8 shows the homology between the I13 element and other *R. solani* strains. The element appears to be specific to anastomosis group 4, although it is not ubiquitous within this anastomosis group as strain PA1 does not contain the element. Much fainter larger bands were also observed on occasion in the strains with homology to the I13 element, which may be due to dimerisation of the plasmids. This is similar to *R solani* AG5 plasmid pRS188 (Jabaji-Hare *et al.*, 1994) Extrachromosomal DNA elements have been detected from strains in other anastomosis groups examined (Miyashita *et al.*, 1990) and also in this study in AG2-2 strain C127 (see Table 4.1), but on the basis of hybridisation, there does not seem to be any significant sequence similarity between the nucleic acid from other strains examined in this study from different anastomosis groups, and the I13 element (see Figure 6.8).

The presence of homologies of I13 in other fungal plant pathogens was also investigated by Southern hybridisation to total nucleic acid extracts. These species all contain dsRNA elements, but study of their extrachromosomal DNA content has not been reported previously. The bacterial plant pathogen *Erwinia caratovora* was also tested together with the yeast *Saccharomyces cerevisiae* killer strain in which the killer phenotype is known to be conferred by dsRNA (Sinclair *et al.*, 1967) Unfortunately no strains which are known to contain excised mitochondrial plasmids were available for analysis. Figure 6.9 indicates that none of the DNA present in any

Figure 6.8. Southern hybridisation of AG4 I13 extrachromosomal element to *Rhizoctonia solani* strains from seven anastomosis groups. Total *R. solani* nucleic acid was isolated from a range of strains by phenol:chloroform extraction and ethanol precipitation as described in Chapter 2. Samples were electrophoresed on 1% agarose (w/v) (**A**) and blotted overnight in 10 x SSC onto Zeta probe membrane. Total I13 extrachromosomal DNA was random prime labelled with ^{32}P α-dCTP and used as a probe. Hybridisation was carried out overnight at 43°C in Church solution containing 50% formamide. After incubation, the blot was washed consecutively in 2 x, 1 x, and 0.5 x SSC containing 0.1% SDS each for 30 minutes, then exposed to photographic film (**B**).

bp
23,130
9,416
6,537
4,361
2,322
2,027
564
A
I13 Element
AG2 SC121
AG2-1 HSV
AG2-1 PS4
AG2-2 C127
AG3 SC222
AG4 C233
AG4 I13
AG4 RH5/T
AG4 PA1
AG5 R470
AG6 FIX1
B
I13 Element
AG2 SC121
AG2-1 HSV
AG2-1 PS4
AG2-2 C127
AG3 SC222
AG4 C233
AG4 I13
AG4 RH5/T
AG4 PA1
AG5 R470
AG6 FIX1

Figure 6.9. Southern hybridisation of AG4 I13 extrachromosomal DNA element to a variety of fungal species. Total fungal nucleic acid was isolated from a range of fungal species, and *E. carotovora,* by phenol:chloroform extraction and ethanol precipitation as described in Chapter 2. Samples were electrophoresed on 1% agarose (w/v) (**A**) and blotted overnight in 10 x SSC onto Zeta probe membrane. Total I13 extrachromosomal DNA was random prime labelled with ^{32}P α-dCTP and used as a probe. Hybridisation was carried out overnight at 43°C in Church solution containing 50% formamide. After incubation, the blot was washed consecutively in 4 x, 2 x and 1 x SSC containing 0.1% SDS each for 30 minutes and then exposed to photographic film (**B**).

E. caratovora

S. cerevisiae

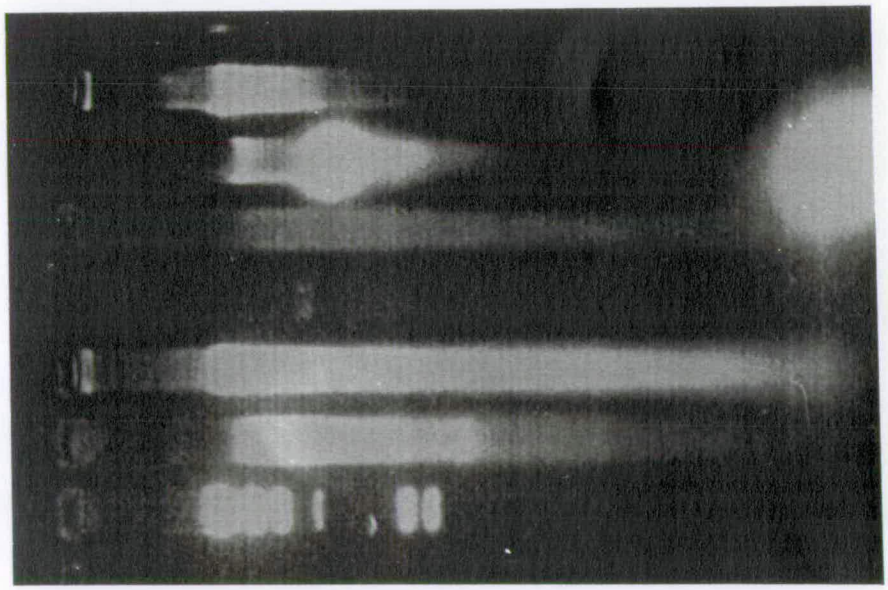
A. foetoides

H. victoriae

O. ulmi

I13

B



E. caratovora

S. cerevisiae

A. foetoides

H. victoriae

O. ulmi

I13

A

23
9.5
6.5
4.3
2.3
2.0

of these strains exhibited significant homology with the AG4 I13 linear DNA element. The element therefore appears to be anastomosis group specific.

6.3. DISCUSSION

Analysis of *Rhizoctonia solani,* AG4 strain I13, has resulted in the identification of an extrachromosomal DNA element from this fungus.

Plasmid-like DNA elements have been characterised from a variety of fungal plant pathogens such as *Fusarium oxysporum* (Kistler & Leong, 1986), *Claviceps purpurea* (Fr.) Tul (Tudzynski & Esser, 1983), *Tilletia caries* (O.C.) Tul (Kim *et al.,* 1990), *Ceratocystis fimbriata* Ell & Halsted (Giasson & Lalonde, 1987) and *Rhizoctonia solani* (Miyasaka *et al.*, 1990; Jabaji-Hare, 1994). These elements are all linear DNA molecules and range in size from 1.9kb to 9.5kb. In the present study, the linear DNA element identified has a predicted size of approximately 2.5kb, so it falls into this size range.

In this study of I13 extrachromosomal DNA, many comparable structural similarities were revealed with other linear DNA plasmids. Such molecules were originally suggested to be linear by their electrophoretic mobility and the lengths of digested plasmid equalled the length of uncut plasmid supporting the linear nature of these molecules (Giasson & Lalonde, 1987; Kim *et al.,* 1990). In the present study, with I13 DNA (probably due to low quality and purity) the enzymes *EcoRI, HindIII, BamHI* and *PvuII* appeared to be unable to digest the element; *SauIIIA* cleaved the element but gave sizes too small to reconstruct the final length of the DNA (Figure 6,4a), but the linear nature of the molecule was suggested by radiolabelling and exonuclease digestion. Exonuclease III digestion of I13 DNA (Figure 6.3) occurred in a similar manner to that of other linear DNA plasmids, including those of *F. oxysporum* (Kistler & Leong, 1986), *T. controversa* (Kim *et al.,* 1990), *C. purpurea* (Duvell *et al.,* 1988), *C. fimbriata* (Giasson & Lalonde, 1987) and *K. lactis* (Kikuchi *et al.,* 1984), and 3' end labelling could be achieved with these molecules, confirming not only the linearity of the molecule, as exonuclease III is known to have no

endonuclease activity (Giasson & Lalonde, 1987), but also the lack of blockage at the 3' end of the molecule. Although exonuclease III does not have endonuclease activity it can digest nicked linear or circular DNA (Sambrook *et al.*, .1989), so the degradation observed in Figure 6.3a may be a result of internal digestion. This would explain why the degradation pattern was not 'stepwise' as found with linearised pBluescript DNA (Figure 6.3b). T_4 DNA polymerase will also label nicked DNA (Sambrook *et al.*, 1989) and, as can be seen if Figure 6.3c, upon labelling and polyacrylamide gel electrophoresis a variety of sizes of labelled fragments were observed, suggesting internal priming. So, although exonuclease III digestion and 3' end labelling data suggest freedom of the 3' ends of the molecule, both enzymes used to ascertain this could also act on nicked molecules. It is, therefore, equally as likely that both exonuclease III digestion and radiolabelling using T_4 DNA polymerase and ^{32}P dCTP, initiated from nicks in the DNA and that the I13 element has blocked 3' and 5' ends making it similar to other known *R solani* plasmids pRS-64 (Miyashita *et al.*, 1990) and pRS188 (Jabaji-Hare *et al.*, 1994).

Blockage at the 3' end of the molecule would also explain the lack of success of the RACE experiment. If the 3' ends were not available for polyadenylation, PCR using the poly T adapter primer would be unsuccessful. Indeed, southern blotting showed this to be the case. The PCR products obtained may be from some chromosomal or mitochondrial DNA which had a polyA region and homology to the second primer used for amplification.

Linearity was further confirmed by hydrolysis with alkali. Linear molecules quickly denature after addition of 0.1N NaOH and cannot be detected after neutralisation. This situation was found in the present study and has also been shown in previous studies with *C. fimbriata* linear extrachromosomal DNA (Normand *et al.*, 1987). The data presented in this thesis, as well as that for all previously characterised linear plasmids described above, may conflict with reports on pRS-64 DNA plasmid from *R. solani* AG4. Upon hydrolysis and neutralisation, pRS-64 remained intact (Miyashita *et al.*, 1990) suggesting a non-linear structure which either was not completely denatured, or was quickly renatured after neutralisation (Barbour & Garron, 1987). The presence of hairpin loops which are easily renatured

and resistant to exonucleases was proposed (Miyashita *et al.,* 1990).

Linear plasmids normally have very specific terminal structures such as cohesive ends, direct or inverted repeats, terminal proteins or hairpins which are required for replication (Kornberg 1980). Indeed, most linear plasmid DNAs have terminal proteins attached to their 5' ends causing blockage so they cannot be digested by λ exonuclease *in vitro* (Meinhardt *et al.,* 1990). In this study I13 DNA was also found to be λ exonuclease resistant even after proteinase k treatment of the molecule to try to remove any terminal proteins which may be bound there. A similar situation has been reported with the 8.2kb plasmid of *C. fimbriata* (Giasson & Lalonde, 1987). In the case of *C. fimbriata* element, there is thought to be covalent binding of the terminal proteins to the DNA. Indeed, with *C. fimbriata*, the proteins remain protective even after treatment with SDS and phenol and digestion with proteinase. AG5 plasmid pRS188 also remained resistant to λ exonuclease after digestion with proteinase (Jabaji-Hare *et al.*, 1994) suggesting its terminal structures, as observed by electron microscopy, are either covalently bound or are not proteinaceous. Strongly bound terminal proteins have also been found linked by a serine to the 5' dAMP of bacteriophage ϕ29 and also human adenovirus (Hermoso & Salas, 1980; Challberg *et al.*, 1980). In both cases SDS, phenol and proteases failed to render the DNA sensitive to λ exonuclease. This is in contrast to the *K. lactis* plasmids in which the terminal proteins could be removed by protease. In the case of *K. lactis,* detection of protein removal was by a band shift upon electrophoresis. However, when the full length *K. lactis* plasmid was used, no band shift was observed, probably because of the small size of the proteins but, when a very small fragment of 39bp from each end was electrophoresed a reduction in size was observed after proteolytic cleavage (Kikuchi *et al.,* 1984).

Other linear plasmids, such as those from *T. controversa*, are sensitive to both exonuclease III and λ exonuclease (Kim *et al.*, 1990), suggesting either a very loose association of a protein with the DNA termini such that it can be removed by SDS or phenol, or that there is an alternative priming and protection mechanism. Thus, there appears to be a variety of mechanisms responsible for protecting linear DNA from degradation. There are also various mechanisms for replication of such molecules.

The structure of pRS-64 was proposed to contain hairpin loops at each end of the molecule, with replication occurring by site specific nicks in a region of homology close to the loop structure. In this model, nicking results in the formation of a free 3' hydroxyl end which can serve as a primer for DNA replication either at one end or from both ends of the plasmid simultaneously (Miyashita *et al.,* 1990). A diagrammatic representation of pRS-64 replication is shown in Figure 1.4. This group also suggested the replication mechanism of the plasmid would lead to dimeric forms. The finding of dimeric forms in pRS188 is consistent with their hypothesis (Jabaji-Hare *et al.,* 1994) and may also be relevant to the I13 element. Plasmids and chromosomal telomeres with hairpin loops have been reported in bacteria, such as *Borrelia burgdorferi* (Barbour & Garon, 1984), and yeasts, such as *Saccharomyces cerevisiae* (Kikuchi *et al.*, 1984), and they can all be distinguished from structures with protein attached to them, by the inability of the latter to label at the 3' end and their resistance to exonuclease III digestion.

Most reported linear DNA plasmids from fungi have no known function. Curing of the plasmids of *C. purpurea* was done by exposure to ethidium bromide. In this case the plasmid was found to be non-essential under laboratory conditions as the only observed phenotypic change was a slightly increased growth rate (Duvell *et al.*, 1988). In the case of *K. lactis,* however, curing resulted in a known phenotype. The plasmid encodes a protein toxin which kills sensitive strains of the same species and other yeast species (Niwa *et al.,* 1981); ultraviolet light-induced curing of plasmids correlated with the loss of the killer character (Niwa *et al.*, 1981; Wesolowsi *et al.,* 1982). With *R. solani,* plasmid pRS-64 was isolated from a weakly pathogenic strain of the fungus and this plasmid was initially thought to be involved in reduction of virulence (Hashiba *et al.,* 1984). However, subsequent studies found DNA plasmids to be widespread in a variety of anastomosis groups in *R. solani* and associated with both highly pathogenic and avirulent strains (Miyashita *et al.,* 1990). Other *R. solani* plasmid-containing strains, AG5 Rh41 and AG4 R36 were shown to be equally as pathogenic on lupins and beans respectively as non-plasmid-containing strains (Jabaji-Hare *et al.,* 1994) so *R. solani* linear DNA plasmids do not appear to be related to virulence.

Southern blotting analysis of these plasmids by Miyasaka and colleagues (1990) and Jabaji-Hare *et al.* (1994) showed plasmid similarity within anastomosis groups but not between them. This was also found to be the case in the present study with I13 extrachromosomal DNA (see Figure 6.9) which showed homology to DNA from two other AG4 strains tested but not to AG2-2 strain C127, as determined by southern blotting. Also, in common with the work of Jabaji-Hare and colleagues (1994) higher molecular weight structures were identified upon probing total DNA with the I13 extrachromosomal DNA element. Jabaji-Hare and co-workers (1994) suggested dimerisation of pRS188 had occurred. This may explain the higher molecular weight bands found occasionally upon blotting with the I13 element.

Miyashita and colleagues suggested that as plasmid-like DNA structures were anastomosis group specific and most anastomosis groups had specific plant hosts, then these DNA structures may determine host specificity. A similar correlation between anastomosis group specific extrachromosomal DNA elements and plant pathogenic specificity has also been reported with *F. oxysporum* (Kistler & Leong, 1986). In the case of *F. oxysporum,* races one and five, which are both pathogens of cabbage, showed homology in their extrachromosomal DNA plasmids, but there was no homology between either of these and race two, a pathogen of radish. The reverse situation also applied in the present study, *Rhizoctonia solani* AG4 I13 is a highly virulent strain which when tested on four plant species (cabbage, carrot, cress and radish) caused damping off. However, this pathogenicity was similar for AG4 PA1, the only strain tested of AG4 which did not contain extrachromosomal DNA when examined by ethidium bromide staining of agarose gels and southern hybridisation. This suggests that the plasmid is not required for host specificity or indeed pathogenicity. It is not particularly surprising that homology exists within anastomosis groups, because the plasmid can be transferred between strains of the same anastomosis group whereas there is an extremely effective anastomosis barrier which probably prevents transfer between anastomosis groups.

A number of these extrachromosomal plasmids appear to be mitochondrially located, such as in *C. purpurea* (Duvell *et al.,* 1988), pRS-64 (Wako *et al.,* 1991) and pRS188 (Jabaji-Hare *et al.,* 1994) but for neither *R. solani* plasmids was the data

conclusive (Jabaji-Hare *et al.*, 1994) due to poor yield of purified mitochondria and the fragility of the mitochondria isolated.

Some linear plasmids are known to integrate into the chromosome, or be excised from chromosomal/mitochondrial DNA throughout the fungal life cycle. Examples of the latter have been reported for *Podospora anserina* (Esser, 1990) and *Neurospora crassa* (Bertrand *et al.*, 1985) senescence plasmids which are excised from the mitochondrial genome at the onset of cell death. Upon excision, the plasmid can replicate autonomously and express its phenotype. If the plasmid is not excised then no senescence will occur. Despite the fact that many of the plasmids are mitochondrial in location, very few show any homology to mitochondrial genes and do not appear to integrate with the mitochondrial genome. (A few plasmids have been shown to be integrated by homology to mitochondrial DNA by southern blotting. These are from *Neurospora crassa* (Bertrand *et al.*, 1985), *Agaricus bisporus* (Robinson *et al.*, 1991) and *Claviceps purpurea* (Tudzynski & Esser, 1986). If the I13 extrachromosomal DNA was of mitochondrial origin, it would be expected that the plasmid would be ubiquitous, as mitrochondrial excised plasmids are usually homologous within a species (eg. the species *R. solani*) instead of anastomosis group specific. Thus, upon probing there would be some homology to mitochondrial DNA, giving more than one band on a southern blot. This was not seen and homology was only found with extrachromosomal elements of other *R. solani* AG4 strains (Figure 6.9).

Due to the limited information on these elements it was hoped that sequence analysis of the I13 linear extrachromosomal DNA element would elucidate a role for the element. However, sequencing of the 638bp fragment contained in plasmid pMC1 gave no information as to the function of this element. Even when the larger hypothetical protein sequences identified by fungal mitochondrial codon usage, ie substituting stop codon UGA with tryptophan, was used for Fasta analysis there was no significant homology with other sequence in the database. The problem was compounded by the fact that most of the linear plasmids from fungal pathogens of plants have not been sequenced and therefore there is very little information in the database for comparison.

One of the key components of these plasmids, if they are autonomous, must be the presence of a DNA polymerase. DNA polymerases have been identified from a variety of eucaryotic linear DNA plasmids: from yeasts such as *K. lactis* (Stark *et al.,* 1984), from filamantous fungi, such as *N. crassa* (Court & Bertrand, 1992) and *C. purpurea* (Oeser & Tudzynski, 1989), and from plants, such as maize (Paillard *et al.,* 1985). All of these are type B polymerases due to their homology to the *pol*B gene of *E. coli* DNA polymerase II. Within these polymerases there are three consensus regions, but even here the homology is very loose. pMC1 does not exhibit significant homology to any of these regions at the protein level. This is not unexpected due to the small size of the fragment sequenced and the fact that outwith the conserved regions of the above polymerases there is no obvious homology between the enzymes. The type B DNA polymerases encoded by extrachromosomal eucaryotic linear DNA plasmids are all approximately 1000 amino acids in length, and those which have been sequenced have broadly similar sizes: 917 and 995 amino acids for maize S1 plasmid DNA polymerase (Paillard *et al.,* 1985) and pGKL1 *K. lactis* DNA polymerase (Stark *et al.,* 1984) respectively, 1097 and 1202 amino acids for *C. purpurea* (Oeser & Tudzynski, 1989) and *A. immersus* (Kempen *et al.,* 1989) DNA polymerases respectively. Therefore the I13 extrachromosomal DNA appears to be just long enough to encode such a polymerase but little else. Although the sequenced fragment contains no homology to type B polymerases, due to lack of available sequence information of linear plasmids of plant pathogenic fungi, it may still be possible that there is a DNA polymerase encoded by the I13 element. Alternatively, a polymerase may be provided from the host genome.

The lack of similarity with any previously sequenced genes or DNA elements, in conjunction with lack of detectable detrimental effects of the dsDNA element on the fungus poses questions as to the function of the element. It is perhaps cryptic. Most eucaryotic extrachromosomal elements, including dsRNA, are thought to be redundant viruses which have lost their ability to infect, but can be maintained in the host, as they do not elicit an antagonistic response. These viruses often utilise the host's replication machinery, such as the *S. cerevisiae* killer virus (Buck, 1979). Fungal viruses are non-infectious so they appear to have lost at least part of their

genome; but they can be transferred upon anastomosis allowing their spread within populations. This would explain the presence of homologous elements in the same anastomosis group (Figure 6.8) which were found in this study. The alternative hypothesis to that of redundant viruses is that the elements may simply represent selfish DNA. The element may have been excised from the fungal chromosome, as it is of no functional use, and persists in the cytoplasm. Due to the nature of the element it is able to persist either by picking up 'useful' genes, or by being present in multiple copies, making removal difficult. The presence of termini which are resistant to degradation would also make elimination more difficult. The identification of similar DNA in three strains of the same anastomosis group makes it unlikely that the element has been repeatedly excised as a piece of redundant DNA, as the presence of identical elements in three different strains is unlikely. However, one excised piece of DNA, from the fungal chromosome, may have been transferred via anastomosis to similar strains.

The selfish DNA hypothesis is based on the principle that certain pieces of DNA are maintained in a cell although they confer no advantage to it (Orgel & Crick, 1980). This contrasts with the theory of natural selection in which useful genes are spread throughout a population, whereas redundant ones are lost. However, one of the limitations of a process of natural selection is that any mechanism which leads to the multiplication of useful DNA will probably also lead to the multiplication of selfish DNA. So, many pieces of otherwise redundant DNA may be efficiently maintained. At present, the I13 DNA element appears to be a piece of selfish DNA, which is either autonomous, or replicated by host genes, this is similar to the report on *R. solani* AG5 pRS188 which has also been partially sequenced and does not contain any homology to other proteins in the database (Jabaji-Hare *et al.*, 1994). At the moment a cryptic function has been assigned to the element but until the complete sequence information for the element is available, it is not possible to exclude a functional potential for the element. It may be large enough to code solely for a DNA polymerase or there may be overlapping reading frames which encode multiple proteins with differing functions. Sequencing of both the I13 element and other linear DNA elements is needed to elucidate such function, if any.

CHAPTER 7

CONCLUDING DISCUSSION

The principal features of the work in this thesis were to examine extrachromosomal genetic elements in *Rhizoctonia solani* and, by investigating anastomosis and vegetative compatibility in the fungus, look at the potential for transfer of these elements within, and between, strains. These points are relevant to understanding the natural movement of extrachromosomal elements in the environment and the population biology of *R. solani.* In addition to this, more information on the function and ability for transfer of extrachromosomal elements may lead to the practical exploitation of these elements, or derivatives of them, to carry genes that will reduce the virulence of *R. solani.* The elements, therefore, have potential in biological control of an economically important plant pathogen.

Transfer is essential for the spread of extrachromosomal elements in *R. solani*, as these elements are naturally non-infectious (Buck, 1986a) and the sexual stage of the fungus is rarely seen in nature. The elements might normally be transferred by cell fusion between hyphae of compatible strains. Anastomosis is therefore the only common method of transfer in *R. solani,* but examination of anastomosis by light microscopy was not sufficient to detect the transfer of subcellular components. It was hoped that fluorochromes would provide a clearer insight into organelle transfer, but the use of fluorochromes in conjunction with ultra-violet light killed the fungus so further work needs to be carried out into developing a simple method for examining anastomosis.

The most efficient method for detecting the movement of extrachromosomal elements would be to label the elements themselves. In this work double-stranded RNA and DNA have been shown to label efficiently with γ-^{32}P d-ATP and α–^{32}P d-CTP respectively so this methodology may be used to monitor transfer between anastomosing strains. Alternatively, the elements could be tagged with a reporter gene, such as the *E. coli hygB* gene which confers resistance to the antibiotic hygromycin B or the *Vibrio* luciferase genes which emits light upon expression. Luminescence systems have the advantage of being viable systems so movement of extracellular elements between cells can be monitored microscopically by examination of light production. In *C. parasitica* cDNA of the extrachromosomal dsRNA element was cloned into a plasmid containing the hygromycin B

phosphotransferase gene as a selectable marker, as well as promoters and terminators from *Aspergillus nidulans.* In this way there was selection of transformed strains and overexpression of the genes for further analysis.

In the present work attempts to reintroduce dsRNA simply by anastomosis into a compatible strain cured of dsRNA were unsuccessful (see Chapter 5). This raises questions as to the natural ease of transfer of these elements. If, as is usual in *R. solani*, the dsRNA is located in older hyphae (Hollings, 1978), then the time for successful transfer may be quite long, as the dsRNA must first be translocated forward in the hyphae before entering the fused tip cells. So transfer may be quite inefficient. One potential method of increasing the rate and efficiency of transfer in appropriate strains of *R. solani* could be by vectorial transfer via a biotrophic mycoparasite such as *Verticillium biguttatum.* Mycoparasites are fungi which parasitise other fungi (Deacon, 1976) and *V. biguttatum* has been shown to reduce black scurf of potatoes caused by *R. solani* anastomosis group 3 strains in experimental field conditions (Van den Boogert & Velvis, 1992). *V. biguttatum* grows slowly when inoculated onto mycelia of *R. solani.* Nevertheless in studies on AG3 strains of *R. solani,* which are most susceptible to parasitism by *V. biguttatum,* even localised establishment of the mycoparasite on the host inoculum caused a marked reduction of sclerotium production in *R. solani* across the host colony (Van den Boogert & Deacon, 1994). These workers also showed that suppression of sclerotium production extended to adjacent colonies of the same *R. solani* strain on agar plates beyond where the two host colonies anastomosed and formed compatible fusions. But when incompatible AG3 strains anastomosed (resulting in localised cell death after fusion) the suppressive effect of *V. biguttatum* on sclerotium production extended only to the colony junction zone, not into the adjacent colony. Microscopic examination of AG3 colonies parasitised by *V. biguttatum* revealed conspicuous cytoplasmic streaming towards the sites of infection by *V. biguttatum* indicative of breakdown of the dolipore septa (J.W. Deacon & D. Scott, unpublished). Moreover, the direction of cytoplasmic streaming in *R. solani* (usually towards tip cells) could be changed in a parasitised colony towards the site of infection by *V. biguttatum.* This 'nutrient sink' effect of *V. biguttatum* may be significant for transporting dsRNA

or even DNA elements between vegetatively compatible strains. It might be exploited experimentally, at least for AG3 strains, but it would be of less value for AG4 strains, which are much less susceptible to *V. biguttatum* (Van den Boogert *et al.,* 1990). It is therefore suggested that further work on transfer of extrachromosomal genetic elements in *R. solani* might focus on AG3, using *V. biguttatum* to facilitate potential transfer.

In this regard, also, the present work revealed a remarkably high incidence of vegetative incompatibility between parent AG4 strains and hyphal tip subcultures of the parent strains. This was found in the two AG4 strains (PA1 and I13), studied in depth, and it did not seem to be related simply to presence or absence of dsRNA, because some dsRNA-free tip subcultures were still compatible with the parent whereas others were incompatible. There was also evidence of weak parent-incompatibility in some of the newly obtained tip subcultures, which became strongly parent-incompatible during further subculturing. Attempts to obtain parent-incompatible AG3 strains by tip subculture were consistently unsuccessful, and Drs P. van den Boogert (pers. comm.) and J. W. Deacon (pers. comm.) have also been unsuccessful in this task. Vegetative incompatibility in fungi is still poorly understood (Cubeta *et al.,* 1993) but it is widely considered to be under control of nuclear genes (Anderson, 1984; Briones & Vilgalys, 1991; Cubeta *et al.,* 1993). Of interest in the present study, the strains (AG4 PA1 and AG4 I13) in which tip-subculturing led frequently to segregation of incompatibility had relatively few nuclei per tip cell whereas strain (AG3 R41) which showed little or no segregation of compatibility during tip-subculture (P. van der Boogert & J.W. Deacon, pers comm.) had many more nuclei per cell. It is possible that tip-subculturing could have generated strains that contained only one (or some) of the nuclear genotypes in the parent dikaryons (heterokaryons) and that this might have led to segregation of compatibility factors. This possibility could be investigated by generation of mutant homokaryons (eg. containing fungicide-resistance genes), perhaps using single-basidiospore cultures, so that the resulting dikaryon crosses would have identifiable nucleotypes. Tip cultures could then be examined for their segregation pattern with regards to resistance and incompatibility. The generation of mutants in *R. solani* is

said to be difficult but might not be so with regard to resistance to inhibitors of melanin biosynthesis (Baldwin, 1984) or some antifungal antibiotics that have been used in Japanese agriculture (Baldwin, 1984).

In the absence of mutants or other means of directly assessing the occurrence of nuclear genotypes, an attempt was made to study the distribution of nuclear numbers in colonies as by DAPI staining and fluorescence these could be easily monitored. This revealed evidence of a degree of local control within the colonies, insofar as there was a tendency for tip cells to have similar nuclear numbers if they arose from the same parent hypha (in clusters). So colonies of AG4 strain PA1 could be considered to be mosaic in terms of nuclear number. Whether or not there were also mosaics of different nuclear types remains unknown. It is notable, however, that Hyakumachi & Ui (1987) recently reported self-incompatible strains of AG2-2, and this self-incompatibility, the basis of which is unknown, is reminiscent of the parent-incompatibility of hyphal tip subcultures found in the present study.

A specific method for introduction of new genes is by protoplast fusion. Protoplasting techniques in *R. solani* have been studied in some detail (Hashiba & Yamada, 1982) and a successful fusion technique has been developed. As anastomosis or vegetative incompatibility is no barrier to fusion of protoplasts a protoplast containing an extrachromosomal dsRNA or DNA element but no nucleus could be fused with protoplasts from strains which do not contain extrachromosomal genes. Hashiba and colleagues (1985) have also reported the transformation of an *R. solani* strain with extrachromosomal DNA element into protoplasts but the report contained little detail and there does not seem to be a direct transformation mechanism available for *R. solani* at the present time, unlike other filamentous fungi such as *Podospora anserina* (Brygoo & Debuchy, 1985), *Gaeumannomyces graminis* (Henson *et al.*, 1988) and *Ustilago maydis* (Banks, 1983) and *Schizophyllum commune* (Monoz-Rivas *et al.*, 1986). As the cell wall presents a large obstacle to the entrance of macromolecules transformation systems are being developed to overcome the barrier. Two novel methods of transfer are the use of DNA-coated macroprojectiles (Klein *et al.*, 1987; Christou *et al.*, 1988) and the use of electric fields (Potter, 1988; Neumann *et al.*, 1992). Macroprojectiles, usually gold or

tungsten pellets, are coated with DNA and the projectile is accelerated. At high velocity the projectile is halted by a stopper plate, but the DNA can continue through holes in the plate and strike the sample. The pellets are projected by chemical (Klein *et al.,* 1987) or electric guns (Christou *et al.,* 1988) and the high velocity enables particles to penetrate the cell wall. With electroporation a brief voltage pulse is used to rupture the plasma membrane and the protoplast remains permeable to small molecules for some minutes (Lindsay & Jones, 1987). Molecules present in medium surrounding the cells may be able to enter the cell at the moment the pulse is applied.

As discussed in some detail in previous chapters there has been no constant phenotype associated with dsRNA in *R. solani,* and the present work is no exception. Other workers have looked specifically at the association of dsRNA with virulence in attempts to exploit the viruses as biological agents for control of *Rhizoctonia* diseases. Unfortunately the presence of dsRNA elements have been correlated not only with reduced virulence (Castano *et al.,* 1978), which would make them potential biological control agents, but enhanced virulence (Finkler *et al.,* 1985). Yet other studies suggest that dsRNA plays no role in *Rhizoctonia* pathogenicity (Zanzinger *et al.,* 1984; Kousik *et al.,* 1994). A recent report by Lakshman & Tavantzis (1994) has shown the appearance of 25kb, 3.7kb and 1.2kb fragments of dsRNA in a spontaneous mutant of a virulent *R. solani* AG3 strain which previously contained 23kb and 6.5kb dsRNA fragments. As well as harbouring these extra dsRNA segments the mutant strain has reduced pigmentation and growth rate and is non-pathogenic on potato. The reduction in pathogenicity coincided with the appearance of the extra dsRNA elements. These new dsRNAs did not cross hybridise with each other or with any of the original dsRNAs suggesting they did not arise from the original dsRNAs. Usually the presence of novel dsRNA elements can be attributed to a deletion in the existing elements. This has been shown for *C. parasitica* (Tartaglia *et al.,* 1986) and *Agaricus bisporus* (Morten & Hicks, 1992), but in the *R. solani* strain the new dsRNAs were larger than the original ones. The spontaneous appearance of the novel dsRNAs has never been reported before but, like *C parasitica,* specific dsRNAs may be responsible for decreased virulence while others might be redundant or aid functions such as replication, integration and persistence.

Thus the assembly of a particular combination of apparently cryptic dsRNA elements within one strain, by chance or otherwise, may control whether virulence is affected in a positive or negative way. This would correlate with Castano *et al.* (1978) where dsRNA was associated with reduced virulence. A second novel finding by Castano *et al.* (1978) was the integration of the 23kb and 3.7kb fragments into the chromosome, although it is unknown if it integrates into the nuclear or mitochondrial genome. This suggests that the virus may use a retrovirus mode of replication which involves a chromosomal copy of the genes. This conflicts with previous data which suggests elements must encode an RNA polymerase for autonomous replication (Finkler *et al.*, 1988).

In accordance with the reports of Zanzinger *et al.*, (1984) and Kousik *et al.*, (1994) and colleagues the dsRNA examined in this study appears to have no role in pathogenicity with both dsRNA-containing and dsRNA-free strains being equally pathogenic on all plant hosts tested (see Chapter 4), but there was sometimes a marked reduction in pectic (both polygalacturonase and pectin lyase) and cellulolytic enzyme activity in dsRNA containing strains (Chapter 4). This reduction may be similar to the down regulation of certain of certain host genes in *Cryphonectria parasitica* (Powell & Van Alfen, 1987; Rigling *et al.*, 1989; Hillman *et al.*, 1990; Carpenter *et al.*, 1992).

In *C. parasitica* the presence of a 12.6kb dsRNA (L-dsRNA) element affects the transcription of specific host genes such as laccase, oxalate, cryparin and two early sporulation genes *vir 1* and *vir 2* (Pfeiffer & Van Alfen, 1992). Laccase has been particularly well studied and is known to be involved in fungal sporulation (Leatham & Stahman, 1981), pigmentation (Clutterbuck, 1972), lignin degradation (Kirk & Shimada, 1981) and pathogenesis (Geiger *et al.*, 1986). The presence of L-dsRNA has been shown to reduce the transcription of the laccase gene by 75-85% (Rigling & Van Alfen, 1991) and affects many cellular processes, effectively debilitating the dsRNA containing strains. The reduction in pectic and cellulolytic enzyme levels in dsRNA containing strains of *R. solani* examined in this study may result from down-regulation of the specific genes. A problem with this work is that with the cultures, upon curing of dsRNA, there was also incompatibility with the

dsRNA-containing strain. This raises the question whether the change in enzyme activity is related to dsRNA or segregation of nuclei upon hyphal tip subculture. Subcultures were isolated which had lost dsRNA but were still compatible with the dsRNA-containing strain (see Chapter 5). These had similar levels of enzyme production to the dsRNA-containing strain suggesting that any changes in enzyme levels were due to nuclear segregation or gene mutation resulting from multiple tip subculture. However, the possibility cannot be excluded that in some of the hyphal tip subcultures, the dsRNA may have integrated into the chromosome or mitochondrial DNA but still expresses its phenotype.

In contrast to dsRNA, hyphal tip subculturing could not cure *R. solani* strain AG4 I13 of an extrachromosomal DNA element. This suggests that it is also present in hyphal tip cells. The inability to cure the cells by other methods such as UV irradiation of heat treatment to 30°C for 7 days suggests either a subcellular location which is protective for the DNA or integration of the element. However, southern blotting of total fungal chromosomal DNA (Figure 6.8) shows only a single band the same size as that used for the probe, suggesting no integration of the element occurs or that no additional chromosomal copies of these elements exist. Many extrachromosomal DNA elements are mitochondrially located eg. *C. purpurea* (Duvell *et al.,* 1988) and *R. solani* pRS-64 (Wako *et al.,* 1991). If the I13 dsDNA element is mitochondrially located, as previously reported for pRS-64 (Wako *et al.,* 1991) and pRS188 (Jabaji-Hare *et al.,* 1994) it may be better protected than the cytoplasmically located dsRNA. This would also require that mitochondrial tip cells would also contain these dsDNA elements.

As with dsRNA the role of extrachromosomal dsDNA elements in *R. solani* is not known. Initially Hashiba and colleagues (1984) found them to be associated with reduced virulence, but subsequent studies have shown these elements to be widespread (Miyasaka *et al.,* 1990; Jabaji-Hare *et al.,* 1994), without specific correlation with virulence. Indeed, in latter studies Jabaji-Hare and co-workers (1994) conclude that the plasmids examined by them, pRS188 and pRS104, cannot be correlated with virulence. It is unknown if the DNA elements are affecting specific cellular processes unrelated to pathogenicity as curing has been a problem

with linear DNA elements. In other fungi, the only element cured previously is from *C. purpurea* (Duvell *et al.,* 1988), with the only phenotypic result being slightly reduced growth rate. No effective 'knock out' experiments can be carried out.

Both dsRNA and dsDNA extrachromosomal elements examined in this study show significant similarity to previously reported elements and although no function can be attributed to the elements at present, further sequence and biochemical information on the elements may help to elucidate this. If the elements do not play an essential role in *R. solani* they may be useful as vectors for introducing new genes into the fungus, as both types of element are stably maintained and do not stimulate an immune response.

The present study has resulted in some useful information on the biology of *R. solani* and the function of extrachromosomal genes in this fungus, but there is still much work needed if these elements are to be useful for the control of *Rhizoctonia* disease. The diversity of species and lack of transfer of the elements both contribute to the problem and much more work into the genetics of the fungus is needed before possible biological control methods can be identified.

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