PHYSIOLOGY AND ECOLOGY OF *IDRIELLA BOLLEYI*, A BIOLOGICAL CONTROL AGENT OF CEREAL ROOT AND STEM BASE PATHOGENS

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Ph.D. Thesis

Edinburgh University

1996

DECLARATION

I declare that this thesis has been composed by myself, and all the work is my own

Lisa Douglas

DEDICATION

To my Grandmother (1899-1995)

ACKNOWLEDGMENTS

I would like to express my gratitude to Jim Deacon for his guidance and supervision over the past 3 years. I would also like to express my gratitude to Mark Hocart for his help and encouragement with much of this work. Thanks to all those who have given me technical help, including members of the Soil Science Department, Edinburgh School of Agriculture, Naresh Magan for his help with the water potential work, Patrick and Laura for their patience in teaching me Noddy's guide to molecular biology, Erland Liljeroth for his much needed guidance with the GUS work.

Thanks to all the people that have made my time in Edinburgh so special and unforgettable. Firstly thanks to my wonderful ex-lab mates, Eirian for always being someone to laugh with, Debbie for always 'partying-on' with me, Ulrike for her encouragement, sound worldly advice and the red wine drinking sessions. Tricia (adopted lab member) for being my faithful film-going friend. Thanks girls for all the good times we had and for your on-going friendship and support. Thanks also to my other one-time lab mates. Dave for insulting me with more derogatory terms than I thought possible, Ade for being a better shot than me when throwing Eppendorfs at Dave, Mette for her great carpet-staining parties, Lorraine for her 'amazing' bouffant hair, Paul for thinking the Jim Rose circus was 'interesting', Kithsiri for torturing us with his curries at lunch time, Richard 'oo la la' for making the lab a golfing hell, Alison for her love of gossip and Sharon for breaking the sound barrier (whooooooppppp!).

Thanks also to all the members of the 'exclusive beer club'. Tony for his friendship, great Mexican feeds and emergency computer help, Richard for proving that you can use the same joke for several years, Kiwi and Jamie for making me laugh so hard, Dan for just always laughing, Geoff for his 'aroma therapy', Hari for her unbounded enthusiasm, Spotty

for being amusingly surreal, Matt for his sock-sucking stories. Thanks also to my other more sober friends, Lynn, Graham, James, Zoe, Jason, Yael, Sally, etc..... and to all my flat mates, Wee Kev, Alison, Gareth, Hazel, Charles, Kate, Jill, Simon and Neil for putting up with any of my bad habits.

A huge thanks to my family for a life-time of support and encouragement that allowed me to achieve my goals.

Finally, a special thanks to Andrew for all his patience and encouragement over the last three years, but most of all just for being there for me through all my Ph.D-induced ups and downs. I couldn't have done it without you.

ABSTRACT

Idriella bolleyi strains, T560, J10 and AB1, differed considerably in their tolerance to water-stress, this was apparent in both osmotic and matric potentials and in all aspects of their growth cycle. The level of tolerance for one strain, T560, was comparable to the take-all pathogen *Gaeumannomyces graminis* var. *tritici,* which is associated with low water-stress tolerance, and another strain, AB1, was comparable to the foot-rot pathogen *Fusarium culmorum,* known to be highly tolerant of drought conditions. Variation in response to osmotic potential was also observed in root and stem base field isolates of *I. bolleyi*. The field isolates differed in the level of mycelial growth and sporulation at potentials of -1.5 MPa (control) and -5.0 MPa. Isolates from the same plant were found to have different levels of sporulation, this may have been related to the degree of microcycle conidiation they exhibited. *Idriella bolleyi* isolates, T560 and AB1, were able to colonise straw in soil at different matric potentials down to -7.0 MPa. Straw colonisation was found to increase from 2 to 8 weeks indicating that both strains were actively growing at all water potentials during this period. A number of other fungal species were isolated from the straw along with *I. bolleyi,* suggesting that a high degree of competition was associated with colonisation. Water potential appeared to be the most influencing factor with regard to the activity of each species. *Idriella bolleyi* could increase its population on unsterilised wheat seeds buried in soil. *Idriella bolleyi* was inoculated onto the seeds as either an alginate or water suspension. For both seed treatments the detectable level of conidia increased exponentially on the seeds over five days, this increase coinciding with the production of young wheat roots. As the seed aged the detectable level of conidia decreased.

A bank of nitrate non-utilising (*nit*) mutants were produced from *I. bolleyi*, their production being influenced by the *I. bolleyi* strain used and the nitrogen source present in the chlorate growth medium used to induce mutation. *Nit* mutant pairings of different

mutational designation often resulted in wild-type growth at the colony junctions indicating complementation and possible heterokaryon formation. However, microscopic examination of DAPI-stained complementing mutants showed that the cells remained uninucleate despite the presence of anastomosing hyphae, suggesting that if a heterokaryon was formed then *I. bolleyi* was unable to maintain the heterokaryotic state, or that the heterokaryotic state was limited. *Nit* mutants showing both vegetative compatibility (complementation) and incompatibility (no complementation) were used in protoplast fusion experiments. Prototrophic colonies were obtained for both crosses suggesting that the protoplasts of the respective strains had fused and that vegetative incompatibility could be overcome using this technique. *I. bolleyi* was successfully transformed with a pGUS5 plasmid and the resulting transformants showed high levels of GUS activity compared to the wild-type strain. Wheat root inoculations enabled visual assessment of the level of colonisation by each transformant. The plant tissue was coloured blue in areas of GUS-active fungal mycelium following incubation in X-gluc solution. Fluorimetric measurements recorded lower levels of GUS activity in transformant-colonised roots and seeds in comparison to transformants in pure culture. This difference may have been related to the overall level of fungal biomass in the two systems. The GUS activity of the transformants remained high after six cycles of conidia production indicating possible integration of the plasmid into the fungal genome. Consistent with this, the transformants deviated significantly from the wild-type strain in terms of radial extension rate, conidium production or conidia shape in culture, again suggesting possible plasmid integration into the genome.

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CHAPTER 1

INTRODUCTION

Idriella bolleyi is a common parasite on the roots and stem bases of cereals and grasses (Sprague, 1950). It is implicated in the biocontrol of several cereal pathogens, including the take-all fungus *Gaeumannomyces graminis* var. *tritici* on wheat (Kirk & Deacon, 1987a) and in golf-course turf (Baldwin *et al.*, 1991), the eyespot fungus *Pseudocercosporella herpotrichoides* (Reinecke *et al.*, 1979; Reinecke & Fokkema, 1981) and *Fusarium* spp. (Reinecke *et al.*, 1979). *Idriella bolleyi* competes for host-derived nutrients that pathogens would use to support the early stages of infection (Kirk, 1984; Deacon, 1991). In particular, *I. bolleyi* excludes pathogen colonisation by competitively exploiting the naturally senescing tissue of cereal roots, coleoptiles and first-formed leaf sheaths (Lewis & Deacon, 1982; Kirk & Deacon, 1987a). *Idriella. bolleyi* has been recognised as being of value as a general-purpose biocontrol agent, reducing the activities of several different pathogens in the particular seasonal or site conditions that favour these.

There is a need to develop economically feasible biological control procedures for use in agricultural production. The problems associated with fungicide use, including pathogen resistance, expense and persistence, have influenced the pendulum of public opinion, which is swinging towards a more natural conservational approach to food production.

Despite many years of research, few biocontrol agents have been developed to a commercial level. It is important that for the future selection and development of new biocontrol agents, much is be learnt from those which have reached a commercial level.

Deacon (1991) outlined the common problems facing the use of biocontrol agents and the factors that need considering when developing a new agent. The addition of any micro-organism to a soil will be useless if the micro-organism is not ecologically suited to that

environment. The natural soil flora will prevent successful establishment and proliferation. To add vast amounts of inoculum to the soil in order to control a pathogen would be economically unfeasible. In view of this, the biocontrol agent should be ecologically suited to the environment which will favour its activities. If a pathogen is to be controlled with reasonable success, the biocontrol agent must be behaviorally similar to the pathogen and so colonise a similar niche or micro-site to that of the pathogen. If a biocontrol system is to be used commercially, production of the inoculum should be economically feasible with the biocontrol agent being produced easily in culture (New & Kerr, 1972; Deacon, 1991).

Ecological attributes relating to the micro-environment of the pathogen are the obvious bases of screening for biocontrol agents, and it is essential that the features of biocontrol agents are in common with those which have been developed successfully (Deacon, 1991).

1.1 Soil-borne root-colonising micro-organisms

Fungal distribution in the soil is related to the soil environment. A greater diversity of species is observed with decreasing depth of soil reflecting the physical and chemical conditions of the soil profile. Changes in the fungal population will be related to such factors as soil aeration, gas composition and nutrient availability (Neville & Webster, 1995). In general fungi will be more numerous around organic matter, and active mycelium is often associated with the presence of plant roots. Away from organic matter and plant roots the fungal activity can be inhibited by the fungistatic properties of the soil (Griffin & Roth, 1979).

Organisms which colonise the roots of plants later become major constituents of the soil micro-flora on organic matter which can then act as an inoculum source for the following season (Bowen, 1979). In a cropping system the build-up of certain micro-flora could be significant if pathogens are present in the population. In the presence of roots the interaction

between micro-organisms will exist particularly in the form of competition for nutrients (Fokkema & van der Meulen, 1976). Shaw & Peters (1994) suggested that in the complicated interactions between fungal species, a pathogen may in fact increase nutrient availability by damaging host tissue and so releasing host nutrients. This in turn may increase the population of non-pathogenic root parasites. An abundance of such a population may help rapid colonisation of new roots and so decrease infection sites for pathogens.

1.1.1 Roots and seeds as micro-habitats for colonisation

The growing plant provides ideal micro-habitats in the form of the rhizosphere and the spermosphere. The plant-microbe associations are complex and in turn the microbe-microbe interactions offer a difficult system for investigation, particularly for those interested in manipulating this environment to reduce colonisation by pathogens and increase activity by competitive or antagonistic micro-flora.

It would be thought that the root surface of plants would be entirely covered from the large number of micro-organisms associated with the soil. However, micro-organisms are not uniformly distributed in soil, but exist in diverse micro-habitats (Parkinson *et al.*, 1963). Microscopy studies showed that as little as 37% of 90 day old *Pinus radiata* root segments were colonised by micro-organisms (Bowen & Rovira, 1976). Root colonisation has been found to be non-random with both fungi and bacteria showing preferential growth along the longitudinal junctions of epidermal cells (Rovira, 1956), and also in association with organic particles and sloughed root cells (Bowan & Rovira, 1976).

Although root colonisation by bacteria has been extensively studied, knowledge of fungal colonisation has been hampered by difficulties from the lack of suitable techniques to study fungal populations. Numerous soil studies have indicated that fungal populations on the

root surface of crop plants change as the plant ages. Fungal populations on young seedlings have been found to be dominated by *Mucor* spp., *Penicillium* spp. and *Trichoderma* spp. The activity of these species is associated with the availability of simple sugars, but as these get used up the incidence of this mixed 'casual' population decreases in favour of species able to degrade cellulose (Garrett, 1951). These species appear to form a more stable population which remain on the roots as the plant continues to mature. This more stable population is often dominated by *Fusarium* spp. and *Gliocladium* spp. (Parkinson & Clarke, 1964; Papavizas & Davey, 1961; Taylor & Parkinson, 1961).

Although fungi are present on the seed, studies have shown that these fungi do not play a significant role in root colonisation (Peterson, 1959; Parkinson & Clarke, 1964). It appears that the rhizoplane (root surface) is mainly colonised by fungi in the surrounding rhizosphere soil (Taylor & Parkinson, 1961).

The association of a fungus in the rhizoplane can be made as a result of overcoming host resistance and finding the right nutritional and environmental conditions for growth. Čatská (1963) believed that the rhizoplane and spermosphere may affect the composition of the colonising micro-flora by the release of exudates. Parkinson *et al.* (1963) also suggested that exudate composition may influence the nature of the micro-flora on the rhizoplane regardless of the existing population within the soil. A mixture of exudates, including sugars, amino acids and organic acids, leak either as active secretions or as plant mucilages from autolysing epidermal cells (Bowen, 1979). Biologically active organic volatiles released by seeds and subterranean parts of plants include alcohols, aromatics, terpenes, ethylene, aldehydes and acetone. These volatiles have been shown to both inhibit and stimulate the germination of various fungal species (Bowen, 1979). The root exudates of seven species of plants belonging to four families showed qualitative differences in the composition of their root micro-flora (Vančura & Hovadîk, 1963). Parkinson *et al.* (1963) found that a plants micro-flora may also change in response to a change in the released exudates as the plant matures.

Other environmental factors may also influence root colonisation. Moisture plays a role in disseminating bacteria and also influences fungal activity. Bacteria require a continuous film of water for movement and discontinuous films of water that may occur in drier soils could limit the movement of such cells to the root surface (Bowen & Rovira, 1976). *Rhizobium trifolii* was limited between -0.004 MPa and -0.04 MPa suction in coarse sand and silt loam respectively, because of the influence of pore size on the continuity of the water film. Zoospores of *Pythium* spp. and *Phytophthora* spp. require water for movement and passive movement to a root may be more important than the movement of its flagella (Griffin, 1972). Although much emphasis has been placed on the ability of fungi to grow through soil (Bowen & Rovira, 1968), activity is also likely to be influenced by the water potential in the soil, the pH and temperature (Parkinson *et al.*, 1963).

Generally most micro-organisms are confined to the surface of the root and have neither a detrimental or a beneficial effect upon the plant. Those micro-organisms that invade the living cortex must overcome the plants defence mechanisms such as the production of chemicals to inhibit microbial growth. Chemicals may be diffusible passing into the rhizosphere or they may be contained in cells prior to infection (Phytoalexins). The nematode *Trichodorus christiei* was found to multiply rapidly on tomato roots but did not feed on asparagus roots. When the plants were grown in pots together and separately, the nematode populations were significantly smaller in asparagus pots alone indicating that a toxic substrate, believed to be a glycoside, diffused into the soil (Baker & Cook, 1974). The phytoalexin tomatin, produced by the tomato plant, is believed to be synthesised in response to attempted invasion by micro-organisms. Macura (1968) suggested that healthy plant roots are not decomposed by the cellulolytic, protolytic and pectinolytic micro-organisms living on the roots due to the production of structural enzyme compounds exuded by the plants. These compounds are believed to be natural inhibitors of cellulase, pectinase and amylase.

1.1.2 Cortical senescence of cereal roots

Competition between ectotrophic cereal root parasites is influenced by the natural progressive senescence of cereal root cortices. The importance of root cortical death (RCD) in relation to fungal colonisation was realised by Holden (1975, 1976). Holden noticed that bacteria occurred deep in the cortex of apparently healthy white roots of wheat only five days old. This finding led to an investigation to assess the rate of cortical cell death using staining. Holden's studies indicated that the rate of cortical death had been grossly under-estimated. In 3-week old wheat and barley plants, 61% and 41% respectively of cortical cells were anucleate in the oldest regions of the seminal roots axes despite the healthy white outward appearance.

Henry & Deacon (1981) found that there was a regular pattern of RCD in cereals, however, the rates differed between cereal types. Henry & Deacon (1981) found that behind the growing tip, cortical cells became progressively anucleate from the outer layers inwards. The innermost cortical cells next to the endodermis remained alive. These results were in agreement with the findings of Holden (1975, 1976). The pattern of cortical senescence was seen to remain the same when cereal plants were grown in sterile and non-sterile conditions. In soil the rate of senescence was not enhanced following inoculation with the weak parasites *Idriella bolleyi* and *Phialophora graminicola* (Kirk & Deacon, 1987b).

From these studies, RCD is believed to be endogenously controlled and to result from a reduced supply of organic nutrients reaching the cortex from the stele. The occurrence of RCD in sterile conditions provides evidence that it is not caused by the activities of micro-organisms (Henry & Deacon, 1981). The pattern of cortical senescence suggests that the cortical cells do not act as an efficient sink compared to the growing tip. The reduction in nutrient supply to the cortex attributable to this, means that if nutrients are to reach the epidermal cortical cells they must first pass through the underlying cells. It is possible that root cortical cells survive for some time after the supply of organic nutrients is removed (Henry &

Deacon, 1981).

1.1.3 Fungal colonisation of cereal roots

In addition to occupying the rhizosphere and the rhizoplane, soil micro-organisms penetrate and colonise the cortical tissues of plants (Nilsson, 1975). Salt (1979) suggested the root-colonising population includes unspecialised facultative parasites able to survive by either colonising roots or as soil saprophytes. Such fungi include *Pythium* spp., *Fusarium* spp., *Rhizoctonia* spp. and *Cylindrocarpon* spp. Some root-colonising fungi are known to be obligate parasites surviving as resting spores in the absence of a host. Such fungi include *Polymyxa* spp., *Lagenocystis* spp. and holocarpic zoosporic fungi.

Those fungi which cause root and stem base disease exert a biological stress which limits plant development. The consequence of such disease results in a reduction in yield which may be of economic significance. The ability of a cereal plant to produce a good yield depends on a healthy root system to support stem and head development. The failure of roots to provide enough nutrients and water during development results in the failure of buds to activate and the formation of leaves and heads on activated tillers (Cook & Veseth, 1991).

A number of fungi have been consistently isolated from both diseased and healthy cereal roots. Salt (1979) isolated *Fusarium* species most commonly from cereal roots, whereas *Colletotrichum* spp. and *Cylindrocarpon* spp. accounted for only 5 to 10% of the isolates. Other species commonly isolated from cereal and/or grass roots include *Cephalosporium* spp., *Microdochium nivale,* (formally *Fusarium nivale*), *Idriella bolleyi, Rhizoctonia* spp., *Periconia macrospinosa, Pythium* spp., *Phialophora* spp., *Gaeumannomyces graminis* var. *tritici* and *G. graminis* var. *graminis* (Deacon, 1974; Salt 1979).

The root-infecting fungi differ in their pathogenicity ranging from aggressive such as *G. graminis* var. *tritici,* to weak parasites including *Phialophora graminicola* and *I. bolleyi.*

Gaeumannomyces graminis var. *tritici* is classed as a major pathogen because it is highly invasive (Holden, 1976). Infection normally occurs in moist conditions with the pathogen spreading from crop residues by ectotrophic runner hyphae. *G. graminis* var. *tritici* penetrates the living epidermis of host roots by hyaline infection hyphae and then invades the intracellular spaces well in advance of other micro-organisms (Holden, 1976). Invasion of the vascular tissue results in the intense chocolate-brown discolouration of the roots (Clarkson & Polley, 1981). *Gaeumannomyces graminis* var. *tritici* is known to be a poor saprophytic competitor and thus benefits from early senescence of root cortices as a food base to increase its inoculum. Once it is established, the fungus goes on to infect the remaining healthy roots.(Kirk, 1984; Kirk & Deacon, 1987a). This type of disease build-up may be important in the early years of monoculture after a break from non-susceptible hosts (Clarkson and Polley, 1981). Take-all infected plants are most obvious near heading where the plants appear uneven in height, irregular in maturity, have few tillers and ripen prematurely. The heads are often sterile and bleached, referred to as 'whiteheads'. In intensive cereal growing parts of the world, crop losses can be up to 10 to 20% in second, third and fourth successive wheat crops (Smith *et al.,* 1988).

Other pathogens that require moist conditions for infection are *Pythium* spp. and a number of *Rhizoctonia* species causing root-rot. *Pythium* rots can be particularly damaging to embryos and seedlings, and are often responsible for the lack of seedling vigor. *Pythium* species can infect a wheat embryo within 48 hours from planting (Cook & Veseth, 1991). Once established on an embryo the fungus is ideally suited to live on soluble sugars and other nutrients from the seed which they solubilize enzymatically. Its movement from the endosperm into the shoots and roots, damage root hairs and young rootlets affecting nutrient uptake. *Pythium* species are ideally suited to infection of the newly germinated embryo, and infection

is believed to be initiated by the leakage of a range of sugars and amino acids from the seed (Cook & Veseth, 1991).

Rhizoctonia disease can occur as damping-off on seedlings or as a root and stem-rot on more mature plants. Damping-off is the most common symptom occurring in cold wet soil. The fungus attacks the growing tip of the seedling which then dies (Agrios, 1988). In contrast, *Rhizoctonia* also causes brown patch disease of turf grass under hot and humid conditions. In cereals *Rhizoctonia cerealis* causes sharp eyespot. The rot forming on the lower stem and leaf sheaths is usually pale with an irregular but roughly elliptical purplish-brown margin (Smith *et al.*, 1988). Plants affected early in their growth may develop 'whiteheads', or lodge, but generally there is a diminution of grain production by infected tillers (Richardson *et al.*, 1976).

In contrast to *Pythium* spp., *Rhizoctonia* spp. and *G. graminis* var. *tritici*, *Cochliobolus sativus* can cause root-rot under dry conditions. *Cochliobolus sativus* can infect plants alone or in combination with *Fusarium* species causing common root-rot of wheat also referred to as dry-land root-rot. Both fungi tend to invade the tiller bases and the sub-crown internodes because they are generally located near the soil surface.

Fusarium species can cause both root and foot-rot in cereal plants. *Fusarium* disease can be caused by either a single *Fusarium* species or by a complex of several *Fusarium* species, and infection can occur at all stages of crop development (Parry *et al.*, 1994). The five *Fusarium* species most commonly involved in the disease complex include *F. culmorum*, *F. avenaceum*, *F. graminearum*, *F. poae* and *Microdochium nivale*, formally classified as *Fusarium nivale* (Mueller, 1977). The geographical distribution of the five species reflect their different optimal temperature and moisture requirements. *Microdochium nivale* is commonly isolated from cereals grown in cooler wetter regions such as Scotland (Rennie *et al.*, 1983). In contrast, *F. graminearum*, *F. avenaceum*, *F. paoe* and *F. culmorum* are more commonly isolated from hotter, dryer areas such as the south central great plains of the USA (Cook,

1968). Infections caused by *Fusarium* spp. include seedling blights, mainly from seed-borne inoculum, and foot-rots in older plants caused mainly by *F. culmorum* and *F. graminearum*. The damaged plant tissue acts as a barrier to water flow which causes the heads to dry up and turn white (Smith *et al.*, 1988).

Pseudocercosporella herpotrichoides causes a destructive foot-rot which is characterised by an eye-shaped lesion that develops on the stem base. Eyespot infection often results in the breakage of the stem resulting in decreased grain production and problems with harvesting because of severe lodging. Spores of *P. herpotrichoides* are produced in the lesion and this production can continue for weeks providing the temperature is cool and there is enough moisture. The fungus can survive on stubble in this form providing inoculum for the following season. The disease is mainly a problem with winter grown wheat where it can cause losses in Europe of up to 5 to 10%, and under continuous wheat cultivation, up to 60%

Cephalosporium gramineum causes *Cephalosporium* stripe of wheat under similar conditions to eyespot disease. Like *P. herpotrichoides*, the fungus uses the residues from the previous crop as a food base. The spores produced during cool, wet periods percolate in soil water down the soil profile until they come into contact with roots. The fungus invades the vascular system mainly via wounds, and is then swept upward in the vascular system by water movement in the plant resulting in the eventual invasion of the stem. Mature plants are usually stunted and die shortly after heading (Smith *et al.*, 1988).

Non-pathogenic root colonisers such as *Phialophora graminicola* and *Idriella bolleyi* are generally weakly invasive. Penetration of the epidermis is limited to senescent or dead cells and the stele is not invaded. *Gaeumannomyces graminis* var. *graminis* and *Phialophora* spp. in comparison, are moderately invasive. These fungi will penetrate cells more rapidly than *P. graminicola* and *I. bolleyi* and have some ability to penetrate living cells. These weak pathogens usually penetrate as far as the inner most cell layer of the cortex and sometimes

invade the stele where they cause local vascular discolouration (Deacon, 1974).

Many root parasites produce cessation structures in the vicinity of living host cells, around the bases of lateral joins and against the inner most cell layer. Cessation structures are believed to be temporary survival structures from which the fungus can resume invasion of the cortex as host resistance declines (Deacon, 1981).

It has been suggested that many weak root parasites may be minor pathogens which limit the yield of crop plants often by forming a disease complex (Salt, 1979). Evidence for this is limited, but some workers have shown that young seedling tissue is especially vulnerable if colonisation becomes heavy. The obligate parasite *Polymyxa* generally shows no visible symptoms of disease but the release of a large number of motile zoospores when the soil is wet, produces sudden heavy infections on young roots (Salt, 1979). Diseases of cereals and grasses from minor pathogens have often been related to poor environmental conditions affecting the host. Root-rot of cereals by *Pythium arrhenomanes* was found to become serious in soils deficient in phosphorus (Vanterpool, 1952). *Idriella bolleyi* has previously been classed as a minor pathogen. At high inoculum levels the fungus was found to cause damage to seedling roots. Damage was attributed to disruption of the stele, particularly the phloem, although most plants recovered quickly after infection (Fitt & Hornby, 1978).

Generally weak parasites may be more beneficial than deleterious because they may be able to control infection by more aggressive pathogens (Salt, 1979; Kirk & Deacon, 1987a). The colonisation of cereal roots by the weak parasites *P. graminicola* and *Idriella bolleyi* may allow these fungi to compete with *G. graminis* var. *tritici* and other root and stem base pathogens resulting in the exclusion of the pathogen and hence biocontrol. The success of biocontrol is dependent on the ratio of competing fungi and early establishment by weak pathogens is crucial (Kirk & Deacon, 1987a).

1.2 Control of soil-borne pathogens

The methods of pest and disease control can be simply categorized as chemical, physical or biological control. Every control management system should maximize a plants ability to defend itself against pests, diseases, weeds and adverse environmental conditions (Cook & Veseth, 1991). In view of this, a single control strategy may not be as effective as using combinations and integrations of chemical, physical and biological controls.

1.2.1 Chemical control

Chemical control of soil-borne disease can be brought about by a number of methods including soil sanitation, seed treatment and occasionally foliar sprays.

Soil sanitation with chemicals is often a major part of integrated pest management programs. Some crops can not be grown profitably unless the soil is first chemically treated. The fields planted to crops which return a high income including cotton, ornamentals, strawberries and vegetables, are routinely treated before use (Lucus *et al.*, 1985).

Sanitation is often brought about by the use of fumigants or non-fumigants. Fumigants produce a gas or vapour in the soil that kills a range of micro-organisms. Non-fumigants are generally applied as a granular formulation which then dissolves in the soil water. Alternatively liquid non-fumigants can be sprayed uniformly over the soil surface. Increased growth responses in wheat have been observed following soil fumigation with methyl bromide and chloropicrin. Despite this, soil sanitation for cereal crops is not an economically feasible system, thus fumigation tends to be limited to small experimental plots (Cook & Veseth, 1991).

Fungicidal chemicals are continually being developed either as seed treatments or as

sprays to control a number of soil-borne diseases including *Rhizoctonia* root-rot (*Rhizoctonia solani*), take-all (*Gaeumannomyces graminis* var. *tritici*) and eyespot (*Pseudocercosporella herpotrichoides*). Chemical seed treatments to control soil-borne root-rots have been limited mainly because roots grow away from the protected seed which leaves them vulnerable to infection. More recently systemically translocated compounds provide enhanced protection at a distance from the seed (Mathre *et al.*, 1986).

Treatment of wheat seed by fumigation is a standard practice in the Pacific North-West. The seed can be treated with a number of chemicals including carboxin and /or thiram, metalaxyl, triadimenol, quintozene and imazilil (Smiley *et al.*, 1990). The success of these chemicals for disease control has been variable. Smiley *et al.* (1990) found little effect with thirteen sterol-inhibiting fungicide treatments on the control of *Rhizoctonia* root-rot. These results were in contrast to those of Cook (1986) who found several fungicides highly toxic to *Rhizoctonia solani* and *Rhizoctonia oryzae in vitro.*

Garcia & Mathre (1987) tested eight sterol-inhibiting fungicides *in vitro* and found that all were inhibitory against *Gaeumannomyces graminis* var. *tritici* at 10μM. In field studies triadimenol and prochloraz were the most effective at rates of 0.3g of active ingredient per kilogram. Similarly Mathre *et al.* (1986) found triadimenol seed treatment delayed symptoms and development of take-all on spring wheat. Yield increases over the untreated controls were significant in both infested and naturally infested plots. Mathre *et al.* (1986) found that triadimenol appeared to have its effect by inhibiting the development of lesions on the seminal roots and sub-crown internodes. As triadimenol does not move downwards in plants, they concluded that the fungicide reduced disease by short-distance diffusion into the soil near the seed, and by translocation upward into the sub-crown internodes. Indeed, Steffens *et al.* (1982) showed that 14 C-labeled triadimenol applied as a seed treatment to wheat and barley, moves to the sub-crown internodes and diffuses into the soil surrounding the seed forming a zone of protection. In contrast to these results, Smiley *et al.* (1990) found that take-all on

winter wheat was strongly but not significantly suppressed by captan and triadimenol treatments, but their results did not lead to consistent improvements in grain yields.

Eyespot disease caused by *Pseudocercosporella herpotrichoides,* has been treated with foliar spray fungicides for many years with a consequential development of fungicide resistance. Originally eyespot was treated with carbendazim-generating fungicides (benomyl and thiophante-methyl) (Hoare *et al.*, 1986). More recently prochloraz, the sterol biosynthesis inhibitor, has been recommended for use in the UK, either alone or in mixtures with carbendazim-generating fungicides. Hoare *et al.* (1986) found that even with a mixture of prochloraz and carbendazim-generating fungicide used at 400g of active ingredient and 250g of active ingredient per hectare respectively, around 90% of isolates were still found to be resistant to carbendazim after three years of use.

Reports of sterol biosynthesis inhibitor-resistance in plant pathology are rare. It is believed that development of resistance is brought about by intense selection pressures (Julian *et al.*, 1994). Imazilil-resistant strains of *Penicillium* species were found in citrus crop packing houses. These were associated with abundant sporulation coupled by intense fungicide use (Angeles-Diaz & Hernadez, 1987). Two reports of *P. herpotrichoides* showing reduced sensitivity were reported in the mid eighties. King *et al.* (1986) found reduced sensitivity of *P. herpotrichoides* to prochloraz in Australia. Gallimore *et al.* (1987) found three isolates from the UK, Germany and Denmark which were able to show weak colony development, however, they appeared to be clearly disrupted by the presence of the fungicide. Three isolates could not be classed as resistant because they were still inhibited below the recommended application rate. More recently isolates from Northern France have been found that can grow on prochloraz *in vitro* at levels of up to 10mg litre^{-1} (Leroux & Marchegay, 1991). This finding has raised concern about the level of fungicide resistance in the field. Julian *et al.* (1994) stated that the development of resistance in the field may be driven by a number of factors including selection pressure exerted by both fungicide usage and in the event of cross-resistance from the

use of other commonly applied compounds.

1.2.2 Cultural control

Cultural methods of control have always been of fundamental importance in traditional farming. With increasing problems from chemical use, including pathogen resistance, food safety and environmental issues, crop management systems are still dependent on the traditional cultural methods of control (Cook & Veseth, 1991). Cultural control encompasses many aspects of crop husbandry including the use of resistant crop varieties, crop rotations, planting management, crop certification, tillage and crop sanitation (Cook & Veseth, 1991).

Along with the removal of volunteer weeds and use of certified seed, there are a number of ways in which the crop can be beneficially managed to reduce disease levels.

1.2.2.1 Crop rotation

Crop rotation has been one of the most traditional methods of preventing disease epidemics within a crop system. Control of disease by crop rotation is usually attributed to death of the pathogen in the absence of a susceptible host as the infested residue decomposes (Rothrock & Langdale, 1989).

Crop rotation has been shown to reduce certain root-rot pathogens. Maxson (1938) found that *Rhizoctonia* root-rot of sugar beet was reduced in three to four year rotations with alfalfa and potato. Likewise, Ruppel (1985) found that growing small grain cereals or corn preceding sugar beet decreased the incidence of *Rhizoctonia* root-rot in sugar beet. Crop rotation has been found to be one of the most effective methods of controlling take-all. Cook

(1981) found that take-all was common following a three year bean/soybean rotation, but was mild following potato, oats or alfalfa. Prew & Dyke (1979) found that oats, clover, broad bean and corn were equally effective in reducing take-all in winter wheat.

Despite the evidence of disease reduction through crop rotation, cereal crops are grown intensively in many temperate areas of the world. Yarham (1981) stated that successful continuous wheat is dependent on efficient husbandry including good seed-bed preparation and good drainage, but more so on the nature of the soil. The discovery of take-all decline allowed growers more confidence to adopt continuous cereal sequences. Take-all decline is either likely to be a modification of the soil micro-flora reducing inoculum activity, or loss of aggressiveness of the inoculum itself (Wong, 1981; Yarham, 1981).

The effect of intensive cereal cropping on other pathogens has not been as devastating as originally predicted. The introduction of an eyespot-resistant wheat variety in the 1950's helped to reduce the incidence of the disease (Yarham, 1979). In the 1960's, a growth regulatory chemical, chlormequat, helped with the production of shorter, thicker straw rendering the plant less vulnerable to lodging (Yarham, 1979).

1.2.2.2. Tillage

Tillage has been shown to influence soil-borne diseases to some extent, however, the effects have been inconsistent (Tinline & Spurr, 1991).

Conventional tillage involves much more soil manipulations than minimum or zero tillage. Tillage methods include the moldboard plough which inverts top soil, including organic crop residues, with the lower non-infected soil. Alternative reduced or zero tillage methods aim to keep soil disturbance to a minimum.

The eyespot pathogen *Pseudocercosporella herpotrichoides* is entirely dependent on host tissue for survival and reproduction. Disease incidence is entirely related to the amount of infested host residue on the soil surface (Cox & Cook, 1962). It was originally believed that stubble-mulching and reduced tillage was likely to favour an increase in the incidence of the pathogen. However, Cook & Waldher (1977) found in an eight year trial in the Pacific North-West, that eyespot incidence was the same or less in stubble-mulched plots than in moldboard ploughed plots.

Some workers have reported that take-all levels are reduced with zero tillage and direct drilling (Brooks & Dawson, 1968), whereas other workers have reported an increase in the disease as a result of this practice (Moore & Cook, 1984). Tinline & Spurr (1991) suggested this may be a result of different cropping systems and different environmental conditions. Tinline & Spurr (1991) found that the incidence of the root-rot pathogen *Cochliobolus sativus* was decreased in spring wheat under zero tillage when compared to conventional tillage. Shallow seeding under reduced tillage decreased the level of disease because of fewer propagules of *C. sativus*.

1.2.2.3 Crop residue

Traditionally crop residues were burnt to destroy a range of organisms including fungi, bacteria and viruses (Lucus *et al.* 1985). More recently legislation stopped this practice and organic residues are now either ploughed back into the soil or left on the soil surface.

Crop residues can provide nutrients in the absence of a living host. In some cases organic residues added to the soil can intensify the microbial activity of soil (Shipton, 1979). The root-rot pathogen *Cephalosporium gramineum* causing *Cephalosporium* stripe of wheat, invades the vascular system of young wheat plants. The infected plant dies after heading but

the fungus persists in the parenchyma. The fungus can persist on dead straw for up to three years (Bruehl, 1975).

Crop residues can have an indirect effect on the incidence of soil-borne disease by increasing the soil surface temperature and moisture. Due to the presence of crop residues, root-rot attack of corn by *Pythium ultimum* was found because *Pythium* species were more active in moist soil (Doupmik *et al.*, 1975). In contrast, minimum disturbance of crop residue was found to reduce the incidence of stalk-rot of sorghum caused by *Fusarium moniliforme* in Western Nebraska. It was believed that the residue maintained a cooler, wetter soil which was less favourable for the pathogen. Likewise, the severity of *Fusarium* foot-rot of winter wheat (*Fusarium culmorum*) in Oregon and Washington was found to be directly related to the water potential. Evaporation of water which had accumulated in the soil profile early in the rainy season was reduced as a result of a dust-mulch created in the summer. Such a management practice successfully reduced the severity of the root-rot disease (Papendick *et al.*, 1973).

1.2.2.4. Time and depth of planting

With many crops high yield can often be obtained by early sowing (Shipton, 1979). Planting date can however, influence disease levels. Early planting of wheat in the Pacific North-West can result in high rates of transpiration in the early summer, predisposing the crop to water-stress and severe attacks of foot-rot caused by *Fusarium culmorum* (Papendick & Cook, 1974).

Huber (1967) found that early planting of wheat in humid areas of the Pacific North-West reduced yields as a result of eyespot and *Rhizoctonia* foot-rots. Greeny (1942) found that the root-rot was reduced by early shallow seeding compared to crops that were sown

late, or too deeply. Greeny (1942) concluded that soil nutrients, temperature, moisture content and aeration play a role in deciding the time, rate and depth of planting at each location to reduce root-rot.

1.2.2.5 Nutrient management

Much research has concentrated on trying to increase plant yields by increasing the availability of certain nutrients. Applications of nitrogen, phosphorus and potassium have increased greatly over the last forty years with the belief that they may promote new root growth or alter the soil pH and so influence disease levels (Yarham, 1979). *Verticillium* wilt of hops was increased as a result of increased nitrogen levels (Sewell & Wilson, 1974). In contrast, Slope *et al.* (1970) found that the incidence and severity of take-all decreased with increasing nitrogen applications due to improved root growth. Huber (1981) stressed that in the spring adequate nitrogen supplied to a cereal crop may help to promote vigorous root growth and thus promote survival of *Gaeumannomyces graminis* var. *tritici* in the autumn. Huber (1981) suggested that wheat and barley in risk situations should ideally be drilled with phosphate and potash only.

The addition of phosphorus in deficient soils controlled symptoms of *Phytophthora cinnamomi* root-rot in *Pinus radiata* (Newhook, 1970). Likewise, potassium was found to reduce corn stalk-rot (*Gibberella raea* and *Gibberella fujikuroi*) when potassium was applied as KCl (Younts & Musgrave, 1958).

1.2.2.6 Host resistance

Host resistance is becoming more and more widely used against a whole range of

fungal, bacterial and viral diseases. Genetic resistance to *Rhizoctonia solani* root-rot has been reported in a number of non-cereal crops including lima beans (Steinswat *et al.*, 1967), snap beans (Dickson & Boettger, 1977) and alfalfa (Barnes *et al.*, 1980). Few reports of resistance against root-pathogens has been found in cereal crops. McDonald & Rovira (1985) claimed to find cereal resistance to *Rhizoctonia solani* root-rot. In contrast, Neate (1989) found very little resistance to this disease in cereals, and so stated that it is unlikely to be enough to warrant using this as a method of disease control in the field. Similarly, differences in wheat cultivar resistance to *Gaeumannomyces graminis* var. *tritici* have been demonstrated, however, there has been large variations in relative performance from one test to another (Jensen & Jørgensen, 1976).

1.2.3 Biological control

Biological control can be defined as the reduction of the amount of inoculum or disease producing activity of a pathogen accomplished by one or through one or more organisms other than man (Cook & Baker, 1983).

The actual mechanisms operating in biocontrol may be complex. Some of which have been recognised as antibiosis, competition and mycoparasitism (Baker, 1968). If biological control is to become more widely used in agricultural production, it is fundamental that the significance of these mechanisms are understood.

1.2.3.1 Antibiosis

Evidence for the role of antibiotics in the soil environment has been difficult to establish. The problems implicating antibiosis in biological control relate to the difficulty

of extracting antibiotics from the soil or the rhizosphere where they might exert their effects (Baker, 1968). Further difficulties have been encountered when trying to replicate *in vivo* and *in vitro* success in field conditions (Fokkema, 1973).

Evidence to suggest that antibiotics and other diffusible compounds may have a role in biocontrol has been strengthened by the use of genetic techniques (Fravel, 1988).

Howell (1987) believed that *Gliocladium virens*, known to be strongly parasitic of *R. solani*, may use antibiotics, notably glioviren, along with mycoparasitism to suppress *R. solani*. Howell (1987) used U.V. radiation to produce a mutant strain of *G. virens* which showed no mycoparasitic activity, but retained the same antibiotic component as the parent strain. The parent and mutant strain had similar efficacy as biocontrol agents of damping-off of cotton seedling disease induced by *R. solani*. Howell & Stipanovic (1983) also compared gliovirin-negative and gliovirin super-producing strains of *Gliocladium virens* in the control of *Pythium ultimum*, and found that the non-producers did not give as much control as the parental strain, however, the super-producers gave better control than the parental strain *in vitro*, but only comparable control with the parental strain *in vivo*.

Pseudomonas fluorescens strain 2-79 is known to produce the antibiotic phenazine-1-carboxylate and this has shown to be active against *Gaeumannomyces graminis* var. *tritici in vitro* (Thomashow & Weller, 1987; 1988). Mutants defective in phenazine production (phz^-) were generated by TN5 insertion and compared with the parental strain to determine the importance of the antibiotic in take-all suppression. Six independent prototrophic phz^- mutants were non-inhibitory to *G. graminis* var. *tritici in vitro*, and produced significantly less control of take-all than the 2-79 phenazine-producing strain on wheat seedlings. Antibiotic synthesis and fungal inhibition were restored in two mutants complemented with cloned DNA containing sequences for the required phenazine production. These studies support the role of the phenazine antibiotic in take-all suppression (Thomashow & Weller,

1987; 1988).

Other antibiotic-like compounds, notably bacteriocins have had remarkable success in disease control. The soil-borne disease crown-gall, caused by *Agrobacterium tumefaciens*, is successfully controlled by *Agrobacterium radiobacter* K84 (Kerr, 1974). Control of *A. tumefaciens* was brought about by the production of agrocin 84. Agrocin 84 acts as an inhibitor of DNA synthesis and so is important in terminating DNA synthesis in the pathogen (Murphy & Roberts, 1979).

Despite the evidence for the production and function of antibiotics, there are technical difficulties in showing that antibiotics can be significant in the bulk of the soil. Once produced antibiotics are likely to diffuse into the soil. They may become microbiologically degraded, or unstable due to soil pH (Baker, 1968). They may also become adsorbed onto soil components (Deacon, 1991). Persistence of antibiotics may occur in the adsorbed condition, but it is more likely that they will be inactive (Brian, 1957). In order for a micro-organism to produce antibiotics, it must be established on a nutrient resource and actively growing. Initial success of an organism in colonising these resources cannot be attributed to antibiotic production (Deacon, 1991). There is evidence to show production of antibiotics is dependent on the presence of specific nutrients. Howie & Suslow (1987) found that the expression of an antifungal gene in *Pseudomonas fluorescens* was enhanced 9-fold by the presence of simple sugars, amino acids and dicarboxylic acids. Antagonistic bacteria were found to consistently produce two or three times as much bacteriocin-like antibiotic when grown on soybean tissue than barley tissue extract.

Howell (1987) emphasised that although antibiosis may play a role in plant disease control, it is highly likely that it is not a mutually exclusive function, but probably occurs in conjunction with other mechanisms such as competition and parasitism. It is most likely that disease suppression will be achieved predominantly through competition for nutritional

micro-sites and that antibiosis is a secondary facilitating mechanism in defense of further intrusion (Baker, 1968; Deacon, 1991).

1.2.3.2. Mycoparasitism

Mycoparasites are common in many natural environments where they may help to regulate the populations of their hosts. A mycoparasite can be defined as a fungus that parasitises another fungus (Boosalis, 1956). It is believed that such fungi may have an important role to play in the biological control of pathogens.

Two distinct classes of mycoparasite, biotrophic and necrotrophic, have been defined by Gäuman (1946). Biotrophic mycoparasites are those characterised by their feeding on living host tissue. They are believed to exist in a physiological balance with the host cells on which they depend for their nutrition (Deacon & Berry, 1992). *Piptocephalis virginiana*, a biotrophic mycoparasite belonging to the order of Mucorales, is known to have a host range encompassing fifteen genera belonging to six families of the order of Mucorales (Berry & Barnett, 1957). Necrotrophic mycoparasites kill their host cells at an early stage during parasitism and then convert to a saprophytic existence to gain nutrients from the dead host cells. *Pythium oligandrum* is a mycoparasite shown to be aggressive to several other fungal species including *Fusarium oxysporum* and *Trichoderma aureoviride* (Jones & Deacon, 1995).

Mycoparasitism involves intimate contact between a host and parasite (Boosalis, 1964). The establishment and association between micro-organisms is still being questioned. How a parasite finds its host is still not fully understood. Butler & King (1951) suggested a role of tropism involving contact stimuli, whereas Barnett & Lilly (1962) believe some form of chemical-elicited tropism is responsible. Although there is some doubt

that mycoparasites do show pre-contact tropism towards their hosts, the lack of available data for statistical comparisons under different conditions of host parasitic combinations is a limitation to the understanding of the phenomenon, thus the responsible factors remain unknown (Deacon & Berry, 1992).

The mechanisms or modes of action of mycoparasites are diverse (Boosalis, 1964). Whatever strategy is used by the mycoparasite, most will stop the host growth within minutes or even prior to contact. Deacon & Berry (1992) observed cessation of growth of *Rhizoctonia solani* and *Pythium aphanidermatum* at distances of 30-300μm by some antagonists. *Gliocladium virens* and *T. harzanium* showed pre-contact abeyance of *P. aphanidermatum* and *Botrytis cinerea* at a distance of 600μm. The host may exhibit explosive lysis or cytoplasmic coagulation or alternatively a mycoparasite may coil around the host or directly penetrate the hyphae. Deacon & Henry (1978) have observed *Pythium oligandrum* coiling around the hyphae of host fungi and in some cases penetrating them. In the case of the pathogens *Fusarium culmorum*, *Botrytis cinerea* and *Rhizoctonia solani*, no pre-contact effect was seen, however, on contact the hosts were penetrated often within ten minutes (Laing & Deacon, 1991).

Boosalis (1964) has shown penetration pegs developing from hyphae in direct contact with the mycelium from the mycoparasite coiling around the host hyphae in the case of *Talaromyces flavus* parasitising *R. solani*. The destructive *Didymella exitialis* penetrates the hyphae of *Gaeumannomyces graminis* var. *tritici* by producing penetrating hyphae, or by boring directly through the host cell wall (Adams, 1990).

There has been many years of research into the action of mycoparasites and few if any have achieved commercial success as introduced inocula. Problems surrounding the use of mycoparasites as biocontrol agents are related to the economic implications resulting from the high rates of application needed, and despite this only low levels of disease control

are obtained (Adams, 1990).

For the suppression of damping-off of carrot, caused by *Rhizoctonia solani*, *Trichoderma harzanium* must be applied at 1500 kg per hectare after methyl bromide fumigation (Strashnow *et al.*, 1985). In order to give significant suppression of *Rhizoctonia* root-rot on sugar beet, Ruppel *et al.* (1983) applied *Trichoderma harzanium* to field plots at a rate of 163 kg of formulation per hectare.

Pythium nunn, a mycoparasite of many soil-borne pathogens, appears *in vitro* to be a promising biocontrol agent, but in the environment it would require a large amount of organic matter to provide it with an energy source. It is possible that the inoculum could be increased if it was applied to the soil with crop residues because *Pythium nunn* cannot produce new resting structures as a result of mycoparasitism (Paulitz & Baker, 1987).

Sporidesmium sclerotivorum appears to have some potential as a biocontrol agent. *Sporidesmium sclerotivorum* is an obligate parasite of *Sclerotinia minor* the cause of root-rot of lettuce. The mycoparasite produces macro-conidia which geminate and parasitise the sclerotia of the pathogen. Because *S. sclerotivorum* can increase its population as it parasitises the host, and because the disease epidemiology, crop, host, pathogen and parasite relationship was known, the original application rate of 23 000 kg/ha could be reduced drastically to just 0.2 kg/ha (Adams, 1990).

1.2.3.3 Competition

Competition is the endeavor of two or more organisms to gain a resource that each requires from the supply of a substrate when the substrate is not sufficient for both. Substrates include high energy carbohydrates, nitrogen and possibly certain growth factors

(Fravel, 1988).

Competition within the soil is likely to be an important factor in the control of plant pathogens. Deacon (1991) suggested that micro-sites or 'windows of opportunity,' which are available for only a limited period, are a major factor in determining the outcome of competition. Such ecological niches will be available to those best adapted to exploit them.

As a large population of micro-organisms will be in a dormant condition within the soil, the ability to germinate and exploit micro-sites will be dependent on available exogenous sources (Lockwood, 1964). Most exogenous nutrients required by micro-organisms will be supplied in the form of exudates from seeds or other subterranean plant organs (Schroth & Hildebrand, 1964). Those micro-organisms requiring simple sugars and carbon constituents of plant material are likely to be primary colonises and will therefore occupy their substrate ahead of competitors. Cellulose and lignin decomposing competitors are likely to be exposed to the full intensity of microbial competition and in such circumstances may produce antibiotics to give them a competitive edge (Garrett, 1970).

The strategy for the use of competition in biocontrol is that one or more essential nutrients in the rhizosphere or rhizoplane must be limiting to inhibit growth (Baker, 1968). The principal limiting factors are carbon, nitrogen, vitamins and iron (Baker, 1968; Lockwood, 1988). *Fusarium solani* f. sp. *phaseoli*, the cause of root-rot of beans, can be controlled by the incorporation of barley straw into the soil. The natural soil micro-flora utilises available nitrogen in order to decompose the amendment, thus the pathogen is unable to infect or penetrate the host due to insufficient amounts of this element for germination (Synder *et al.*, 1965). In other circumstances the limitation of carbon for germination of *F. solani* f sp. *phaseoli* was demonstrated as being the principal inhibiting factor in soil (Cook & Schroth, 1965). Toussoun & Synder (1961) found that a combination of amino acids and porous porcelain seed models, stimulated 12% chlamydospore germination of *F. solani* f.

sp. *phaseoli* compared with no germination in a water control.

Iron is essential for the germination and growth of fungi. Some *Pseudomonas* spp. produce siderophores which have a high affinity for iron. These bacteria can act in direct competition with fungi by chelating any free available iron in the rhizosphere. In iron limited conditions some *Pseudomonas* spp. have been shown to suppress particular soil-borne diseases by depriving the pathogen of iron for growth (Campbell, 1989).

The weak parasites *Phialophora graminicola* and *Idriella bolleyi* have been shown to control take-all in grasslands and cereal monocultures. These fungi colonise the naturally senescing root cortices and control the pathogen by competitive niche exclusion (Kirk & Deacon, 1987a, 1987b). The success of one commercially available biocontrol agent has been attributed to the ability of the biocontrol agent to exploit the same 'opening' as the pathogen. *Peniophora gigantea* is able to control the pathogen *Heterobasidion annosum* on pine stumps because it colonises the stumps naturally in competition with *H. annosum* and excludes it. Routine inoculation of pine stumps with the biocontrol agent in Britain totalled 62, 000 hectares in 1973 (Deacon, 1983).

1.3 Biological control of cereal root and stem base pathogens

Weak parasites have been shown to successfully compete with *Gaeumannomyces graminis* var. *tritici* in field conditions and this is particularly notable when the inoculum level of the pathogen is low (Wong, 1981). Hypovirulent strains of *Gaeumannomyces graminis* var. *tritici* are known to exist naturally with virulent strains in wheat fields in several countries (Asher, 1981). In France hypovirulent strains of *G. graminis* var. *tritici* were isolated from take-all decline soils. In pot experiments these strains were shown to reduce take-all disease. Similarly in field trials hypovirulent strains of *G. graminis* var.

tritici were able to prevent the spread of pathogenic *G. graminis* var. *tritici* from an inoculated point (Wong, 1981).

Gaeumannomyces graminis var. *graminis* is known to be non-pathogenic to many grasses and cereals (Walker, 1981). *Gaeumannomyces graminis* var. *graminis* was used in Australia to precolonise wheat roots. The precolonised roots were protected from infection by *G. graminis* var. *tritici* in field conditions and consequently grain yields and dry weights were significantly greater than for unprotected plants (Wong, 1981). The biological control of *G. graminis* var. *tritici* by *Phialophora graminicola* has been seen in a number of studies. *Phialophora graminicola* is abundant in almost all British grasslands, but is relatively uncommon on cereal roots (Deacon, 1973, 1974; Kirk & Deacon, 1987a). *Phialophora graminicola* is considered to be a successful parasite of grass roots because it is non-pathogenic and thus exists in a harmonious relationship with its host. In contrast, a pathogen such as *G. graminis* var. *tritici* is regarded as a less efficient parasite due to its destructive infection (Garrett, 1970; Deacon, 1973). Deacon (1973) found that high populations of *P. graminicola* could be built-up rapidly in grass leys and this population could be carried onto successive wheat crops resulting in restricted colonisation by *G. graminis* var. *tritici*. Balis (1970) and Scott (1970) emphasised that the degree of control is dependent on how much of the root system is in contact with *G. graminis* var. *tritici*. It is feasible that short term leys (1 year) could be useful to control take-all in intensive cereal rotations, however, once a population has been carried over to wheat in the field it cannot increase its population on the roots of cereals (Deacon, 1973). This factor emphasises the need for high populations of the antagonist to be established initially during grass leys.

In view of these successes, Wong (1981) emphasised that control may only be effective in the early years of monoculture or following a break crop when take-all levels are reduced. Control is unlikely at the onset of severe take-all.

More recently, in search for alternative biocontrol agents of take-all and other root and stem base diseases, attention has focused on *Idriella bolleyi* because it is commonly found on roots and stem bases of graminaceous hosts (Sprague, 1948, 1950; Murray & Gadd, 1981), and so shares the same ecological niche as many root and stem base pathogens.

Despite the claims that *I. bolleyi* may be a minor pathogen at high inoculum levels on seedling roots (Fitt & Hornby, 1978), it is likely to be more beneficial than deleterious because it may control infection by more aggressive pathogens (Kirk & Deacon, 1987a). Control of *G. graminis* var. *tritici* was obtained by using dispersed *I. bolleyi* inocula. Wheat roots showed vascular discolouration in the presence of *G. graminis* var. *tritici* alone, but the number of roots infected decreased as the inoculum of *I. bolleyi* increased. Control was significant at an inoculum ratio (*I. bolleyi:G. graminis* var. *tritici*) of 10:1. Highly significant results were also obtained using layered *I. bolleyi* inocula. The establishment of *I. bolleyi* in the root regions below the seed prevented the upward spread of take-all, but spread of the disease to the lower areas of the root was not prevented (Kirk & Deacon, 1987a).

1.4 The commercial application of *Idriella bolleyi*

Although many of the root-parasitising fungi have been shown to control take-all in field conditions, there are major disadvantages in developing these as biocontrol agents. *Phialophora graminicola* and *G. graminis* var. *graminis* cannot be found at high inoculum levels on cereal roots in field conditions (Deacon, 1973). Many of these fungi do not produce germiable spores in culture and hence an inoculum could not be produced cheaply in a delivery system feasible for commercial use (Kirk & Deacon, 1987a).

Idriella bolleyi appears to have many advantages over the other ectotrophic root parasites which favours its development as a biocontrol agent. *Idriella bolleyi* is found frequently on stem bases of cereals, therefore is ecologically suited to the environment where control is required (Sprague, 1948; Murray & Gadd, 1981). *Idriella bolleyi* is known to sporulate readily in the exponential phase of its growth in submerged liquid culture, and is exceptional in this respect as few fungal control agents sporulate readily under such circumstances (Lascaris & Deacon, 1994). *Idriella bolleyi* has simple growth requirements which can be satisfied by glucose, nitrate nitrogen, thiamine and various other minerals (Lascaris & Deacon, 1994). The simple nutritional requirements of the fungus enable it to be easily grown in cheap liquid substrates such as molasses, a by-product of sugar manufacturing. This has important implications for commercial production.

The use of submerged liquid fermentation is preferable to the use of solid media in manufacturing as it allows the biocontrol inoculant to be scaled-up with ease (Jabudansa *et al.*, 1993). Jabudansa *et al.* (1993) showed that biomass production is feasible in liquid culture, and that the conidial fraction of *I. bolleyi* biomass has the greatest potential for formulation in a biocontrol inoculum. Jabudansa *et al.* (1993) found that although mycelium will be produced from conidia, this is more likely to occur in nutrient rich environments and less so in a nutrient limited rhizosphere.

Idriella bolleyi conidia can tolerate being dried onto seeds (Lascaris & Deacon, 1991). Chao *et al.* (1986) emphasised that a seed-borne inoculum may be important for water-facilitated transport down the rhizosphere thus increasing the dispersal of the inoculum. Seed-borne biocontrol agents may have the opportunity to be the first colonisers of roots (Parke, 1990). The ready sporulation of *I. bolleyi* under limited nutrient conditions will facilitate rapid proliferation in the rhizosphere, this may be advantageous to the biocontrol agent when competitors are growing in the mycelial form. The importance of *I. bolleyi* in competitive niche exclusion of root and stem base pathogens is enhanced by the

strong tropic responses shown in the conidial germ tubes which emerge towards dead or senescing root hairs, but emerge away from living root hairs (Allan *et al.*, 1992).

Field trials have shown that seed-applied *I. bolleyi* can improve yields of wheat by up to one tonne per hectare in first and second wheat crops (M.A.F.F., unpublished). It is thought that *I. bolleyi* may be able to control a range of root and stem diseases including *G. graminis* var. *tritici* (Kirk & Deacon, 1987a), *Pseudocercosporella herpotrichoides* causing eyespot (Reinecke *et al.*, 1979; Reinecke & Fokkema, 1981), and *Fusarium* foot and root-rot (Reinecke *et al.*, 1979), so therefore have a wider application in the future.

1.5 Strain improvement of biological control agents

Trends in biological control are changing from manipulating the environment to increase the level of naturally occurring biocontrol agents, to the use of specific biocontrol agents. There is more emphasis on improving strains to give them a better adaptability to differing environmental conditions and hence a greater competitive ability against other micro-flora.

Generally some form of genetic manipulation is used to try and improve strains. The most commonly used techniques include transformation, genetic recombination through protoplast fusion, UV irradiation and chemical mutagenesis. The traditional methods of UV irradiation and chemical mutation are becoming less favourable in comparison to transformation and protoplast fusion because they often result in unwanted detrimental mutations (Hocart & Peberdy, 1989).

Protoplast technology enables whole genomes to be crossed and this has been valuable for those seeking to produce variability among progeny (Harman & Straz, 1991).

Sometimes this technology is used to try and combine two useful attributes from different strains with the hope that a single superior strain will be produced (Harman & Straz, 1991). Transformation has the advantage that specific genes can be added or deleted. Through gene cloning, certain genes can be used in different species regardless of their taxonomic grouping (Tilburn *et al.*, 1983).

Despite much effort to improve fungi for use as biocontrol agents for plant pathogens, very little success has been achieved. However, these technologies have been successfully used for a number of industrial and medical purposes showing the potential of genetic manipulation.

L-phenylalanine ammonia lyse (PAL) catalyses the deamination of L-phenylalanine to trans-cinnamic acid and ammonia in a variety of plants and micro-organisms. PAL has a number of industrial and medical uses including treatment of certain mouse neoplastic tumors, quantitative analysis of serum phenylalanine and the treatment of phenylketonuria (Orndoff *et al.*, 1988). A chemical mutagen, ethyl methansulfonate, was used in order to produce mutants with increased PAL production in the yeast species *Rhodotorula graminis*. Mutants were obtained that produced PAL levels four to five times greater than the wild-type (Orndoff *et al.*, 1988).

In agriculture a strain improvement program was developed to increase extracellular phytase production by *Aspergillus niger*. About 75% of the total phosphorus in cereals, legumes and seeds exist as phytic acid phosphorus. Phytic acid phosphorus is essentially unavailable to monogastric animals and so has anti-nutritive properties. Mutants of *A. niger* with increased phytase production were obtained following UV irradiation. These mutants may be valuable for enzymatic treatment of animal feed and could therefore reduce phosphorus supplementation by increasing the bio-availability of the natural phosphorus in the feed (Chelius & Wodzinski, 1994).

Genetic engineering has been successful for the construction of protein over-producing fungal strains, such strains having value in the food industry. In particular, *Aspergillus* spp. have been used to insert multiple copies of specific cloned protein-producing genes using transformation. This procedure has been shown to considerably increase protein production (Verdoes *et al.*, 1995).

Sulphated carbapenem antibiotics having a dimethyl group on the eighth carbon are uniquely produced by *Streptomycetes griseus* sub species *cryophilus*. These antibiotics are highly stable compared to other sulphated carbapenem antibiotics, and have strong antimicrobial properties towards a range of gram-positive and gram-negative bacteria. Genetic recombination was successfully performed to obtain super-producers of sulphated carbapenem antibiotics by crossing sulphate transport-negative mutants of *S. griseus* and a sulphate transport-positive parent using polyethylene glycol protoplast fusion (Kitano *et al.*, 1985).

These examples of strain improvement provide some hope that these techniques will eventually be applied successfully to biological control agents for use in plant disease control.

With regard to strain improvement, Cook (1992) has emphasised the need to customize strains by selecting those adapted to local environments, and if required to introduce specific genes for biocontrol activity just as crop cultivars have had specific genes introduced to combat specific combinations of diseases. Cook (1992) also stressed that in the attempt to customize biocontrol agents, the exchange of microbial germplasm or genes nationally and internationally may allow more effective biological control over a wider range of soil conditions. In view of this, it is also necessary that there are logical and reasonable regulations to permit the release of strains with new traits or combinations for commercial use.

1.6 Aims of the work of this thesis

The aim of the work in this thesis was to further contribute to the development of *Idriella bolleyi* as a potential biocontrol agent of cereal root and stem base pathogens. In particular, emphasis was placed on understanding more about the physiology of *I. bolleyi* and of its behaviour and limitations as a biocontrol agent.

Because *Idriella bolleyi* was recognised as being of value as a general-purpose biocontrol agent reducing the activity of a number of pathogens, inter-strain variability to water-stress *in vitro* and *in vivo* was of interest. This work aimed to evaluate the possibility that strains might be targeted for the control of particular pathogens, or that strains mixtures could be used to improve biocontrol consistency over sites and seasons. With the need for a more 'customised' approach to biocontrol, part of this work also aimed to evaluate the feasibility and potential of strain improvement through mixed strain inoculum or by the use of techniques to bring about genetic recombination between two strains with desirable traits.

It has been established that *I. bolleyi* can sporulate on sterile seeds in perlite and go on to colonise the root system. However, little was known about its ability to increase its inoculum level on unsterilised seed in competition with the natural soil micro-flora. This work aimed to understand more about the competitive ability of *I. bolleyi* against other micro-organisms, as early rapid colonisation of roots would be of importance to the success of a seed-borne biocontrol agent. In view of this, a more desirable genetic marker was required to produce an accurate method for assessing the level of fungal biomass in plant tissue. This work aimed to introduce such a marker and evaluate its potential for use in studying fungal-fungal interactions *in vivo*.

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

All defined chemicals (unless otherwise stated) were purchased from Sigma (Poole, Dorset, UK), British Drug Houses (BDH; Poole Dorset, UK) and Fisons (Loughborough, UK). Enzymes for DNA manipulations were purchased from Boehringer Mannhiem. Enzymes for protoplast production were purchased from John Sturge Ltd. (Selby, North Yorkshire, UK), Pollock & Poole Ltd. (Woodley, Reading, UK) and Attila Jozsef University (Szeged, Hungary).

2.1.1 Fungal cultures

Fungal cultures were obtained from the Edinburgh University culture collection as follows: *Idriella bolleyi* strain T560 from barley roots, Southern England (Dr G.A. Salt, 1974); *Idriella bolleyi* strain AB1 from Flax, Northern Ireland (Black & Brown, 1986); *Idriella bolleyi* strain J10 from wheat Edinburgh (Dr J.J. Kirk, 1977). *Idriella bolleyi* strain T560-R2 and strain J10-R2 were produced as carbendazim-resistant strains from the parental strains T560 (Lascaris, 1990) and J10 (Dr J.J. Kirk, 1977). Also used were *Fusarium culmorum* (strain CD9, Edinburgh University culture collection), *Gaeumannomyces graminis* var. *tritici* (strain LD1, from wheat near Edinburgh, 1992) and *Phialophora* sp. (Strain IMI 187786, from maize, Edinburgh University culture collection).

Strains were maintained on Potato Dextrose Agar (PDA; Section 2.1.2) at 25°C for periods of up to four weeks. *Idriella bolleyi* strain AB1 was sub-cultured at regular intervals (every fourth sub-culture) onto Oatmeal Agar (Section 2.1.2) to prevent the culture from sectoring. For long-term storage, all strains were stored as mycelial blocks in 10 ml of sterile distilled water contained in Universal bottles, and kept at 4°C. Root and stem base isolates of *I. bolleyi* (Section 3.3.4) were maintained on Oatmeal Agar at 25°C for periods of up to four weeks; nitrate non-utilising (*nit*) mutants of *I. bolleyi* (Section 4.3.1) were maintained on Nitrate Medium (Section 2.1.2) at 25°C for periods of up to four weeks; GUS-transformed strains of *I. bolleyi* were maintained on PDA medium supplemented with Hygromycin B (100μg ml^{-1}) at 25°C for periods of up to two weeks. For long-term storage, *I. bolleyi* root and stem base isolates, nitrate non-utilising mutants and GUS transformed strains were stored as mycelial blocks (2mm x 2mm) in a 10% glycerol solution (Section 2.1.3) contained in screw-cap Eppendorfs (1.5 ml). These were snap-frozen in liquid nitrogen and kept at -80°C.

2.1.2 Growth media

Distilled Water Agar (WA). Water agar (Oxoid No.3), 20g; distilled water, 1 litre.

Potato Dextrose Agar (PDA). Potato Dextrose Agar (Oxoid), 39g; distilled water, 1 litre.

Potato Dextrose Broth (PDB). Potato Dextrose Broth (Oxoid), 24g; distilled water, 1 litre.

Oatmeal Agar. Oats (Scots porridge oats), 20g; agar (Oxoid No.3), 15g; distilled water, 1 litre. The oats were brought to the boil in 500 ml of distilled water and simmered for 1 h, then filtered through 2 layers of cheese cloth. The volume was made up to 1 litre with distilled water. Oxoid No.3 agar was added prior to autoclaving.

3% Molasses Medium. Cane molasses, 30g; distilled water, 1 litre.

Standard Liquid Medium. Na_2HPO_4, 0.75g; $MgSO_4.7H_2O$, 0.12g; NH_4NO_3, 0.4g; KH_2PO_4, 0.75g; NaCl, 0.1g; yeast extract (Oxoid), 0.1g; malt extract (Oxoid), 1g; glucose, 1.8g; distilled water, 1 litre.

Czapek-Dox Liquid Medium. Czapek-Dox Liquid Medium (Oxoid), 49g; distilled water, 1 litre.

Minimal Medium. $NaNO_3$, 2g; KH_2PO_4, 1g; $MgSO_4.7H_20$, 0.5g; KCl, 0.5g; Trace elements, 0.2ml (citric acid, 5g; $ZnSO_4.H_20$, 5g; $FeSO_4.7H_2O$, 4.75g; $CuSO_4.5H_2O$, 0.25g; H_3BO_3, 0.05g; distilled water, 95 ml); thiamine hydrochloride, 0.4mg; glucose, 20g; agar (Oxoid No.3), 20g; distilled water, 1 litre.

Basal Medium. $MgSO_4.7H_2O$, 2g; K_2HPO_4, 1g; KCl, 0.5g; thiamine hydrochloride, 0.4mg; glucose, 20g; agar (Oxoid, No.3), 20g; tap water, 1 litre.

Asparagine Medium. Asparagine, 2g; $MgSO_4.7H_20$, 0.5g; K_2HPO_4, 1g; KCl, 0.5g; thiamine hydrochloride, 0.4mg; glucose, 20g; agar (Oxoid No.3), 20g; tap water, 1 litre.

Nitrate Medium. $NaNO_3$, 2g; $MgSO_4.7H_20$, 0.5g; KCl, 0.5g; KH_2PO_4, 1g; glucose, 20g; thiamine hydrochloride, 0.4mg; agar (Oxoid No.3), 20g; tap water, 1 litre.

Complete Regeneration Medium. Sucrose, 205.2g; Potato Dextrose Agar (Oxoid), 39g; distilled water, 1 litre.

Minimal Regeneration Medium. Sucrose, 205.2g; $NaNO_3$, 2g; KH_2PO_4, 1g; $MgSO_4.7H_2O$, 0.5g; KCl, 0.5g; Trace elements, 0.2ml (citric acid, 5g; $FeSO_4.7H_20$, 4.75g; $ZnSO_4.7H_20$,

5g; $CuSO_4.5H_2O$, 0.25g; H_3BO_4, 0.05g; $NaMoO_4$, 0.05g; distilled water, 95ml); thiamine hydrochloride, 0.4mg; agar (Oxoid No. 3), 20g; distilled water, 1 litre.

Sabouraud Dextrose Broth. Glucose, 40g; Mycological peptone (Oxoid), 10g; distilled water, 1 litre.

Luria Broth (LB Broth). Tryptone, 10g; bacto yeast extract, 5g; NaCl, 10g; distilled water, 1 litre. After the broth was autoclaved, filter sterilised solutions of thiamine hydrochloride, 35 mg and glucose, 1g, prepared in dH_2O, were added.

Luria Agar (LB Agar). Tryptone, 10g; bacto yeast extract (Difco), 5g; NaCl, 10g; agar (Oxoid No.3), 15g; distilled water, 1 litre. After the medium was autoclaved, filter sterilised solutions of thiamine hydrochloride, 35 mg and glucose, 1g, prepared in dH_2O, were added.

SOC Medium. Bacto-tryptone (Difco), 20g; bacto-yeast extract (Difco), 5g; NaCl, 0.5g; glucose, 20mM; distilled water, 1 litre. The solutes were shaken until dissolved and then 10 ml of 250 mM KCl was added. The pH was adjusted to 7.0 with 10 M NaOH. Just prior to use, 5 ml of sterile 2 M $MgCl_2$ was added.

All culture media were sterilized by autoclaving at 20psi, 121°C for 15 minutes.

2.1.3 Solutions

50 mM Sodium Maleate Buffer. Maleic acid, 5.8g and NaOH, 2g were dissolved separately in 250ml distilled water. $MgCl_2.6H_2O$, 81.5g was added and the solution was made up to

a volume of 1 litre with double distilled water. The solution was adjusted to a pH 5.8 with a NaOH solution (5 or 6 pellets of NaOH dissolved in 200ml of distilled water).

0.4 M Magnesium Chloride Stabilizer. $MgCl_2$, 81.5g was dissolved in 1 litre of distilled water.

Phenol. Water saturated phenol was equilibrated to pH 8.0 for DNA manipulations. 0.5 M Tris Cl (pH 8.0) and 8-hydroxyquinoline (0.1% v/v) were added with magnetic stirring, until the solution reached the correct pH. The phenol solution was stored in the dark at -20°C.

3 M Sodium Acetate Buffer. Sodium acetate, 408.3g was dissolved in 500 ml dH_2O then adjusted to the appropriate pH with glacial acetic acid. Distilled water was added to give a final volume of 1 litre.

Tris Cl. Tris base (tris (hydroxymethyl) amino methane) was dissolved in dH_2O and the pH was adjusted to the required value by adding concentrated hydrochloric acid. dH_2O was added to give a concentration of 1 molar.

EDTA. A Stock solution of 0.5M EDTA (ethylenediaminetetra amino acid, disodium salt) was prepared in dH_2O. The pH was adjusted to 8.0 with 10 M NaOH.

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

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

Sodium Dodecyl Sulphate (SDS). SDS was prepared as a 10% stock solution. SDS (electrophoresis grade), 100g was dissolved in 900 ml of dH_2O by heating to 68°C. The pH was adjusted to pH 7.2 by adding a few drops of concentrated HCl. The volume was adjusted to 1 litre with dH_2O. The solution was diluted to a 1-fold concentration prior to use. The stock solution was stored at room temperature.

STE. A solution of STE was prepared as a 20-fold stock solution by adding NaCl, 5.84g; Tris base, 7.8g and 2µl of 0.5M Na_2EDTA to dH_2O to give a total volume of 1 litre.

Ethidium Bromide. A stock solution was prepared by dissolving 1g of ethidium bromide in 100 ml dH_2O, and the solution was stored in the dark at room temperature.

Glycerol solution. Glycerol, 100ml was dissolved in 1 litre of distilled water.

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 dH_2O), 6 mM disodium EDTA and 0.025% bromophenol blue (w/v in dH_2O) dissolved in 6 x TBE. The solution was stored at room temperature.

2.2 Methods

2.2.1 Experimental soil

Soil collected from the Kings Buildings site (Edinburgh University, Edinburgh, UK) was air-dried and sieved (4 mm mesh) and stored in plastic bags at 4°C.

2.2.1.1 Soil pH

Soil pH was measured in a soil-double distilled water mixture (1:2 v:v).

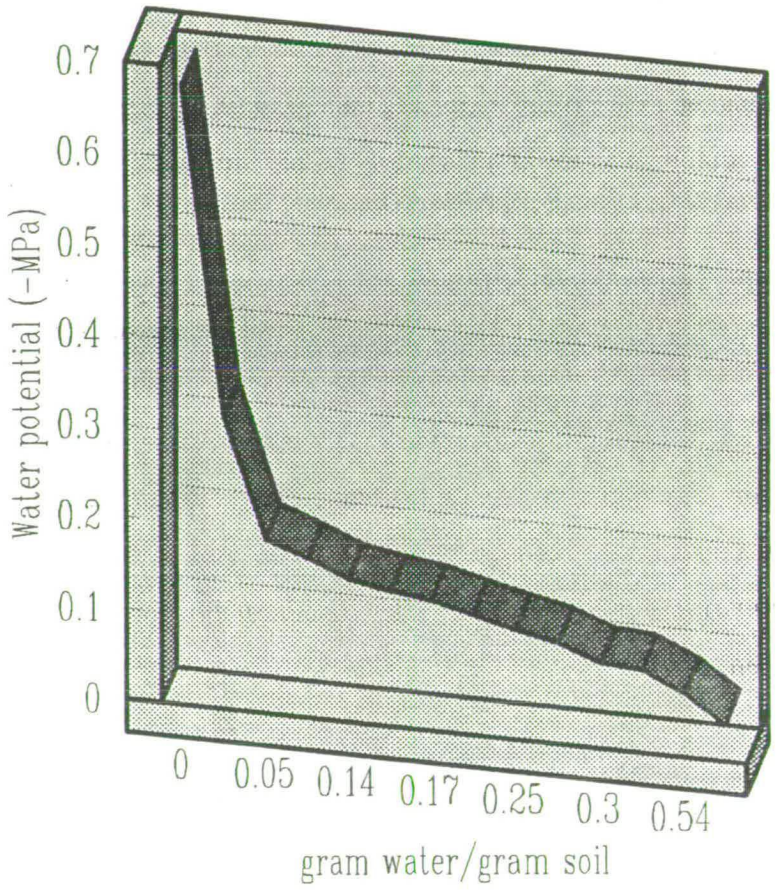
2.2.1.2 Soil field capacity

Field capacity of the soil was calculated by placing soil into a pre-weighed soil sample retaining ring consisting of a metal ring (4 cm diameter) with a fine mesh bottom. The retaining ring was filled to the top with soil. The retaining ring and the soil were re-weighed and placed on a draining rack held in a tray. The soil was saturated with distilled water and the apparatus was sealed in an air-tight plastic bag to prevent evaporation. The soil sample was left to drain for 48 h. The soil and retaining ring were re-weighed and placed in a pre-heated oven at 80°C and dried to constant weight. The water content of the soil was calculated (g H_20/g soil).

2.2.1.3 Water release characteristic

A water release characteristic was done on a porous suction plate (constructed by the Soil Science Department, Edinburgh University), which produced matric potentials of -0.01 MPa to -0.05 MPa. Higher matric potentials between -0.14 MPa and -1.5 MPa, were done using a 15 bar ceramic plate extractor (Soil Moisture Equipment, Co. Santa Barbara, California), connected to a compressed air cylinder filled to 137 bar max. at 15°C (Boc. Guildford, Surrey). Matric potentials below -1.5 MPa were done using a filter paper method (McInnes & Weaver, 1994). Figure 2.1 shows the water release characteristic for soil at the Kings Buildings site, University of Edinburgh.

Figure 2.1: Water release characteristic of Kings Buildings soil, University of Edinburgh, UK. (Field capacity = 67g H_2O per 100g soil).



For the porous suction plate, soil obtained from the Kings Buildings site, prepared as described (Section 2.2.1), was placed into a pre-weighed retaining ring (Section 2.2.1.2) and brought to saturation. The soil samples were placed in the porous suction plate and left to equilibrate at the desired water suction for 4 days. At 4 days, three retaining rings containing the soil were removed, re-weighed immediately and then placed in an oven at 80°C and dried to constant weight. The water content of the soil was calculated (g H_2O/ g soil). This procedure was repeated for a range of suction pressures. Three replicate soil samples were used for each suction.

For the ceramic plate extractor, soil samples were prepared in retaining rings as described above, and brought to saturation with distilled water. The 15 bar ceramic plate was completely immersed in water and soaked for 24 h prior to use. The retaining rings containing the soil were placed on top of the ceramic plate and the equipment was tightly sealed. The Plate was brought to the desired pressure using the connecting compressed air and left to equilibrate. Upon equilibration the equipment was opened and the soil cores were reweighed and then placed directly back onto the plate and the equipment was resealed. After sealing the plate was brought to the next desired pressure. This procedure was repeated for a range of pressures. After equilibration at the highest pressure (15 bar), the soil cores were removed from the plate, re-weighed, then placed in an oven at 80°C and dried to constant weight. The water content was calculated. (g H_2O/g soil). Six replicate soil cores were used in total for all pressures.

The filter paper method (McInnes & Weaver, 1994) was used to determine the matric potential of two samples of soil prepared as in section 2.2.1, that had been adjusted to water contents which were below that recorded at 15 bar (0.091 g H_20/g soil).

Soil samples were adjusted to the water contents of 0.08 g H_20/g soil and 0.04g H_20/g soil, by mixing the soil and water in a plastic bag. The plastic bag containing the soil

was heat-sealed and placed at 20°C and the soil was left for 48 h to equilibrate. The bags were then opened and each soil sample was immediately placed into a metal container (9 cm diameter and 4 cm depth). Each can was filled with soil to a 2 cm depth and then using forceps, a single filter paper (ashless Whatman No.42, 9.0 cm circle) was placed on top of the soil in each container and the container was then filled to maximum capacity with more soil. The soil-filter paper combination was sealed into the container using Polycell heat-shrinking film. The film around the container was heated using a hair dryer to create an air-tight seal. The film was further secured around the container with plastic electrical tape. The container was placed directly into a polystyrene box, which was sealed with a polystyrene lid and then placed into a Gallenkamp controlled-environment incubator at 20°C and left for 7 days to allow the filter paper and soil to reach the same water potential. After equilibration, the metal container was removed from the box and the film seal around the container was broken. Using forceps the filter paper was removed from the container and excess soil was brushed off with a paint brush. The filter paper was placed into an oven-dried can and the lid was closed. The filter paper and can combination were weighed, the lid was then opened and the can and filter paper were placed into an oven at 105°C for 3 h. This process was carried out as rapidly as possible to prevent water-loss from the filter paper by evaporation. After drying, the lid of the can was replaced and the can and paper were allowed to cool before re-weighing. The water content of the equilibrated filter paper was calculated using the equation:

$$\text{Water content (Kg Kg}^{-1}) = \frac{(\text{wet paper mass + can mass}) - (\text{dry paper mass + can mass})}{(\text{dry paper mass + can mass}) - \text{can mass}}$$

The relationship between water content and water potential for ashless Whatman No.42 has been determined (Al-Khafaf & Hanks, 1974), (Figure 2.2). Using the curve for this relationship, the water potential of the filter paper was determined. The water potential

for the two soil samples were determined as -5.0 MPa (0.08g H_2O/ g soil) and -7.0 MPa (0.04g H_2O/g soil).

2.2.2 Surface sterilisation of plant material

Plant material was surface sterilised by shaking for 20 min on a Gallankamp flask shaker in 1% sodium hypo-chlorite. The plant material was then rinsed in three changes of sterile distilled water and one change of sterile distilled water containing antibiotics (streptomycin sulphate, 50µg ml^{-1}; penicillin G, 50µg ml^{-1}). The plant material was blotted dry on sterile filter paper and plated on to the appropriate medium.

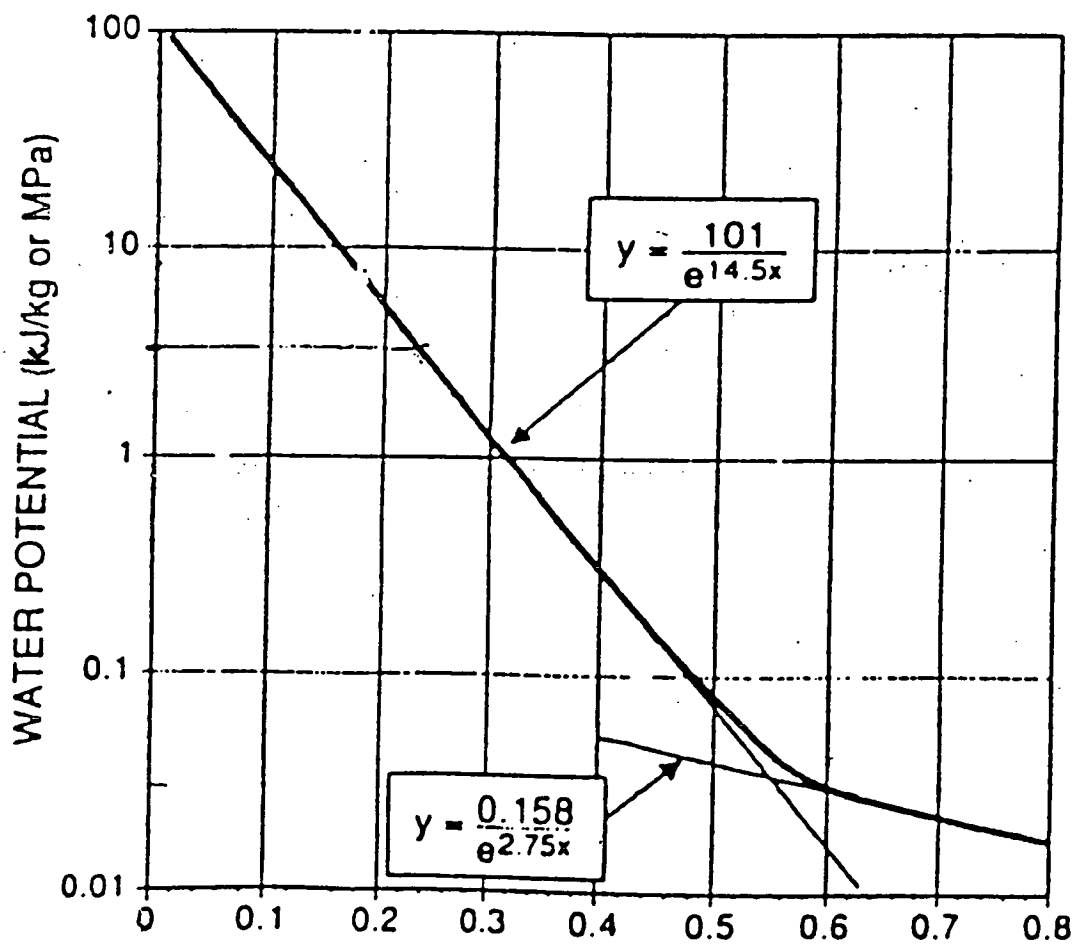
2.2.3 Conidial suspensions

Conidial suspensions of *I. bolleyi* were obtained by flooding 8-day PDA cultures with 10 ml sterile distilled water, then dislodging the spores by rubbing the surface with a glass rod. The suspensions were transferred to sterile centrifuge tubes and washed three times in distilled water by centrifugation (3000 x *g* for 4 min). The spore number was determined with a haemocytometer and the final suspension was adjusted to the desired concentration in distilled water.

2.2.4 Production of imazilil-resistant strains of I. bolleyi

An imazilil strain of *Idriella bolleyi* (AB1-IZ) was generated as follows. Conidial suspensions of *I. bolleyi* strain AB1 were prepared as in Section 2.2.3. The spore suspension was adjusted to c. 7 x 10^4 spores ml^{-1}. Conical flasks (200 ml) containing 100

Figure 2.2: Water potential-water content relationship for ashless Whatman No.42, 9.0 cm circles.



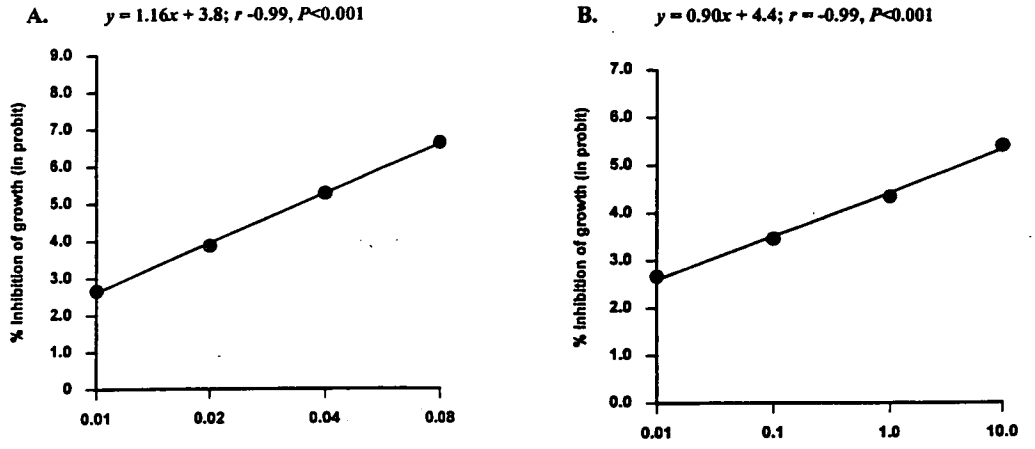
ml of 3% Molasses Medium (Section 2.1.2) was inoculated with 5 ml of the spore suspension. The flask was shaken on an orbital shaker at 170 rpm in the dark at 25°C. At five days, 50 ml of the culture was transferred into another conical flask (200 ml) containing 50 ml of 3% Molasses Medium. Imazilil was added to the flask at a concentration of 1μg ml^{-1}. The flask was incubated for five days as previously described, then 50 ml of the flask contents was again transferred into 50 mls of fresh 3% Molasses Medium and imazilil was again added at a concentration of 1μg ml^{-1}. This process was repeated until reasonable fungal growth was observed in the medium at this concentration of fungicide. The concentration of fungicide was then increased to 2μg ml^{-1} and the cultures were grown as described above. The concentration of imazilil in the culture medium was gradually increased by 1μg ml^{-1} each time until good fungal growth was observed at fungicide concentration of 10μg ml^{-1}. Using a sterile inoculating loop this culture was then streaked onto PDA medium (Section 2.1.2) containing imazilil at 10μg ml^{-1}. After 3 days incubation a single colony was removed from the PDA plates using a sterile scalpel and transferred to a fresh PDA plate containing imazilil at 10μg ml^{-1}. The imazilil-resistant strain was maintained by regular sub-culturing onto fresh PDA containing imazilil at 10μg ml^{-1}.

The imazilil-resistant strain was compared to the wild-type strain for growth on PDA plates containing imazilil at 0.01, 0.02, 0.04 and 0.08μg ml^{-1} for the wild-type and 0.01, 0.1, 1.0, 10.0μg ml^{-1} for the fungicide-resistant strain. The colony diameter was measured after 7 days and the ED_{50} (estimated dose for 50% reduction in growth) was calculated as 0.4μg ml^{-1} for the wild-type strain and 6.5μg ml^{-1} for the fungicide-resistant strain (Figure 2.3).

2.2.5 Fluorimetric β-glucuronidase assay

Mycelium from *I. bolleyi* was harvested onto pre-weighed Whatman filter paper No.7 and the fresh weight was determined. The mycelium was transferred to a motar on ice and

Figure 2.3: Percentage reduction of linear growth rate (expressed as probit) of *I. bolleyi* against a logarithm of imazilil concentration for A, the wildtype, and B, an imazilil resistant mutant.



ground with liquid nitrogen. Extraction buffer (0.1 M Na_2HPO_4 (Ph 6.8); 0.5 mM β-mercaptoethanol; 0.5mM EDTA; 1% NaCl) was added in a 1:10 w/v ratio, and the suspension was mixed with a high speed homogeniser for 2 min. The extract was clarified by centrifugation at 13 000 x *g* for 10 min on a Sorvall RC-5B refrigerated super-speed centrifuge, using a Sorvall GSA rotor. Extract samples (50 µl) were added to 0.45 ml of extraction buffer containing 0.44 mg ml^{-1} of MUG (4-Methylumbelliferyl glucuronide; Sigma) as a substrate of glucuronidase. The samples were incubated in the dark for 90 min at 37°C. After incubation 200µl were removed and the reaction was stopped by the addition

of 1.8 ml of 0.2 M $NaCO_3$. The fluorescence of each sample was measured on a Perkin-Elmer LS-5B Luminesence Spectrometer. Three readings were taken for each extract.

The GUS activity of inoculated and uninoculated roots was measured using the method described above. In order to calculate the volume of extraction buffer required, the fresh weight of the roots was determined prior to macerating.

2.2.6 *Transformation of* Escherichia coli *with pGUS5*

Escherichia coli cells (2µl) and 1µg of plasmid DNA (pGUS5) was placed in an ice cold electroporation cuvette (Bio-Rad). The cuvette was placed in a Bio-Rad Gene Pulser and pulsed with 240 volts for 2 sec. The electroporated cells were transferred to a sterile Eppendorf and re-suspended in SOC medium (Section 2.1.3) and incubated for 1 h at 37°C. Serial dilutions of the cells were made following incubation and aliquots (100µl) of each dilution were plated onto LB agar containing ampicillin at 50µg ml^{-1}. The plates were incubated at 37°C for 24 h. Control (untransformed) cells were also plated onto the same medium and incubated as above. After 24 h, cells growing on the LB plates were inoculated into an Eppendorf containing 1 ml of LB broth containing ampicillin at 50µg ml^{-1}, and stored as a freezer permanent culture at -70°C.

2.2.7 *Plasmid (pGUS5) Extraction from* Escherichia coli

Freezer permanent pGUS5 transformed cells of *Escherichia. coli* (2µl) were inoculated into a 5 ml flask of LB broth containing ampicillin at 50µg ml^{-1} and grown overnight at 37°C on an Denley orbital shaker. The flask contents were transferred to a 1 litre flask containing 500 ml of LB broth containing ampicillin at 50µg ml^{-1}. The flask was incubated overnight at 37°C on a flask shaker (New Brunswick Scientific Co., controlled

environment incubator shaker). After incubation the cells were spun down at 13 000 x *g* (4°C) on a Sorvall RC-5B refridgerated centrifuge using a Sorvall GSA rotor for 15 min. The pellet was resuspended in 6 ml of lysis buffer (25mM Tris, pH 7.5; 10mM EDTA, pH 8.0; 15% Sucrose) containing 100µl of lysozyme (2mg ml^{-1}; Sigma). SDS (1%) in 12 ml of 0.2 M NaOH, was added to the cells which were then mixed by gentle inversion and put on ice for 15 min. 3 M sodium acetate (7.5 ml) at pH 4.6 was added, and the cells were again mixed by gentle inversion and placed on ice for a further 15 min. The cells were spun at 13 000 x *g* (4°C) for 10 mins. The supernatant was transferred to a fresh sterile centrifuge tube by filtering through a thin layer of cotton wool in a filter funnel. Rnase (10mg ml^{-1}; Sigma) (75µl) was added to the cells which were then incubated at 37°C for 40 min. An equal volume of isopropanol was added and the cells were spun at 22 000 x *g* (4°C) for 20 min. The pellet was resuspended in 5 ml of double distilled water. The total volume was split into ten Eppendorf tubes (0.5 ml each). An equal volume of phenol (pH 8.0) was added to each tube. The tubes were spun on a microfuge at 15 000 x *g* for 2 min. The upper aqueous layer was removed into a fresh tube taking care not to collect any solution at the interface. An equal volume of chloroform was then added to this aqueous solution, the sample was thoroughly mixed by vortexing and spun for 1 min at 15 000 x *g*. The upper fraction was decanted into a fresh tube and the phenol-chloroform extraction was repeated twice more. On the final extraction, the upper layer from each tube was removed and pooled into a single centrifuge tube and the total volume was measured. DNA was precipitated from the solution by the addition of 0.1 volume of 3 M sodium acetate buffer, (pH 5.0) and 2.5 volumes of absolute ethanol. The solution was incubated at -80°C for 10 min and then the solution was spun at 15 000 x *g* for 3 min. The ethanol was drained off and then the pellet was dried under vacuum for 10 min. The pellet was resuspened in 1.6 ml H_2O and 0.4 ml of NaCl was added. The contents were mixed by gentle inversion. A 13 % solution of Polyethylene glycol 8000 (2 ml) was added and the contents were mixed by gentle inversion. The solution was placed on ice for 1 hour then spun at 20 000 x *g* (4°C) for 10 min. The pellet was washed with 70% ethanol and re-spun (x2) at 20 000 x *g* (4°C) for 10 min. The

pellet was dried under vacuum and then resuspended in 500 µl of TE solution and stored at -80°C.

2.2.8 Restriction digests

Restriction digests were carried out over 16 h at 37°C with a commercially available enzyme, using the supplied buffer (Boehringer mannheim). Plasmid pGUS5 (10µg) was digested in a total volume of 20µl containing 1 x restriction enzyme buffer, dH_2O and 10 units of restriction enzyme.

2.2.9 Agarose gel electrophoresis

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

2.2.10 Video Microscopy

Observation chambers were prepared by immersing coverslips (44 x 64 mm) into molten water agar and draining off the excess to create a thin coating. The coverslips were directly laid flat onto solidified water agar plates and allowed to set, after which they were inoculated as required. Following an appropriate period of incubation, the coverslips

were removed from the agar plates and inverted onto a chamber consisting of a microscope slide (76 x 51 mm) to which a rectangle of glass capillary tubes, 2 mm high, had been glued. To prevent drying of the coverslip, the capillary tubes were coated in Vaseline to create a seal. The back of the coverslip was wiped clean prior to observation.

Observations using bright field microscopy, were done on a Leitz Orthoplan microscope with x70 oil-immersion objective. A colour video camera Panosonic S-VHS F15 was attached to the microscope and to a Panosonic S-VHS AG-6720 video recorder and Panosonic BT-M1420-PY colour monitor on which the observations were recorded. Photographs of observations were recorded using a Mitsubishi P61-8 video copy processor attached to the video recorder, or on a Leitz Orthomat 35 mm fully automatic camera connected to the microscope.

Observations using fluorescence microscopy were done with a x70 oil-immersion lens on a Leitz Orthoplan microscope equipped with a Ploemopak 2.1 fluorescence vertical illuminator giving UV light from a mercury vapour lamp. Filter block A (BP 330-385nm exciting filter) and filter block H (BP 390-490 nm exciting filter) giving excitation in the ultra-violet and violet blue range respectively were used for fluorescence observations. A Panosonic VW CL-350 colour video camera was attached to the microscope and to a Toshiba DV80B video recorder and Sony Triniton 1460UB colour monitor on which observations were recorded. Photographs were taken using a Leitz Orthomat 35 mm fully automatic camera connected to the microscope.

Video recordings were made on Master Broadcast S-VHS videotapes and photographs taken with the Leitz Orthomat 35 mm automatic camera on Kodak Ektrachrome P800/1600 film for fluorescence microscopy, or Fujicolor 400 super G for bright field photographs

CHAPTER 3

EFFECTS OF ENVIRONMENTAL CONDITIONS ON THE BEHAVIOUR OF *IDRIELLA BOLLEYI*

3.1 Introduction

The use of beneficial micro-organisms for the biological control of root-infecting pathogens has much potential to an agricultural system (Cook and Baker, 1983). In order for a seed-applied biocontrol agent to be successful, it must colonise the root system and proliferate there (Lascaris & Deacon, 1991). In view of this, little is known about the environmental conditions within the soil that may influence the spread of these organisms in the rhizosphere. Cook & Dunniway (1980) have stressed the importance of water-relations with regard to the life-cycles of soil-borne fungi. Water has a major effect on the activity of fungi (Ayres & Boddy, 1986) and this includes pathogens and their biocontrol agents (Cook & Baker, 1983).

Disease development on plants is associated with moisture levels, for example *Fusarium* foot-rot of cereals is typically associated with drought stress and *Fusarium culmorum* and related *Fusaria* are highly tolerant of water stress *in vitro* (Cook & Christen, 1976). Conversely, the take-all fungus infects in wetter conditions and is intolerant of water stress *in vitro* (Cook & Christen, 1976). Because of the diversity of conditions associated with disease development, it is fundamental to establish the environmental conditions

affecting the activity of biocontrol agents, as this will influence their overall success in disease control.

We recognised that *I. bolleyi* might be of most value as a general-purpose biocontrol agent, reducing the activities of several different pathogens in the particular seasonal or site conditions that favour these. We therefore studied the inter-strain variability of *I. bolleyi* to water stress *in vitro* and *in vivo*, so that strains may be targeted for control of particular pathogens (Deacon, 1991) or strain mixtures might be used to improve the consistency of biocontrol over sites and seasons.

Along with influencing the growth and activity of fungi, water is believed to be involved with the dispersal of micro-organisms in the rhizosphere. Dispersal of micro-organisms has important ecological implications in increasing the rate of active spread and colonisation (Chao *et al.*, 1986). Soil water has been shown to influence the distribution of seed-applied *Pseudomonas fluorescens* in soil (Parke *et* al., 1986) and Krauss & Deacon (1994b) have shown the movement of fungal spores of *Mucor hiemalis* down the rhizosphere of groundnut plants (*Arachis hypogaea*) in a tropical soil profile. *Idriella bolleyi* is known to sporulate readily in submerged liquid culture, facilitating its use as a biocontrol agent (Deacon, 1988). More recently Lascaris and Deacon (1991) showed that *I. bolleyi* could also sporulate abundantly on sterile wheat seeds grown in unsterile perlite, resulting in the colonisation of the root system by newly formed spores carried in percolating water. From these results they suggested that early growth on the seed is necessary for the establishment of a high population of *I. bolleyi* in the rhizosphere.

Following on from the work of Lascaris & Deacon (1991), *I. bolleyi* was examined for its ability to produce spores on unsterile seeds, in unsterile soil, under competition from other microflora. It is thought that this work may lead to a more accurate

estimation of the degree at which *I. bolleyi* could proliferate in a normal competitive environment.

The work in this chapter is a further contribution to the development of *I. bolleyi* as a potential seed-applied biocontrol agent.

3.2 Methods

3.2.1 In vitro *effects of water potential on* I. bolleyi

Three randomly chosen strains of *I. bolleyi* T560, J10 and AB1, were examined for their tolerance to water-stress in osmotically and matrically adjusted media. The media were adjusted to different water potentials from -0.1 MPa to -5.0 MPa, using either KCl or Polyethylene glycol (PEG) 8000 (Sigma). The concentrations were as follows (g l^{-1}): For KCl, -0.1 MPa (none), -1.1 MPa (10.06), -1.9 MPa (17.90), -3.0 MPa (28.44), -3.9 MPa (36.50), -5.0 MPa (47.56); for PEG 8000: -0.1 MPa (none), -1.1 MPa (300), -1.9 MPa (414), -3.0 MPa (530), -3.9 MPa (595), -5.0 MPa (650). The water potentials of the adjusted media were measured with an osmometer for osmotic potential (KCl-adjusted media) or a thermocouple psychrometer for matric potential (PEG-adjusted media).

The strains were tested for the effects of water-stress on linear growth, biomass production, spore germination and sporulation. Where possible, comparative tests were made with two cereal pathogens which have been intensively studied for responses to water stress: *Fusarium culmorum*, which is known for its drought tolerance, and *Gaeumannomyces graminis* var. *tritici* which has low water stress tolerance. A *Phialophora* sp. from maize was also used for comparison in some experiments.

3.2.1.1 Colony extension rates

Agar discs (5 mm) were taken from the margins of 7 day-old colonies of all fungi on PDA medium (Section 2.1.2) and placed centrally on PDA (Section 2.1.2) plates osmotically adjusted with KCl, or into polyethylene glycol-supplemented PDB (Section 2.1.2) using the concentrations described in Section 3.1.2. PEG had to be used in the liquid medium because it prevented gelling of agar. All dishes were incubated at 25°C and colony diameters were recorded daily for 7 days. The dishes containing PEG-supplemented liquid medium were handled carefully to avoid disturbance of the mycelia, and growth was measured by placing the plates onto a marked grid. For comparison with *I. bolleyi*, *Fusarium culmorum*, *Gaeumannomyces graminis* var. *tritici* and *Phialophora* sp. were also used in this experiment. In all cases radial growth rates were calculated as mean ± SEM for three replicates, but for ease of comparison they are presented as percentage growth in the control (-0.1 MPa).

Statistical differences between strains of *I. bolleyi* were calculated in two ways. On PDA where control growth rates of strains were almost identical, the measured growth rates of *I. bolleyi* strains at each water potential were subjected to analysis of variance. For the liquid medium, where the control growth rates of the strains differed, each replicate at each PEG concentration was expressed as percentage of control growth rate and the percentages were subjected to analysis of variance after arcsine transformation; however, data for -1.1 MPa could not by analyzed because they exceeded 100% for *I. bolleyi* strain J10.

3.2.1.2 Biomass Production

Conical flasks (250 ml) containing KCl- adjusted Standard Liquid Medium (Section 2.1.2) were inoculated with single PDA discs (5 mm) of *I. bolleyi* then incubated for 7 days

at 25°C on an orbital shaker at 125 rpm. The flask contents were then filtered under vacuum so that the biomass was retained on pre-weighed 9.0 cm Whatman filter paper circles. These were then dried to constant weight at 80°C to obtain biomass yields. There were three replicate flasks per treatment. The same procedure was tried for PEG-adjusted medium, but abandoned because PEG was retained on the filter papers used to collect the biomass, precluding accurate measurement.

3.2.1.3 Spore Germination

Conidial suspensions of *I. bolleyi* were prepared as described in Section 2.2.3. The spore suspension was adjusted to c. 15 x 10^5 spores ml^{-1} using a haemocytometer. The spore suspension (1 ml) was mixed into 9 ml of Standard Liquid Medium adjusted to different water potentials with KCl or PEG 8000 (Section 3.2.1.). Drops of the suspensions were transferred to sterile glass coverslips which were then inverted onto glass rings on microscope slides and sealed with Vaseline to prevent drying. The hanging drops were incubated at 25°C for 14 h, which had previously been found to be the optimum time for assessment of germination when the germ tube length equaled or exceeded the length of the spore. Standard errors were always very low, so for ease of comparison the data are presented as percentage germination.

3.2.1.4 Sporulation in liquid culture

Conical flasks (250ml) containing 50 ml of Standard Liquid Medium adjusted to different water potentials with KCl or PEG 8000 (Section 3.2.1), were inoculated with spore suspensions of *I. bolleyi* prepared as in Section 2.2.3, so that the final spore concentration in the flasks was c. 5 x 10^5 spores ml^{-1}. The flasks were incubated at 25°C on an orbital

shaker at 120 rpm. Aliquots (0.5 ml) of the media were removed aspectically every 24 h for 7 days and spore numbers were assessed with a haemocytometer. There were three replicate flasks per treatment.

3.2.2 Effect of water potential on straw colonisation in unsterile soil

This experiment involves the use of fungicide tolerant *Idriella bolleyi* strains, T560-R2 (Section 2.1.1) and AB1-IZ (Section 2.2.4), to compare their ability to colonise pieces of straw, buried in unsterilized soil adjusted to different water potentials.

Oatmeal-sand inoculum was prepared using 100g of dried, sieved (300μm mesh) sand, 3g sieved oatmeal (100μm mesh) and 14 ml distilled water in 250ml conical flasks. After thorough mixing, the flasks were plugged with cotton wool, and autoclaved at 121°C for 45 minutes. Each flask was inoculated with an 11 mm PDA disc of *I. bolleyi* strain AB1-IZ or strain T560-R2, taken from the colony margin of a 14 day old colony, using a No.7 cork-borer. The flasks were incubated in the dark at 25°C for 21 days, shaken at four day intervals to mix and disperse the fungal growth.

Dried mature barley straw was cut into 2 cm pieces, in lots of 500, and together with 25 ml of distilled water placed in 500 ml capacity flasks. The flasks were autoclaved twice at 121°C for 25 minutes.

Unsterilised dried, sieved soil (Section 2.2.1) was weighed in lots of 120g into polyethylene bags, and adjusted to different water potentials (shown as -MPa) by addition of known quantities of sterile distilled water (g H_2O per g soil), as follows: (0.14) -0.6 MPa; (0.091) -1.5 MPa; (0.07) -5.0 MPa; and (0.04) -7.0 MPa. The soil and water in each bag was thoroughly mixed. Fifteen replicates were used for each water content. After the addition of water, the bags were heat-sealed and the soil was left to equilibrate for 48 hours.

After 21 days, the oatmeal-sand inoculum was tipped into trays. One tray contained inoculum of strain T560-R2, another contained AB1-IZ. A third tray received one flask of strain T560-R2 inoculum and one flask of strain AB1-IZ inoculum, together with an equal amount (2 flasks) of dried sterilised sand. The tray contents were thoroughly mixed, so that, weight-for-weight, the mixture contained the same amount of inoculum of each fungus as in the trays of the single strains.

Lots of 46 sterilised straw pieces were rolled in each oatmeal-sand inoculum until the straw surface was uniformly coated. Each lot of 46 coated straw pieces was placed into a bag of equilibrated soil and the bag was re-sealed. The straws were mixed in the soil to disperse them evenly, and the bags were then incubated in the dark at 20°C. After 2 days, 14 days and 42 days, each bag was opened to retrieve some straw pieces. Twenty three straws were removed from each bag at day 2, then the bags were re-sealed. These straws were cut in half (1cm pieces), one half was discarded and the other half of each straw was washed thoroughly in tap water over a sieve, then surfaced sterilised (Section 2.2.2). The straw pieces were blotted dry on sterile filter paper, then halved longitudinally. One of the longitudinal halves was plated onto Water Agar (Section 2.1.2) containing antibacterial antibiotics (50µg ml^{-1} streptomycin sulphate; 50µg ml^{-1} Penicillin G). The other longitudinal half was plated onto the same agar containing fungicide (carbendazim at 10µg ml^{-1} for strain T560-R2; imazilil at 2µg ml^{-1} for strain AB1-IZ). However, straws treated with the AB1-IZ/T560-R2 mixed inoculum were cut into quarters then one quarter was plated onto antibiotic water agar, one quarter onto carbendazim agar, one quarter onto imazilil agar (concentrations as above), and the remaining quarter was discarded.

The agar plates were incubated in the dark at 25°C for 14 days, after which the agar surrounding the straw pieces was examined microscopically for growth of *I. bolleyi* and other fungi.

For the straws harvested at 14 days, 23 straw pieces were removed and cut in half, then 23 halves were placed back into the soil and the bags were re-sealed. The other 23 halves were washed and plated as described above. At 42 days the remaining 1cm pieces were sampled and treated as described above.

During each sampling, 2g of the soil was removed from one replicate bag at each water potential and was placed in a pre-weighed container. The container and soil sample were then placed in an oven at 80°C and dried to constant weight to obtain dry weights of the soil. Water loss from the soil in the bags was calculated at each sampling time.

A conidial suspension of *I. bolleyi* was prepared from a 7 day old PDA culture of strain T560-R2, (Section 2.2.3). Centrifuged spores were resuspended in either 15ml of sterile distilled water or 15ml of sterile 1% Sodium alginate. Each suspension was transferred to sterile universal bottles and the spore number of each suspension was adjusted to a concentration of c. 10×10^6 spores ml^{-1} using a haemocytometer.

3.2.3 *Conidia production on wheat seed buried in unsterile soil.*

A uniform size of wheat seed was obtained by placing seeds into a 4 mm sieve to separate small seeds and fragments. The seeds were counted into lots of 280 and one lot was surface sterilised (Section 2.2.2) and blotted dry in sterile petri-dishes containing sterile filter paper. The sterilised seeds were divided into 2 lots of 140 and one lot was inoculated with *I. bolleyi* strain T560-R2 by immersing into the prepared aqueous conidial suspension. The seeds were rolled for 10 min on a Denley Spiromix 10 to ensure thorough coating of the seeds. The seeds were removed from the suspension using sterile forceps and placed in a sterile petri-dish and dried for 1 h with the lid half off, in a laminar-flow cabinet, and in close vicinity to a lit Bunsen burner. The second lot of 140 seeds were immersed into the 1%

sodium alginate conidial suspension and rolled on a Denley Spiromix 10 for 10 min, as for the aqueous spore suspension. The seeds were removed from the 1% sodium alginate using sterile forceps and placed directly into a 0.25% calcium chloride solution to create a calcium-alginate gel coating. The seeds were removed from the calcium chloride solution and washed in sterile distilled water, then blotted dry on sterile filter paper. This process was repeated twice more, but in spore free 1% sodium alginate, so that the seeds were coated with one gel layer containing spores and two outer spore-free sodium alginate - $CaCl_2$ layers. The mean spore loading for both the aqueous conidial suspension and the 1% sodium alginate conidial suspension were calculated from the loss of volume after the inoculation of the seeds.

Another lot of 280 unsterilized seeds were divided into 2 lots of 140 and one 140 lot was immersed in an aqueous conidial suspension and the remaining 140 lot was immersed into a 1% sodium alginate conidial suspension. The seeds were treated in the same way as the sterilised seeds.

Controls were prepared for sterile and unsterilised seeds as described above, but the seeds were placed into either spore-free sterile distilled water or spore-free 1% sodium alginate.

The seeds were potted in plastic cups containing 80g of unsterilised, dried, sieved soil (Section.2.2.1), which had been brought to 30% saturation, sealed in polythene bags and left to equilibrate for 48 h. Five seeds were placed in each cup at a depth of 2 cm and the pots were sealed in polythene bags to prevent water loss. The seeds were harvested at 0, 1, 3, 5, 8, 14 and 25 days. Four replicate cups were removed for each treatment, including the controls, at each sampling. The 5 seeds were removed from each replicate cup and the excess soil was shaken off, but the surrounding soil on the seed was left untouched. The 5 seeds from each replicate cup were placed into 10 ml of sterile distilled water and

shaken for 20 min on a Gallankamp flask shaker at maximum speed for 20 min. Serial dilutions were made for each replicate and 0.1 ml of the appropriate dilution was plated onto one-quarter strength PDA supplemented with KCl, (30.0 g l^{-1}) carbendazim (50 µg ml^{-1}) and antibacterial antibiotics (streptomycin sulphate 50µg ml^{-1}; Penicillin G 50µg ml^{-1}), and spread with a sterile glass spreader. Three plates were used per replicate. The plates were incubated in the dark at 25°C for 3 to 4 days and the number of colonies were counted, with the assumption that one colony was derived from a single spore.

3.2.4 *Variation between field root and stem base isolates of* I. bolleyi *to water potential*

Cereal plants were collected from three sites around Edinburgh along with soil samples from each site. The soil pH was measured in a 1:2 ratio of soil with distilled water. Soil field capacity was measured (Section 2.2.1.2).

The roots and stem bases of the cereal plants, from each site were cut up and surface sterilised (Section 2.2.2). The surface sterilised plant material was plated onto Water Agar (Section 2.1.2) supplemented with antibacterial antibiotics (streptomycin sulphate, 50µg ml^{-1}; Penicillin, G 50µg ml^{-1}) incubated at 25°C , in the dark. The plates were checked at regular intervals for the growth of *I. bolleyi* from the plant tissue. Isolates of *I. bolleyi* observed growing out from the plant material, were removed with a sterile inoculating loop and streaked onto fresh water agar plates amended with KCl (28.44 g l^{-1}) to reduce contamination by *Mucor* spp. Isolates were re-streaked onto fresh unsupplemented Water Agar plates until a pure culture was obtained. If a pure culture of *I. bolleyi* was obtained, the isolate was streaked onto a fresh unsupplemented PDA plate. Those isolates where contamination could not be eradicated were discarded.

Spore suspensions of each isolate were made up (Section 2.2.3) and streaked onto PDA. After 2 days a colony deriving from a single spore was removed and plated onto a fresh PDA plate. The subsequent experiment was carried out using cultures derived from a single spore of each isolate.

A universal bottle containing 15 ml of Standard Liquid Medium (-0.1 MPa) (Section 2.1.2) was inoculated with a mycelial disc (0.5mm) from the margin of a four day old Oatmeal Agar culture (Section 2.1.2) of an *I. bolleyi* field isolate. This process was repeated for all isolates. Three replicate bottles were used for each isolate. The bottles were incubated at 25°C in the dark on a Denley orbital mixer for 4 days. At 4 days, the number of conidia in each bottle was counted using a haemocytometer and the bottle contents were filtered onto 7.0 cm Whatman filter paper No.3. The filter papers were placed in an oven at 80°C, dried to constant weight and the total dry biomass weight of each bottle was calculated. This method was repeated for all isolates using Standard Liquid Medium that had been osmotically adjusted to -3.9 MPa with KCl (36.5 g l^{-1}). Three replicates bottles were used for each isolate at -3.9 MPa.

3.3 Results

3.3.1 In vitro *effects of water potential on* I. bolleyi

Three strains of *I. bolleyi* (T560, AB1 and J10) were tested for the effects of water stress on the three stages of activity that are most relevant to biocontrol, namely spore germination, hyphal growth and sporulation. The effects of osmotic potential was tested by the adjustment of growth media with KCl, whereas the effects of matric potential was tested by the adjustment of growth media with polyethylene glycol 8000 (Section 3.2.1).

3.3.1.1 Colony extension rates

The three strains of *I. bolleyi* (T560, AB1 and J10) were grown at 25°C on PDA plates supplemented with KCl. Colony diameter was recorded after 7 days and the radial growth rate was calculated as mean ± SEM for three replicates.

The three strains of I. *bolleyi* grew at roughly similar rates on the control agar plates at colony radial extension rates of 0.30, 0.30 & 0.29 mm 24 h^{-1} for strains AB1, T560 and J10 respectively. But their responses to amendment of the agar with KCl differed markedly (Figure.3.1). Strain AB1 grew at undiminished rate down to at least -5.0 MPa and its growth rate was enhanced by more than 100% at intermediate water potentials compared with the control. Strain T560 showed a rapid and progressive fall in colony extension rate with increasing water-stress, and grew very slowly at -3.0 MPa and lower water potentials. Strain J10 was intermediate in behaviour; at -3.0 MPa its extension rate was reduced to about half the control value and it made little growth at -3.9 MPa and lower. In comparison with these strains, *Fusarium culmorum* showed almost no reduction in colony extension rate down to -3.9 MPa but its rate dropped markedly at -5.0 MPa. *G. graminis* var. *tritici* and *Phialophora* sp. were poorly tolerant of KCl, making little growth at -3.0 MPa and none at -3.9 MPa.

Polyethylene glycol 8000 prevented agar from gelling, so it was added to Potato Dextrose Broth and colony extension was measured by placing the petri-dishes containing this broth onto a marked grid. The colonies were measured and radial growth was calculated as mean ± SEM for three replicates.

Fusarium culmorum was the most tolerant of increasing water-stress, its extension at -5.0 MPa being reduced to about half the control value, and *G. graminis* var. *tritici* was the least tolerant, its growth being stopped at -3.9 MPa. *Phialophora* sp. was not included in the experiment. The three strains of *I. bolleyi* showed intermediate behaviour between

Figure 3.1: Radial extension on agar osmotically adjusted with KCl, as a percentage of control (-0.1 MPa). Extension rates of controls (mm 24 h^{-1}, means ± SEM for 3 replicates) were: *Fusarium culmorum* (●) 4.3 ± 0.2; *Gaeumannomyces graminis* var. *tritici* (■) 3.0 ± 0.6; *I. bolleyi* AB1 (△) 3.6 ± 0.6; *I. bolleyi* (○) 3.6 ± 0.6; J10 (□) 3.4 ± 0.2; *Phialophora* sp. (▲) 3.4 ± 0.1. Asterisks denote significant differences in growth between all *I. bolleyi* strains at each water potential based on analysis of variance of actual growth measurements.

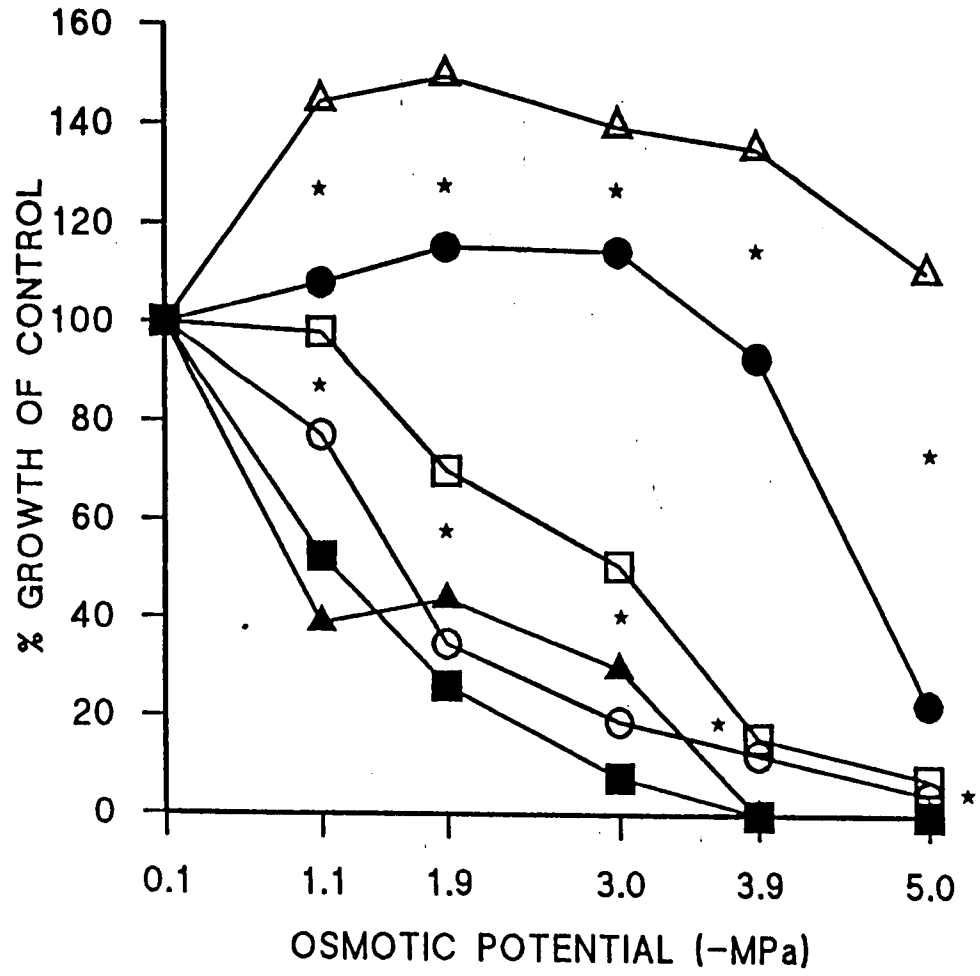
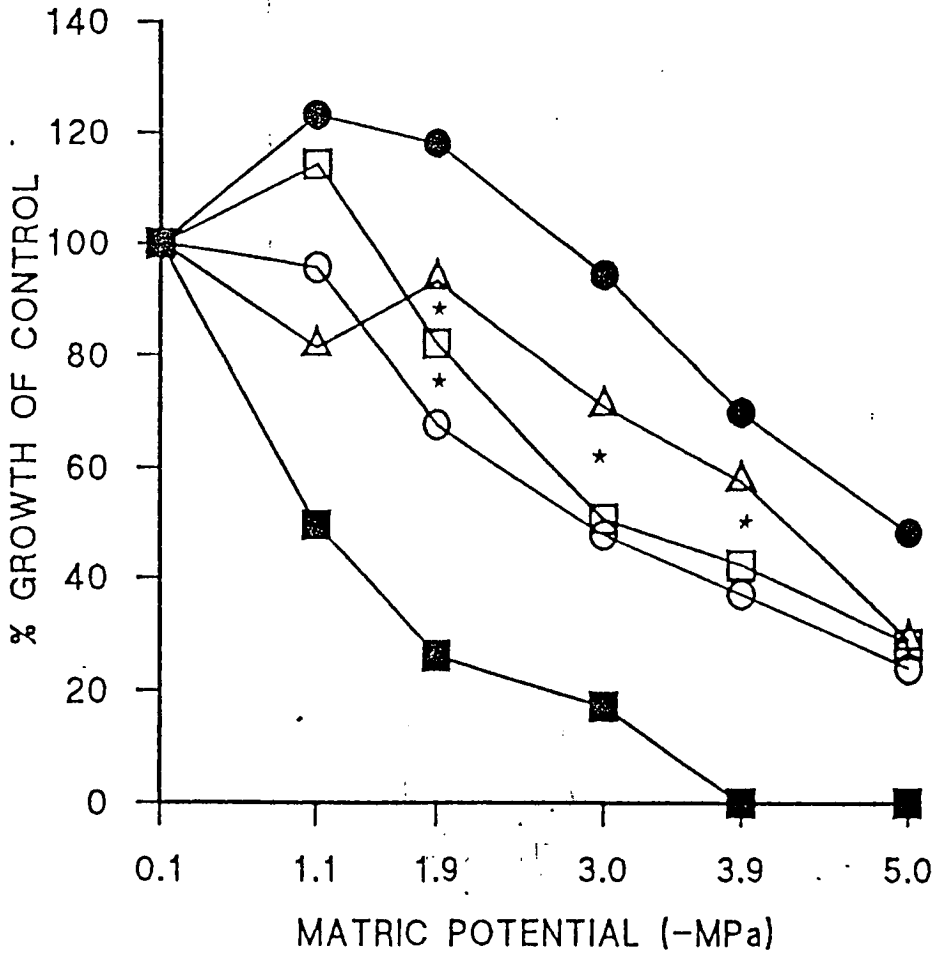


Figure 3.2: Radial extension in medium adjusted with PEG 8000 as a percentage of control (-0.1 MPa). Extension rates of controls (mm 24 h^{-1}, means ± SEM for 3 replicates) were: *Fusarium culmorum* (●) 1.2 ± 0.3; *Gaeumannomyces graminis* var. *tritici* (■) 1.4 ± 0.6; *I. bolleyi* AB1 (△) 1.3 ±0.1; *I. bolleyi* T560 (○) 1.1 ± 0.5; *I. bolleyi* J10 (□) 1.0 ± 0.1. Significant differences between *I. bolleyi* strains at each matric potential shown by asterisks, based on analysis of variance of arc-sine-transformed data (not applicable to -1.1 MPa).



that of *F. culmorum* and *G. graminis* var. *tritici* and they differed less from one another than in KCl-adjusted medium. Nevertheless, strain AB1 was the most stress tolerant approaching the tolerance of *F. culmorum*, and strain T560 was the least tolerant (Figure 3.2) in KCl-adjusted medium. Nevertheless, strain AB1 was the most stress-tolerant,

3.3.1.2 Biomass production in shake-culture flasks

Flasks containing Standard Liquid Medium adjusted osmotically with KCl were inoculated with PDA discs of *I. bolleyi* and incubated for 7 days at 25°C on an orbital shaker. The flask contents were filtered onto paper and the biomass oven dry weight was determined.

It was possible to obtain biomass estimates only in KCl-adjusted liquid medium because PEG was retained on the filter paper used to retrieve the biomass. As shown in Figure 3.3, all three strains of *I. bolleyi* produced similar biomass yields in the control flasks. The final biomass yields of strains AB1 and J10 were affected little by KCl down to -5.0 MPa, but growth of strain T560 was markedly reduced at -3 MPa and lower.

3.3.1.3 Spore germination in KCl or PEG-supplemented liquid medium

Flasks of liquid medium adjusted to different water potentials with either KCl or PEG 8000 were inoculated with conidial suspensions of *I. bolleyi*. Drops of the medium containing the conidia were transferred to sterile coverslips and inverted onto glass rings sealed to microscope slides in order to create hanging drops. The drops were examined for germination of the conidia after 14 h at 25°C.

Germination of conidia of the three strains of *I. bolleyi* after 14h (Figure 3.4) broadly reflected the differences obtained from measurements of colony extension. Each strain was more tolerant of increasing concentrations of PEG than of KCl. With either type of supplementation, strain AB1 germinated well down to at least -5.0 MPa; strain J10 was intermediate in response; strain T560 showed poor water-stress tolerance, its germination at -3.0 MPa being reduced to less than one-third of the control value and being inhibited completely at -5.0 MPa.

3.3.1.4 Sporulation in liquid culture

Conical flasks containing Standard Liquid Medium adjusted to different water potentials with KCl or PEG 8000 were inoculated with spore suspensions of *I. bolleyi.* The flasks were incubated on an orbital shaker and aliquots (0.5 ml) were removed aseptically every 24 h for 7 days, and spore numbers were assessed with a haemocytometer.

Spore production in liquid culture varied markedly between strains and between differently supplemented media (Figures 3.5 to 3.10). In the controls (-0.1 MPa) all three strains began to sporulate after 2-3 days and gave maximum spore yields ranging from c. 6 to 18 x 10^6 spores ml^{-1} in the culture medium. In media osmotically adjusted with KCl (Figures 3.5 to 3.7) all three strains of *I. bolleyi* produced at least some spores down to -5.0 MPa. Strain AB1 produced significantly more spores at -1.9, -3.0, and -3.9 MPa than in the controls at some stages of culture growth, and even at -5.0 MPa its spore production was no lower than in the controls. Strain T560 produced significantly more spores at - 1.1, -1.9 and -3.0 MPa than in the controls. Strain J10 initially produced more spores in all KCl-supplemented media than in the control, but final spore yields of this strain were similar for all treatments.

Figure 3.3: Dry weights of *I. bolleyi* AB1 (△), T560 (○) and J10 (□) in liquid medium osmotically adjusted with KCl (Mean ± SEM for three replicates).

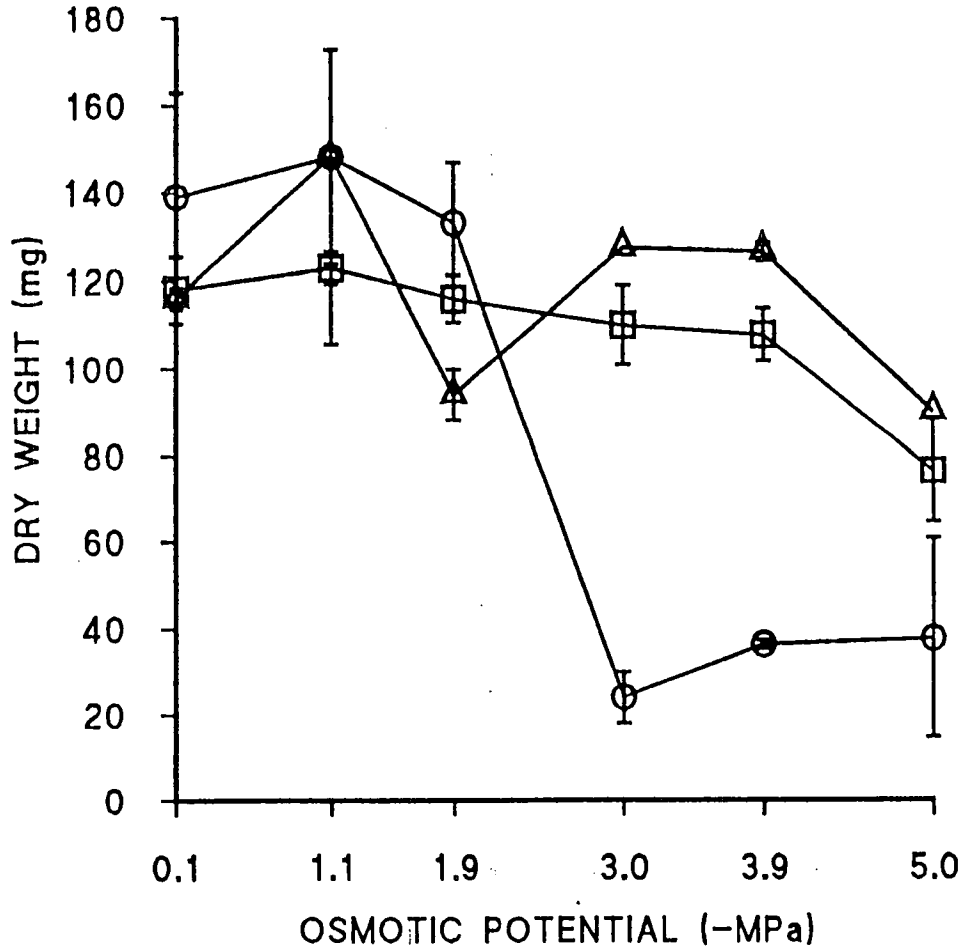
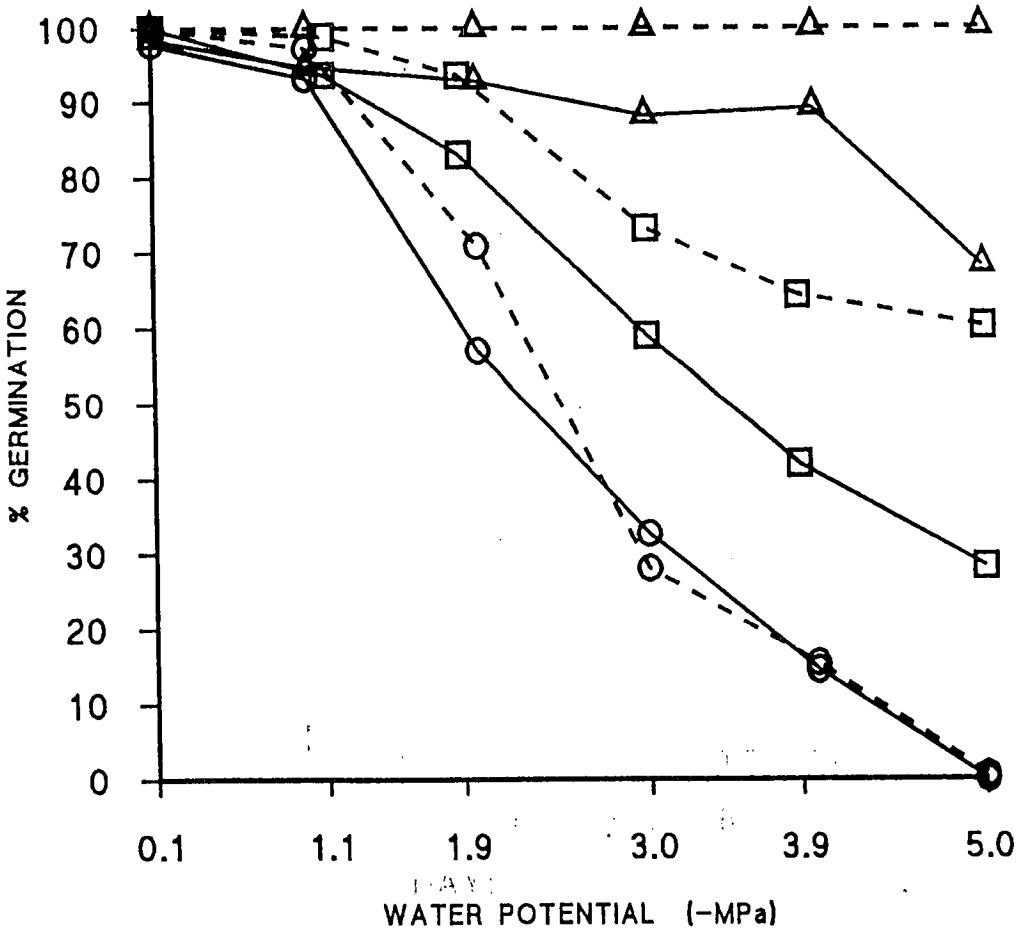


Figure 3.4: Percentage germination of *I. bolleyi* AB1 (Δ), T560 (O) and J10 (□) at different osmotic potentials (KCl, solid lines) or matric potentials (PEG 8000, broken lines). Data based on counts of 200 conidia in each of three replicates. Standard errors (not shown) conidia in each of three replicates. Standard errors (not shown) calculated for the original data were smaller than the symbol sizes here.



In PEG-adjusted media (Figures 3.8 to 3.10) almost all levels of applied water-stress significantly reduced the amount of sporulation by each strain, although this might in part have involved an effect of viscosity. Notably, however, strain AB1 still produced some spores at -3.0, -3.9 and -5.0 MPa whereas sporulation by strains J10 and T560 was suppressed at these water potentials. It was noticed that all three strains differentiated to produce chlamydospores in response to matric potentials of -1.1 MPa and lower; the cultures progressively melanized with time. This was not seen in the controls or in KCl-adjusted media.

3.3.2 Effect of water potential on straw colonisation in unsterile soil

The complex design of this experiment enabled comparisons to be made in different ways between the different types of medium used for isolation, between the different soil water potential and between the different *I. bolleyi* strains in mixed and single inoculum of strains.

At each sampling time the water loss from the bags was negligible showing that the water potential remained constant in the bags over the 6 week period of the experiment.

Tables 3.1 and 3.2 show the isolation of *I. bolleyi* and other fungi from straws plated onto Water Agar with antibacterial antibiotics, but not containing fungicides so there was no selection for individual *I. bolleyi* strains.

Disregarding the effect of soil water potential, at the 2 week sampling (Table 3.1), *Idriella bolleyi* strain T560-R2 (single inoculum) was isolated from a total 338 of the 460

Figure 3.5: Spore production by *I. bolleyi* (AB1) in liquid medium adjusted with KCl; means of three replicates. Bars above the graphs show 5% L.S.D. between treatments at each time; nsd = no significant difference.

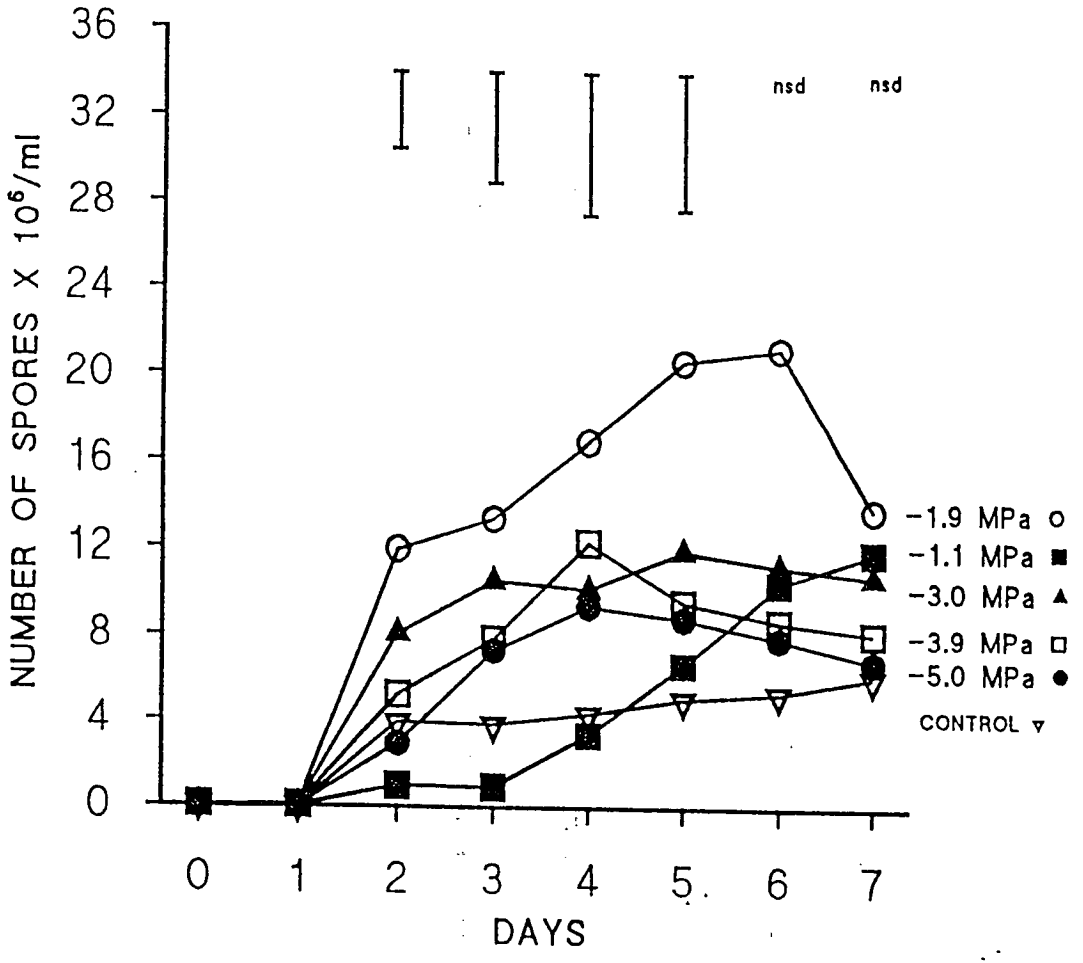


Figure 3.6: Spore production by *I. bolleyi* (T560) in liquid medium osmotically adjusted with potassium chloride. Means for three replicates; bars show 5% L.S.D. between treatments at each time; nsd = no significant difference.

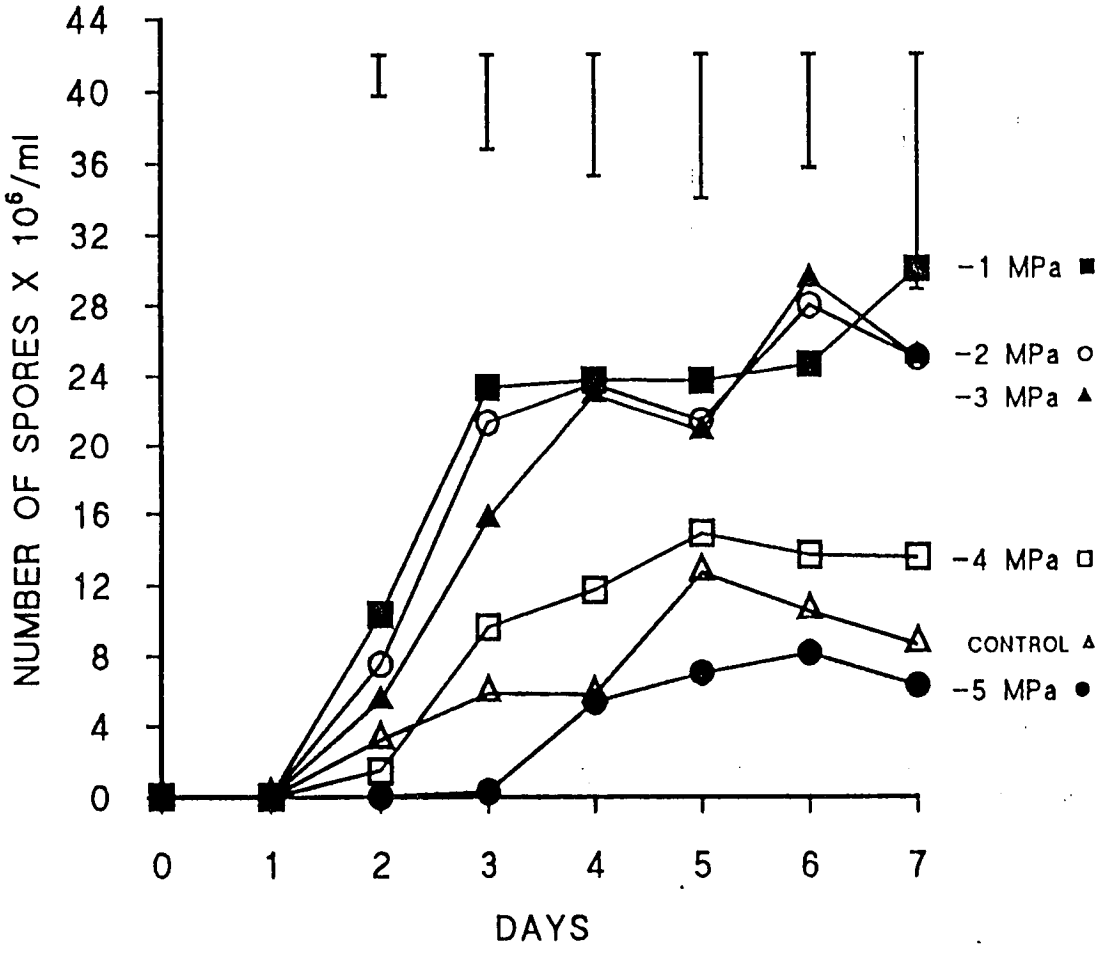


Figure 3.7: Spore production by *I. bolleyi* (J10) in liquid medium adjusted with KCl; means for 3 replicates. Bars above the graphs show 5% L.S.D. between treatments at each time; nsd = no significant difference.

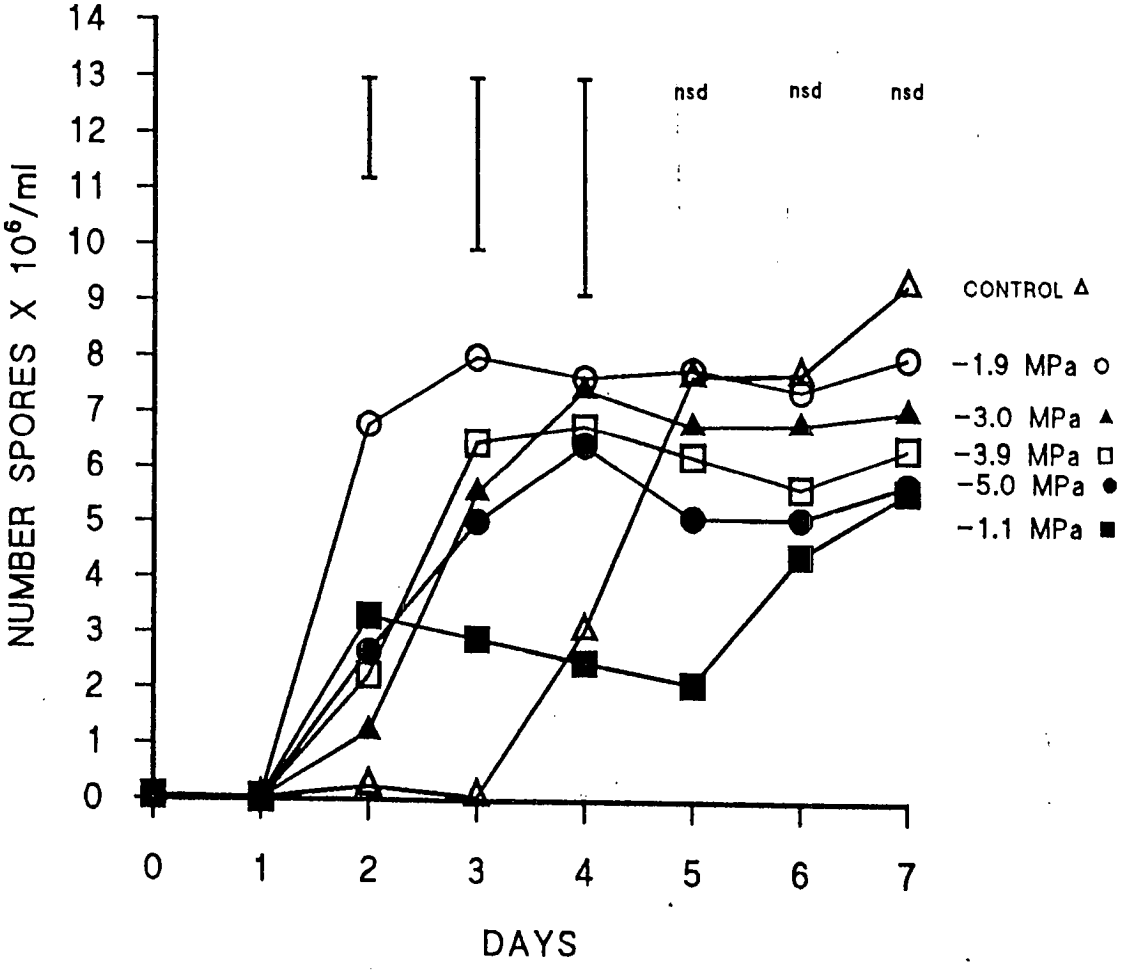


Figure 3.8: Spore production by *I. bolleyi* (AB1) in liquid medium adjusted with PEG 8000, means of three replicates. Bars above graphs show 5% L.S.D. between treatments at each time; nsd = no significant difference.

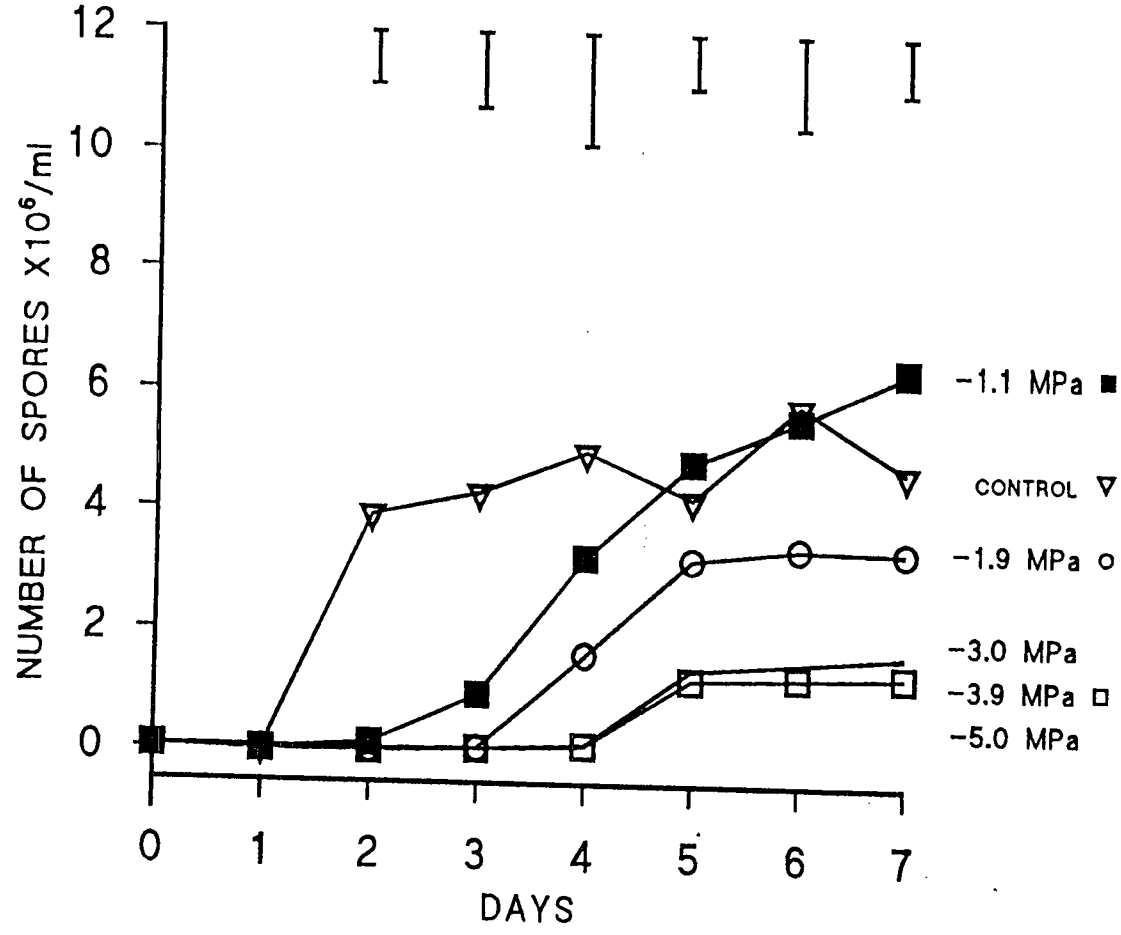


Figure 3.9: Spore production of *I. bolleyi* (T560) in liquid medium matrically adjusted with PEG 8000. Means of three replicates; bars show 5% L.S.D. between treatments at each time; nsd = no significant difference.

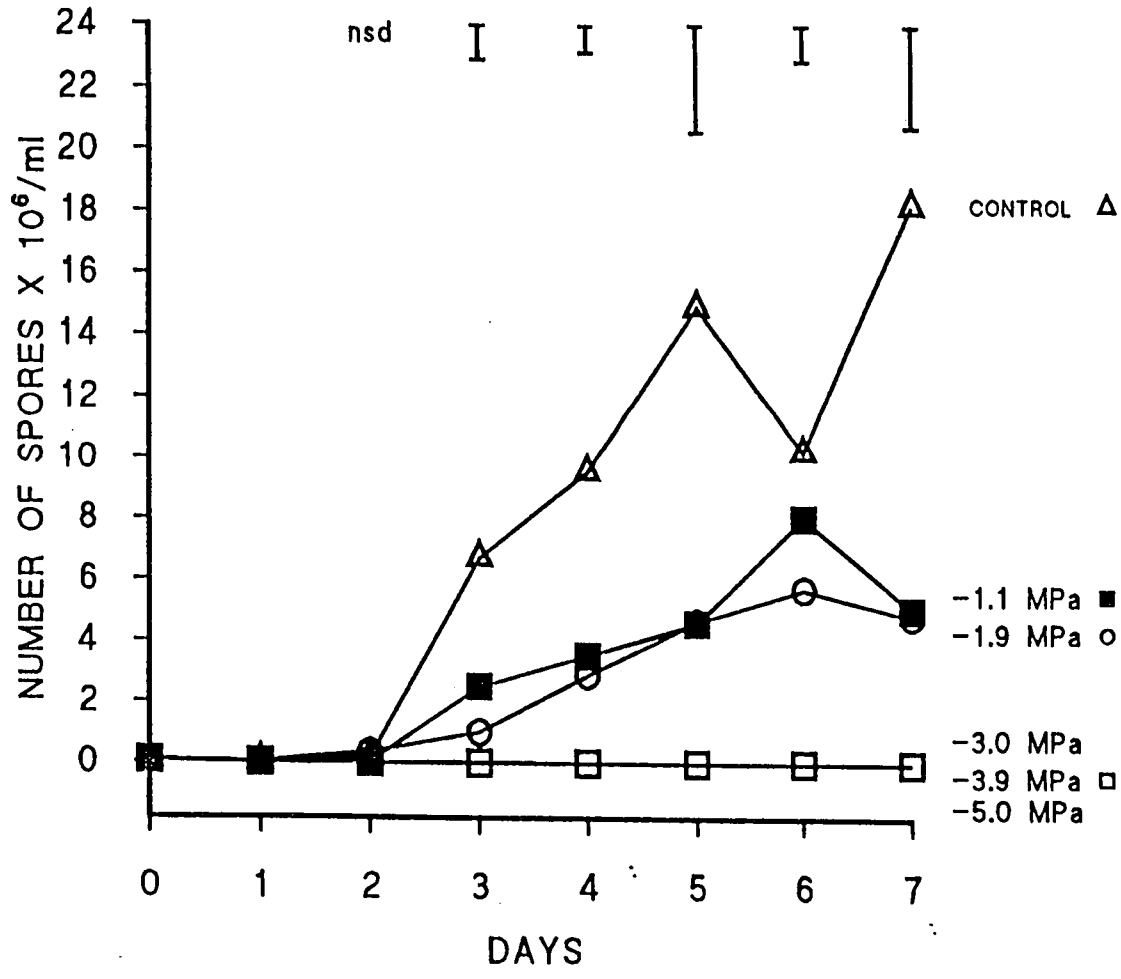
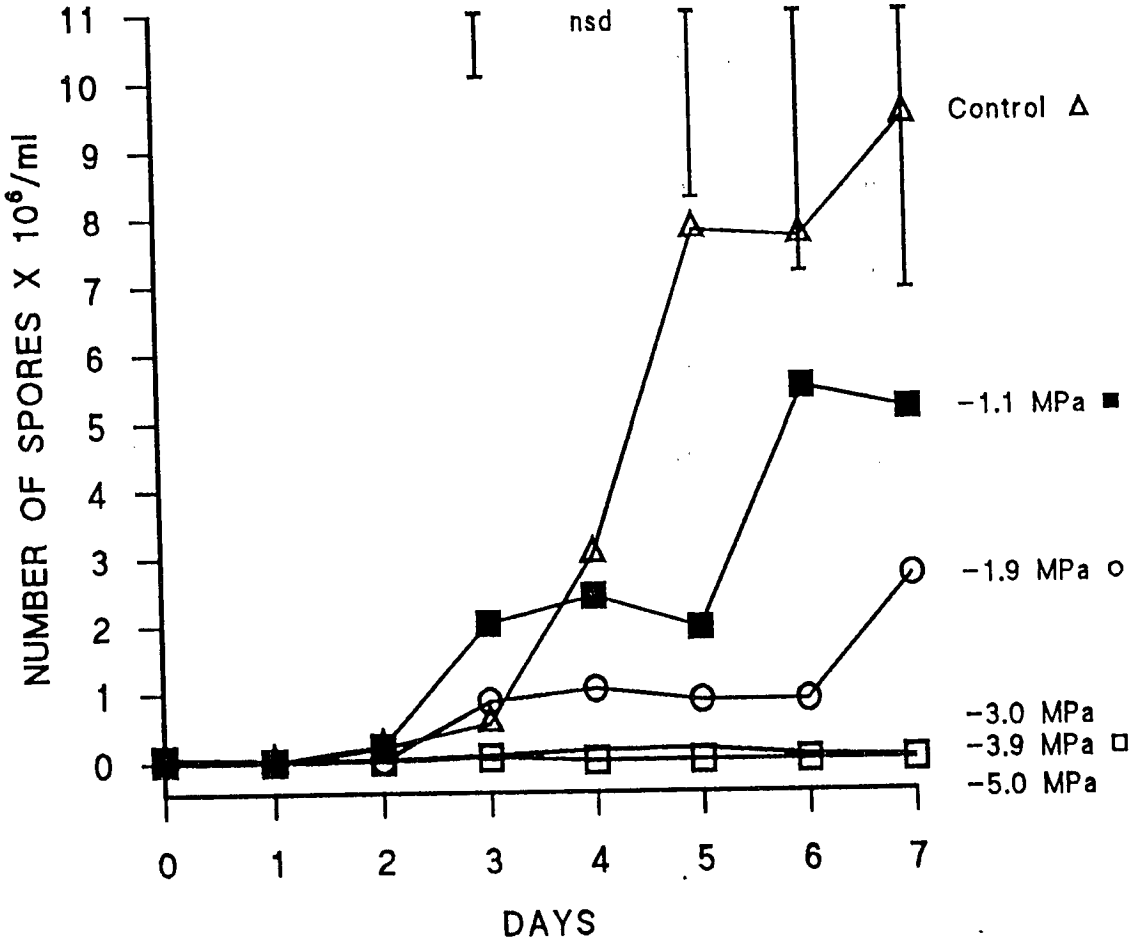


Figure 3.10: Spore production of *I. bolleyi* (J10) in liquid medium matrically adjusted with PEG 8000. Means of 3 replicates; bars show 5% L.S.D. between treatments at each time; nsd = no significant difference.



straws plated, compared with 291 of the 460 straws for strain AB1-IZ (single inoculum) and 312 of the 460 for the combined inoculum treatment. These values do not differ significantly by chi-squared analysis, indicating that the use of Water Agar without fungicides enabled the two *I. bolleyi* strains to be retrieved to similar degrees from the single strain inoculated straws. It shows also that dual inoculation of straws had no effect on the frequency of isolation of *I. bolleyi* compared with single strain inoculated straws.

At the 6 week sampling (Table 3.2) the frequency of recovery of *I. bolleyi* was higher for all straw treatments, and at all soil water potentials, compared with at 2 weeks. This difference between straw colonisation at 2 and 6 weeks was significant at -7.0 MPa (P = 0.01), at -5.0 MPa (P = 0.001), at -1.5 MPa (P = 0.001) and at -0.6 MPa (P = 0.01) by chi-squared analysis. Indeed, *I. bolleyi* was recovered from 91% of all straws plated at the 6 week sampling, compared with only 68% of straws at the 2 week sampling.

Disregarding the different *I. bolleyi* isolates, there were two noticeable findings concerning the different soil water potentials. First, at the 2 week sampling, the total number of straws from which *I. bolleyi* was recovered was remarkably similar at all soil water potentials: - 232, 238, 238 and 233 from soils of -0.6, -1.5, -5.0 and -7.0 MPa respectively. Second, this pattern had changed by the 6 week sampling, when *I. bolleyi* was recovered from 289 straws at -0.6 MPa water potential, compared with 325, 333 and 306 at -1.5, -5.0 and -7.0 MPa respectively. However, analysis by chi-squared indicated that these differences were not significant. So *I. bolleyi* showed, generally, the least ability to increase its level of colonisation between 2 and 6 weeks at the lowest level of imposed water-stress

A comparison of the difference in the total numbers of straw colonised at each water potential in the 4 week interval between samplings showed that the increase in the total numbers of straws colonised over 4 weeks was significant (P = 0.02) by chi-squared analysis,

at different water potentials, even though the total numbers of straws colonised at 6 weeks was not significantly different between potentials using chi-squared analysis.

Using agar unsupplemented with fungicide enabled a high number of other fungal species to be isolated along with *I. bolleyi* (Tables 3.1 and 3.2). A total of 14 species of fungi were isolated, eight of which were isolated frequently; these included *Fusarium* sp., *Trichoderma* sp., *Gliocladium* sp., *Cephalosporium* sp., *Mucor* sp., *Pythium oligandrum*, *Alternaria* sp. and *Cladosporium* sp. The remaining 6 species were isolated rarely so have been grouped together and referred to as 'other species'. No attempt was made to identify these fungi to species level, except of for *Pythium oligandrum*, but the *Gliocladium* sp. predominantly was *G. roseum* or one of the closely related species (*G. catenulatum*).

Disregarding any effects from the *I. bolleyi* inoculum on the straw and the effects of water potential, no fungi except *Mucor* sp. was isolated at the 2 day sampling time. The total straws colonised by *Mucor* sp. at 2 days was 14. All fungi were isolated from the straw at both 2 and 6 weeks sampling time. Pooling the data for the different treatments at 6 weeks sampling, the number of straws colonised by these fungi had risen for all species with the exception of *Pythium oligandrum* and *Mucor* sp., where the number of straws colonised actually decreased. The straw colonisation was significantly higher ($P = 0.01$; chi-squared analysis) at 6 weeks than at 2 weeks for all species with the exception of *Pythium oligandrum*, *Mucor* sp. and the 6 'other' species. The incidence of *Mucor* sp. had significantly decreased at 6 weeks ($P = 0.01$; chi-squared analysis).

Considering the effect of water potential on colonisation by the fungi other than *I. bolleyi*, there are several points of note. *Fusarium* sp. was isolated predominantly at the lower potentials, -7.0 MPa and -5.0 MPa. At 2 weeks straw colonisation by *Fusarium* sp. at -7.0 MPa was significantly higher with respect to the other water potentials (-0.6 MPa and -1.5 MPa),

Table 3.1: Total number of isolations of fungi on water agar, from straw pieces retrieved after 2 weeks from soil held at different water potentials; data pooled for 23 pieces of straw in each of 5 replicates (maximum 115).

I. bolleyi inoculum:	T560-R2				T560-R2 + AB1-IZ				AB1-IZ			
Soil water potential (-MPa)	-7.0	-5.0	-1.5	-0.6	-7.0	-5.0	-1.5	-0.6	-7.0	-5.0	-1.5	-0.6
Idriella bolleyi	91	86	86	75	68	73	90	81	74	79	62	76
Fusarium sp.	92	74	63	76	95	31	26	14	115	71	39	36
Trichoderma sp.	3	14	48	25	6	8	12	39	4	32	56	30
Gliocladium sp.	21	13	6	1	35	22	16	2	24	33	2	8
Cephalosporium sp.	0	45	96	82	2	42	60	66	0	23	57	89
Mucor sp.	0	0	6	10	1	1	1	10	0	0	5	3
Alternaria sp.	0	0	0	0	4	0	5	3	5	7	0	0
Cladosporium sp.	0	0	0	0	2	2	1	0	0	4	0	7
Pythium oligandrum	0	0	2	3	0	12	4	15	0	0	7	8
Other spp.	1	0	0	0	0	0	3	2	0	0	1	0
Total	**208**	**232**	**307**	**272**	**213**	**191**	**218**	**232**	**222**	**249**	**229**	**257**

Table 3.2: Total number of isolations of fungi isolated on water agar, from straw pieces retrieved after 6 weeks from soil held at different water potentials; data pooled for 23 pieces of straw in each of 5 replicates (maxium 115).

I. bolleyi inoculum:	T560-R2				T560-R2 + AB1-IZ				AB1-IZ			
Soil water potential (-MPa)	-7.0	-5.0	-1.5	-0.6	-7.0	-5.0	-1.5	-0.6	-7.0	-5.0	-1.5	-0.6
Idriella bolleyi	102	109	113	104	102	111	99	97	104	113	113	88
Fusarium sp.	103	74	34	17	99	99	22	15	82	100	40	42
Trichoderma sp.	0	15	28	71	5	10	32	45	1	17	81	85
Gliocladium sp.	90	81	27	19	81	101	64	18	72	82	50	17
Cephalosporium sp.	0	36	84	55	0	19	96	58	0	37	91	41
Mucor sp.	0	0	4	5	0	0	1	11	0	0	0	0
Alternaria sp.	0	9	0	0	0	3	2	1	0	0	1	2
Cladosporium sp.	5	3	0	0	0	3	0	0	2	2	1	0
Pythium oligandrum	0	0	0	1	0	2	1	0	0	0	0	0
Other spp.	0	2	0	2	1	0	1	0	0	12	0	10
Total	300	329	290	274	288	348	318	245	261	363	377	285

($P = 0.01$; Two-way anova), (Appendix table 3.1). At 6 weeks colonisation at both -7.0 MPa and -5.0 MPa, was significantly higher than at the -1.5 MPa and -0.6 MPa potentials ($P = 0.01$; Two-way anova), (Appendix table 3.2). *Gliocladium* sp. showed a similar pattern of straw colonisation as *Fusarium* sp. At 2 weeks, straw colonisation by *Gliocladium* sp. was significantly higher at -7.0 MPa than at the other water potentials; also colonisation at -5.0 MPa was significantly higher than at -0.6 MPa ($P = 0.01$; two way anova), (Appendix table 3.3) but there was no significant difference between -5.0 MPa and -1.5 MPa. At 6 weeks straw colonisation by *Gliocladium* sp. was significantly higher at -7.0 MPa and -5.0 MPa with respect to -1.5 MPa and -0.6 MPa ($P = 0.01$, two way anova), (Appendix table 3.4).

Trichoderma sp. and *Cephalosporium* sp. showed an opposite pattern of occurrence, with predominant isolation at the -0.6 MPa and -1.5 MPa water potentials. For both *Trichoderma* sp. and *Cephalosporium* sp. at 2 weeks, colonisation was significantly higher at both -1.5 MPa and -0.6 MPa than at the lower water potentials ($P = 0.01$; Two-way anova), (Appendix tables 3.5 and 3.7). At 6 weeks the same pattern of colonisation was observed for *Trichoderma* sp. as at 2 weeks, but with *Cephalosporium* sp. the number of straws colonised was significantly different between all potentials ($P = 0.01$; Two-way anova), (Appendix tables 3.6 and 3.8). In marked contrast to *Fusarium* sp. and *Gliocladium* sp., the incidence of *Trichoderma* sp and *Cephalosporium* sp. was extremely low at -7.0 MPa, indicating that these fungi were intolerant of the highest level of water-stress.

Cladosporium sp. was isolated from straws in low numbers at all potentials, and at 2 and 6 weeks there was no significant difference between colony numbers at each potential.

Alternaria sp. was found at a low incidence at all water potentials. No significant difference was found at 2 weeks. At 6 weeks the incidence of *Alternaria* sp. was greatest but the low overall number of occurrences precluded chi-squared analysis.

Mucor sp. and *P. oligandrum* were seen mainly from straws at the -0.6 MPa and -1.5 MPa potentials. At 2 weeks, the incidence of *Mucor* sp. was significantly higher at -0.6 MPa than at the other potentials ($P = 0.01$). At 6 weeks, colonisation was significantly higher at -0.6 MPa and -1.5 MPa than at -5.0 MPa and -7.0 MPa ($P = 0.01$ Two-way anova). The occurrence of *P. oligandrum* was too low at 6 weeks for statistical analysis, but at 2 weeks its occurrence differed markedly at different water potentials ($P = 0.01$, Two-way anova), being highest at -0.6 MPa, and it did not grow from straws that had been held in soil at -7.0 MPa.

It is not known if inoculation of straws with *I. bolleyi* had a direct influence on straw colonisation by the other fungal species, as no straws without *I. bolleyi* were used in the experiment. However, disregarding the effects of soil water potential, there were differences in the detection of other fungi relating to the *I. bolleyi* inoculum used to inoculate the straw.

At 2 weeks sampling, colonisation by *Fusarium* sp. was significantly lower on straws inoculated with the T560-R2/AB1-IZ mixed strain inoculum than with either of the single strain inocula ($P = 0.01$; Two-way anova) (Appendix table 3.1). At 6 weeks, however, there was no such difference (Appendix table 3.2).

Overall, the incidence of *Trichoderma* sp. from straws inoculated with the mixture of *I. bolleyi* strains was lower at both 2 and 6 weeks than for straws inoculated with single strains of *I. bolleyi*. The difference in incidence of *Trichoderma* sp. between the mixed inoculum and the single inoculum of strain AB1-IZ was significant at both 2 and 6 weeks ($P = 0.01$; two way anova), but not between the mixed strain inoculum and the single strain inoculum of T560-R2 at either sampling time (Appendix tables 3.5 and 3.6).

At 2 weeks, levels of *Cephalosporium* sp. were significantly higher on straws inoculated with single strain T560-R2 than with either single strain AB1-IZ or the mixed

strain inoculum of *I. bolleyi* ($P = 0.01$; Two-way anova), (Appendix table 3.7); however, there was no such difference at 6 weeks (Appendix table 3.8).

Pythium oligandrum was significantly higher at 2 weeks on straws inoculated with the mixture of *I. bolleyi* strains than straws inoculated with either strain AB1-IZ ($P = 0.05$; chi-squared analysis) strain T560-R2 ($P = 0.001$; chi-squared analysis). But *P. oligandrum* was rarely encountered at the 6 week sampling. At 6 weeks, *P. oligandrum* levels were significantly higher on straws inoculated with the mixed strain inoculum, than with the AB1-IZ and T560-R2 single strain inoculums ($P = 0.01$; Two-way anova.).

3.3.2.1 Effects of different isolation media

Plating of straws on Water Agar (Tables 3.1 and 3.2) allowed the isolation of *I. bolleyi* irrespective of strain, however, agar supplemented with fungicide was also used to selectively isolate each specific inoculant strain. This enabled comparisons of straw colonisation by the different *I. bolleyi* strains to be made at different water potentials, as well as allowing the effectiveness of the selective media to be assessed. The results are shown in Tables 3.3 and 3.4.

No *I. bolleyi* was recovered from straws harvested at 2 days on either Water Agar or fungicide-supplemented agar, thus this data was not shown in Table 3.3 and 3.4. Overall, the results from these tables show that *I. bolleyi* could grow from all straws that had been buried at all water potentials, after both 2 and 6 weeks. There were some differences in the degree of growth by different strains of *I. bolleyi*, but the differences were relatively small and were confined mainly to the 2 week sampling. For example, at this time the incidence of recovery of *I. bolleyi* from straws inoculated with strain AB1-IZ was consistently lower on both Water

Table 3.3: Number of straws (means of 5 replicates, 23 straw pieces per replicate) colonised by *Idriella bolleyi* after 2 weeks burial in unsterilised soil at different water potentials. Isolations of *I. bolleyi* were on different agar media: carbendazim agar to select for strain T560-R2, imazilil agar to select for strain AB1-IZ, data for fungicide-free water agar (from Tables 3.1 and 3.2) are given for comparison.

Isolation medium	*Idriella bolleyi* strain used as inoculum	Soil water potential (-MPa)							
		-7.0	-5.0	-1.5	-0.6	Row mean	SED	5% LSD	1% LSD
Water Agar	T560-R2	18.2	17.2	17.2	15.0	**16.90**	**0.98**	**1.96**	**2.60**
Water Agar	AB1-IZ	14.8	15.8	12.4	15.2	**14.55**			
Water Agar	T560-R2/AB1-IZ	13.6	14.6	18.0	16.2	**15.60**			
Carbendazim Agar	T560-R2	17.8	20.2	20.2	14.0	**18.05**			
Imazilil Agar	AB1-IZ	15.8	16.2	19.8	14.0	**16.45**			
Carbendazim Agar	T560-R2/AB1-IZ	19.2	19.2	18.0	20.0	**19.10**			
Imazilil Agar	T560-R2/AB1-IZ	15.0	18.0	15.8	13.2	**15.50**			
	Column mean	**16.34**	**17.31**	**17.34**	**15.37**				
	SED	**0.74**							
	5% LSD	**0.89**							

Table 3.4: Number of straws (means of 5 replicates, 23 straw pieces per replicate) colonised by *Idriella bolleyi* after 6 weeks, burial in unsterilised soil at different water potentials. Isolation of *I. bolleyi* were on different agar media: carbendazim agar for the selection of strain T560-R2, imazilil agar for the selection of strain AB1-IZ, data for fungicide-free water agar (from Tables 3.1 and 3.2) are given for comparison.

Isolation medium	*Idriella bolleyi* strain used as inoculum	Soil water potential (-MPa)							
		-7.0	-5.0	-1.5	-0.6	Row Mean	SED	5% LSD	1% LSD
Water Agar	T560-R2	20.4	21.8	22.6	20.8	**21.40**	**0.59**	**1.18**	**1.57**
Water Agar	AB1-IZ	20.8	22.6	22.6	17.6	**20.90**			
Water Agar	T560-R2/AB1-IZ	20.4	22.2	19.8	19.4	**20.45**			
Carbendazim Agar	T560-R2	22.2	23.0	21.8	22.2	**22.30**			
Imazilil Agar	AB1-IZ	21.2	21.2	21.2	21.2	**21.20**			
Carbendazim Agar	T560-R2/AB1-IZ	20.8	22.0	21.8	18.4	**20.75**			
Imazilil Agar	T560-R2/AB1-IZ	19.8	22.2	20.8	22.0	**21.20**			
	Column mean	**20.80**	**22.14**	**21.51**	**20.23**				
	SED	**0.45**							
	5 % LSD	**0.89**							

Agar and Water Agar supplemented with imazilil, than the recovery of *I. bolleyi* from straws inoculated with strain T560-R2 on Water Agar or Water Agar supplemented with carbendazim. There was also lower recovery of *I. bolleyi* from the straws inoculated with the mixture of strains when plated on imazilil-supplemented agar than on carbendazim-supplemented agar. These results must reflect the differences in growth of the two *I. bolleyi* strains, because strain T560-R2 could not grow on imazilil-supplemented agar, and strain AB1-IZ could not grow on carbendazim-supplemented agar. However, there was no consistent difference between strains T560-R2 and AB1-IZ in their pattern of occurrence at different water potentials: both strains were able to grow from the majority of straws held in soil at all water potentials. Comparison of the data in Tables 3.3 and 3.4 also show that the *I. bolleyi* strains could be retrieved at least as frequently on the respective fungicide-supplemented agar as on Water Agar. At both sampling times this frequency was slightly higher on fungicide-supplemented agar. This was probably because the presence of fungicide markedly reduced the incidence of other fungi on the agar, as was noted during assessments of the plates.

It was noted that the only other fungal species isolated on fungicide-supplemented agar was *Mucor* sp. *Mucor* sp. was isolated on the carbendazim-supplemented agar, isolated at 2 days, 2 weeks and 6 weeks. The total number of colonies of *Mucor* sp. isolated on carbendazim-supplemented agar, (total from both combined strain and single strain T560-R2 carbendazim plates) irrespective of water potential, was 16, 36 and 38 respectively. *Gliocladium* sp. was the only other fungal species to be isolated on the imazilil-supplemented agar, at 2 weeks and 6 weeks, and the total number of straws colonised (total from both combined strain and single strain AB1-IZ imazilil plates) irrespective of water potential, was 161 and 432 respectively. As in the isolation of these same fungi on unsupplemented agar, *Gliocladium* was mainly isolated at the lower water potentials, -5.0 MPa and -7.0 MPa,

whereas *Mucor* sp. was isolated at the higher potentials, -0.6 MPa and -1.5 MPa. (This data is not presented in Tables 3.3 and 3.4).

3.3.3 Conidia production on wheat seed buried in unsterile soil

Idriella bolleyi was examined for its ability to sporulate on sterile and unsterile wheat seeds, following their inoculation with either an aqueous or a sodium alginate-calcium chloride conidial suspension. The seeds were buried in unsterile soil and harvested at regular time intervals. Spore numbers from the seeds were determined after shaking the seeds in sterile distilled water and plating serial dilutions onto carbendazim-supplemented agar. The pattern of sporulation on the seeds could be followed, and the ability of *I. bolleyi* to compete with a natural competing microflora could be determined.

Idriella bolleyi spores could be detected from both sterile and unsterile seeds, for both water and alginate inoculation treatments at all sampling times. However, the spore number was seen to vary over the sampling time.

3.3.3.1 Seed germination and seedling development

All seeds had begun to germinate by the 24 h sampling, and they had produced several seminal roots by 3 days. By 25 days, the seedlings were at growth stage 11 (Zadoks scale), and the seeds at this stage were extremely soft and breaking apart. There was no effect of inoculation treatment on seedling development.

3.3.3.2 Recovery of *I. bolleyi* conidia from wheat seeds retrieved from unsterilised soil

From planting of the wheat seeds, the level of detectable conidia on the seeds showed the same pattern for the different treatments, only the timing of this pattern was slightly different between treatments (Table 3.5). At zero days, spore loadings on the seed were between 30 to 40 x 10^4 spores $seed^{-1}$. Spore loadings were not significantly different between treatments. At 24 h, detectable spore number for all treatments had fallen to between 2 to 5% of the original spore loadings. The rapid drop in spore number was followed by a sharp increase in number resulting in a peak of spore production which was then followed by a steady decline in spore numbers.

Two-way analysis of variance showed that there was no significant difference in spore numbers between the four treatments at each sampling time and no effect of the treatment on spore number over the assessment period (Table 3.5). Pooled data for sterile and unsterile alginate and sterile and unsterile water treatments, showed no significant difference with regard to the method of spore loading. Pooled data (Table 3.6).

Spore numbers in both the sterile and unsterilised treatments began to increase on day three after the initial decline at 24 h. Spore production on sterile seeds increased for both water and alginate treatments, peaking at 5 days. The spore number for the sterile alginate treatment at 5 days was 65.6 x 10^4 spores ml^1, this value being higher than the original seed loading recorded at 50 x 10^4 spores ml^{-1} The sterile water treatment peaked at 41.9 x 10^4 spores ml^{-1}, at 5 days, which was lower than the original spore loading at 50 x 10^4 spores ml^{-1}.

The detectable spore number on both the sterile alginate and sterile water treated seeds began to decline at some point after day 5, this being evident with spore number being lower on day 8. Numbers continued to decline for both sterile treatments, and by day 14, the spore number for the sterile alginate treatment was 50.75 x 10^4 spores ml^{-1}, 77% of the spore

Table 3.5: Number of detectable *Idriella bolleyi* spores (strain T560-R2) recovered from wheat seeds (either sterile or unsterile) after inoculation with a conidial suspension of *I. bolleyi* (*I. bolleyi* suspensions were either in distilled water or sodium alginate-$CaCl_2$ solution), and after burial in unsterile soil at 20 °C. The seeds were sampled at time intervals (shown below) and shaken in 10 ml distilled water. Serial dilutions of the water were made and plated on to ¼-strengh PDA supplemented with KCl (36.5 g l^{-1}), Carbendazim (50µg ml^{-1}) and antibacterial antibiotics (strepotmycin sulphate 50µg ml^{-1} and Penicillin G 50µg ml^{-1}). One colony was assumed to derive from a single spore. (mean for 4 replicates, five seeds per replicate). All mean spore numbers to be multiplied by 10^4 $seed^{-1}$.

	Sample time of seeds								
Seed Treatment	**Day 0**	**Day 1**	**Day 3**	**Day 5**	**Day 8**	**Day 14**	**Day 25**	**Row mean**	**SED**
Unsterilised alginate	40.00	0.72	5.67	10.42	15.50	7.56	2.00	**11.69**	**5.46**
Unsterilised water	32.50	1.12	5.84	9.59	26.50	9.49	0.91	**12.28**	
Sterilised alginate	30.00	0.98	13.42	65.50	50.75	5.07	1.27	**28.30**	
Sterilised water	50.00	2.71	9.25	41.91	36.10	10.92	0.13	**21.57**	
Column mean	**38.13**	**1.38**	**8.54**	**31.85**	**32.21**	**8.26**	**1.07**		
SED	**7.22**								
5% LSD	**14.4**								
1% LSD	**19.22**								

Two-way analysis of variance (P =0.05) and (P= 0.01)

Table 3.6: Pooled data for the number of detecatable *Idriella bolleyi* spores (strain T560-R2) recovered from wheat seeds (either sterile or unsterile) after inoculation with a conidial suspension of *I. bolleyi* (*I. bolleyi* suspensions were either in distilled water or sodium alginate-$CaCl_2$ solution), and after burial in unsterile soil at 20°C. The seeds were sampled at time intervals (shown below) and shaken in 10 ml distilled water. Serial dilutions of the water were made and plated on to ¼-strength PDA supplemented with KCl (36.6 g l^{-1}), carbendazim (50µg ml^{-1}) and antibacterial antbiotics (streptomycin sulphate 50µg ml^{-1} and Penicillin G 50µg ml^{-1}). One colony was assumed to derive from a single spore. (The data for both water and alginate treatments are pooled for sterilised and unsterilised seeds; Means for 8 replicates, five seeds per replicate). All mean spore numbers to be multiplied by 10^4 $seed^{-1}$.

	Sample time of seeds										
Seed Treatment	**day 0**	**day 1**	**day 3**	**day 5**	**day 8**	**day 14**	**day 25**	**Row mean**	**SED**	**1% LSD****	**5% LSD****
Unsterilised seed	36.25	0.92	5.75	10.00	21.00	8.52	1.45	11.98	**13.50**	**35.91**	**27.0**
Sterilised seed	40.00	1.85	11.33	53.71	43.43	7.99	0.90	12.28	**3.32.**	**8.83**	**6.63**
P* value	**ns**	**ns**	**ns**	**0.01**	**ns**	**ns**	**ns**	**ns**			

* T-test between means in columns: significantly different at *P* value shown; ns = no significant difference between means.

** One-way analysis for data in rows at ($P = 0.01$) and ($P = 0.05$)

number recorded at peak spore production. Spore number for the sterile water treatment was 36.1 x 10^4 spores ml^{-1}, 86% of the spore number recorded at peak production. Spore number for both treatments continued to fall from day 8 to day 25, and spore number at 25 days was 1.27 x 10^4 spores ml^{-1} and 0.13 x 10^4 spores ml^{-1} for the sterile alginate and the sterile water treatments respectively.

Spore production on the unsterilised seeds increased at a slower rate over time for both the alginate and the water treatments, reaching a peak at 8 days, 3 days slower than that recorded for the sterile seeds. Spore numbers at the point of peak production were lower than those on the sterile seeds. At 8 days mean spore number in the unsterilised alginate treatment was 15.5 x 10^4 spores ml^{-1}, only 39% of the original spore loading recorded at 40.0 x 10^4 spores ml^{-1}. The unsterilised water treatment peaked at day 8, with a detectable spore loading of 26.5 x 10^4 spores ml^{-1}, 81% of the original spore number which was recorded at 32.5 x 10^4 spores ml^{-1}.

Detectable spore number declined for both the unsterilised alginate and the water treatments after 8 days, this being evident by a lower spore number for both treatments at day 14. Spore number at 14 days for the unsterile alginate treatment was 49% of the peak production at 7.56 x 10^4 spores ml^{-1} and spore number for the unsterile water treatment was 36% of the peak spore production at 9.49 x 10^4 spores ml^{-1}. Spore number continued to decline for both treatments and at 25 days spore number was 2 x 10^4 spores ml^{-1} and 0.91 x 10^4 spores ml^{-1} for the unsterile alginate and the unsterile water treatments respectively.

No *I. bolleyi* spores were recovered on the fungicide-supplemented PDA from the uninoculated control seeds at any of the sampling times, showing that all *I. bolleyi* spore forming colonies on the fungicide-supplemented PDA derived from the original inoculum.

3.3.4 Variation between field root and stem base isolates of I. bolleyi *to water potential*

Field isolates of *I. bolleyi* were obtained from both the root and stem bases of mature cereal plants from three sites around Edinburgh. The isolates were examined to see if they varied in their response to water stress. It was thought that if a large degree of variation was observed, this may indicate that isolates are showing different ecological adaptions. Strain variability to water-stress may allow strains to be targeted for the control of particular pathogens or that mixed strain inoculum may be used to improve the consistency of biocontrol over sites and seasons.

Isolates were obtained from the roots and stem bases of cereal plants, collected at 3 sites around Edinburgh, and were compared for their biomass production and sporulation in Standard Liquid Medium osmotically adjusted with KCl (-3.9 MPa) and also in Standard Liquid Medium, without KCl (control).

3.3.4.1 Site description

Soil from each site was tested for its pH and the field capacity of each soil was determined (Section 2.2.1.1), (Table 3.7).

All soils were found to be acidic, however, site T was found to have the lowest pH of the 3 sites. Water content at field capacity varied for all three sites with site T having the greatest water holding capacity and site B having the least.

3.3.4.2 Isolation of *I. bolleyi* from the root and stem bases of cereal plants

Idriella bolleyi was successfully isolated from the root and stem bases of cereal plants obtained from three sites around Edinburgh (sites B,C and T).

Spore number for the sterile water treatment was 36.1 x 10^4 spores ml^{-1}, 86% of the spore number recorded at peak production. Spore number for both treatments continued to fall from day 8 to day 25, and spore number at 25 days was 1.27 x 10^4 spores ml^{-1} and 0.13 x 10^4 spores ml^{-1} for the sterile alginate and the sterile water treatments respectively.

Table 3.7 Soil and field description for the isolation of *I. bolleyi* from mature cereal roots and stem bases from three sites around Edinburgh (sites B, C and T).

Soil	Field description	Soil pH	Water content at field capacity (g H_2O/ 100 g soil)
B	*Winter wheat*	6.58	50g H_2O/100g soil
C	*barley/grass undersown*	6.75	61g H_2O/100g soil
T	*barley/grass undersown*	5.40	80g H_2O/100 g soil

Only two isolates were obtained from winter wheat plants at site B, with one isolate being obtained from the stem base and one from the roots.

3.3.4.3 The effect of osmotic potential on growth and sporulation of root and stem base isolates

For growth in Standard Liquid Medium with or without osmotic adjustment with KCl, there was a large amount of variation between individual isolates in terms of biomass production (Table 3.8). Biomass ranged from as little as 6.63 mg (Isolate T-1(1)S from stem base) to as much as 22.30 mg (Isolate T-3(1)S from stem base) at -0.1 MPa, and from 9.67 mg (Isolates T-1(4)S and T-4(2)S from stem base) to 28.67 mg (Isolate T-3(1)S from stem base) at -4.0 MPa. Two-way analysis of variance of the data showed highly significant differences ($P = 0.05$) between isolates and between applied water potentials, with better growth overall at -4.0 MPa than at -0.1 MPa. Indeed, only 6 of the total 26 isolates grew poorer at - 4.0 MPa than at - 0.1 MPa and often this difference was small in such cases.

For sporulation in liquid medium, with or without osmotic adjustment with KCl, a large amount of variation was observed between the isolates (Table 3.9). Sporulation at -0.1 MPa ranged from 1.87 x 10^6 spores ml^{-1} (Isolate C-3(2)S from stem base) to 12.23 x 10^6 spores ml^{-1} (Isolate C-1(1)R, from roots) and sporulation at -4.0 MPa ranged from 0.31 x 10^6 spores ml^{-1} (Isolate C-3(2)R from roots, to 6.24 x 10^6 spores ml^{-1} (Isolate C-4S from stem base). Two-way analysis of variance of the data showed that there was highly significant differences for sporulation between the isolates and between the applied water potential. Unlike the biomass production overall sporulation was seen to decrease at -4.0 MPa with respect to -0.1 MPa. Out of 26 isolates, only 9 isolates had higher sporulation at -4.0 MPa than at -0.1 MPa, however, these differences were again small in such cases.

A high production of biomass at -4.0 MPa, did not always correspond to a high level of spore production at -4.0 MPa. From 20 isolates which had higher biomass production at -4.0 MPa than at -0.1 MPa, only 9 of those isolates had sporulation levels at -4.0 MPa which exceeded spore production at -0.1 MPa. From these 9 isolates, 7 were from the stem base and 2 were from the root.

It was observed that some of the isolates showing very low biomass production at -0.1 MPa, often produced some of the highest biomass yields at -4.0 MPa, (Isolates C-4S, C-1(1)R, C-3(2)S, C-2(1)R, T-4(1)S, T-3(2)R and T-2R). Also, some isolates showing very high biomass production at-0.1 MPa showed some of the lowest biomass production at -4.0 MPa, (Isolates C-3(1)R, C-4(1)R, T-1(4)S, and T-3(1)R).

A similar occurrence was observed for spore production. Those isolates showing very high sporulation at -0.1 MPa showed very low sporulation at -4.0 MPa (Isolates T-1(1)S, T-1(2)S, T-3(1)R, T-3(2)R, C-2(1)R and B-1(1)S). Also some isolates showing the opposite pattern of very high sporulation at -4.0 MPa showed some of the lowest sporulation at -0.1 MPa (Isolates T-1(2)S, T-2R, T-3(1)S, T-3(2)S, C-3(1)S, C-3(2)S and B-1(1)R).

Isolates from the roots and stem base of the same plants were compared for each site. Isolates from site B, plant 1, (B-1(1)R and (B-1(1)S), showed a similar level of biomass production to each other at both -0.1 MPa and -4.0 MPa. Isolates from site C, plants 1, 2, 3 and 4, all showed a similar level of biomass production at -0.1 MPa. All these isolates showed a similar increase in biomass production at -4.0 MPa with the exception of C-3(2)R, where biomass production was a lot lower than its counterparts from the same plant. At site T, there was more variation between the isolates from the same plant with respect to sites C and B. Isolates from site T, plants 1 and 4, showed a different level of biomass production at both -0.1 MPa and -4.0 MPa. One isolate, T-1(4)S, from plant 1, showed a decrease in biomass production at -4.0 MPa with respect to -0.1 MPa, while its counterparts from the same plant showed an increase in biomass production at -4.0 MPa with respect to -0.1 MPa.

Differences in sporulation became apparent between isolates from the same plant. Isolates at site B, plant 1 (B-1(1)R and B-1(1)S) both showed a decrease in sporulation at

Table 3.8: Dry weight (mg) of total biomass of *I. bolleyi* root and stem base isolates from 3 field sites (T,C and B), grown in standard liquid medium, adjusted osmotically with KCl, (Mean for 3 replicates at 4 days incubation at 25°C).

Isolate number and isolate origin *	Osmotic potential of liquid medium (- MPa)		Row mean	SED	5% LSD
	-0.1 MPa	-4.0 MPa			
T-1(1)S	6.63	10.67	**8.65**	**1.85**	**3.63**
T-1(2)S	9.97	11.67	**10.82**		
T-1(3)S	10.97	14.33	**12.65**		
T-1(4)S	14.97	9.67	**12.32**		
T-3(1)S	22.30	28.67	**25.48**		
T-3(2)S	11.63	17.00	**14.32**		
T-4(1)S	8.97	17.00	**12.98**		
T-4(2)S	11.30	9.67	**10.48**		
T-4(3)S	6.87	20.20	**13.43**		
T-2R	8.30	28.33	**18.32**		
T-3(1)R	18.97	13.00	**10.98**		
T-3(2)R	8.63	15.00	**11.82**		
C-2S	9.63	17.67	**13.65**		
C-3(1)S	11.96	20.00	**15.98**		
C-3(2)S	9.87	22.67	**16.27**		
C-4S	6.63	18.00	**12.32**		
C-1(1)R	7.63	18.33	**12.98**		
C-1(2)R	9.53	17.33	**13.43**		
C-2(1)R	9.30	18.67	**13.98**		
C-2(2)R	11.63	15.67	**13.65**		
C-3(1)R	14.43	12.33	**13.38**		
C-3(2)R	8.97	10.33	**9.65**		
C-4(1)R	20.00	12.00	**16.00**		
C-4(2)R	11.30	11.33	**11.32**		
B-1(1)S	10.53	11.33	**10.93**		
B-1(1)R	11.30	10.67	**10.98**		
Column mean	**10.86**	**15.82**			
SED	**0.52**				
5% LSD	**1.02**				

Two-way analysis of variance ($P = 0.05$)

* **e.g. T-1(1)S** = Isolate from site T, plant 1, stem base; number in brackets denotes more than one replicate from the same source

Table 3.9: Conidia production (x 10^6 spores ml^{-1}) by *I. bolleyi* root and stem base isolates from 3 field sites (T,C and B), grown in standard liquid medium, adjusted osmotically with KCl,(mean for 3 replicates for 4 days incubation at 25°C).

Isolate number and isolate origin *	Osmotic potential of liquid medium (- MPa)		Row mean	SED	5% LSD
	-0.1 MPa	-4.0 MPa			
T-1(1)S	7.90	0.63	**4.26**	**0.88**	**1.74**
T-1(2)S	6.13	1.16	**3.65**		
T-1(3)S	2.82	2.84	**2.83**		
T-1(4)S	2.43	0.94	**1.68**		
T-3(1)S	3.03	5.29	**4.16**		
T-3(2)S	2.53	3.84	**3.19**		
T-4(1)S	5.10	4.10	**4.60**		
T-4(2)S	4.06	1.04	**2.55**		
T-4(3)S	4.83	5.34	**5.09**		
T-2R	2.80	3.71	**3.27**		
T-3(1)R	3.93	0.74	**2.34**		
T-3(2)R	4.53	1.18	**2.86**		
C-2S	2.00	0.90	**1.45**		
C-3(1)S	2.53	3.82	**3.18**		
C-3(2)S	1.87	3.74	**2.81**		
C-4S	6.13	6.24	**6.19**		
C-1(1)R	12.23	5.77	**9.00**		
C-1(2)R	5.30	1.51	**6.81**		
C-2(1)R	6.80	2.33	**9.13**		
C-2(2)R	6.20	4.90	**5.50**		
C-3(1)R	3.35	0.75	**2.05**		
C-3(2)R	3.92	0.31	**2.12**		
C-4(1)R	5.17	5.80	**5.48**		
C-4(2)R	3.22	3.54	**3.38**		
B-1(1)S	6.08	3.94	**5.01**		
B-1(1)R	4.25	1.74	**2.99**		
Column mean	**4.58**	**2.93**			
SED	**0.24**				
5% LSD	**0.48**				

Two-way analysis of variance ($P = 0.05$)

*** e.g. T-1(1)S** = Isolate from site T, plant 1, stem base; number in brackets denotes more than one replicate from the same source

Table 3.10: Number of spores showing microcycle conidiation (x 10^4 spores ml^{-1}), from *I. bolleyi* root and stem base isolates from 3 field sites(T, C and B), grown in standard liquid medium, adjusted osmotically with KCl (means for 3 replicates for 4 days incubation at 25°C).

Isolate number and origin *	Osmotic potential of liquid medium (-MPa)	
	-0.1 MPa	-4.0 MPa***
T-1(1)S	0.0	2.00
T-1(2)S	0.0	0.0
T-1(3)S	0.0	3.0
T-1(4)S	0.0	0.3
T-3(1)S	0.0	31.0
T-3(2)S	0.0	4.0
T-4(1)S	0.0	21.0
T-4(2)S	0.0	3.0
T-4(3)S	0.0	17.0
T-2R	0.03	9.0
T-3(1)R	0.0	3.0
T-3(2)R	0.0	3.0
C-2S	0.0	8.0
C-3(1)S	0.0	25.0
C-3(2)S	0.0	25.0
C-4S	0.0	22.0
C-1(1)R	0.0	52.0
C-1(2)R	0.0	6.0
C-2(1)R	0.0	14.0
C-2(2)R	0.03	14.0
C-3(1)R	0.03	0.3
C-3(2)R	0.0	0.7
C-4(1)R	0.0	2.0
C-4(2)R	0.03	5.0
B-1(1)S	0.0	4.0
B-1(1)R	0.0	0.0
SED	-	9.29
5% LSD	-	19.13

*** One-way analysis of variance ($P = 0.05$)

* e.g. **T-1(1)S** = Isolate from site T, plant 1, stem base; Number in brackets denotes more than one isolate from the same source

-4.0 MPa with respect to -0.1 MPa. Isolates from site C, Plants 1, 2, 3 and 4 showed different plants 1 and 2 showed a decrease in sporulation at -4.0 MPa with respect to -0.1 MPa, however, all isolates from Plant 4 showed an increase in sporulation at -4.0 MPa with respect to -0.1 MPa. From plant 3, out of 4 isolates, 2 isolates (from the stem base, isolates C-3(1)S and C-3(2)S), showed an increase in sporulation at -4.0 MPa, whilst the other 2 isolates (from the roots, isolates C-3(1)R and C-3(2)R) showed a decrease in sporulation at -4.0 MPa with respect to -0.1 MPa. At site T, there was much variation between isolates for sporulation at both -0.1 MPa and -4.0 MPa. Isolates from site T, plants 1, 3 and 4 all showed a lower sporulation level at -4.0 MPa with respect to -0.1 MPa with the exception of isolate T-4(3)S where sporulation increased at -4.0 MPa. Both isolates from plant 5 and the only isolate from plant 2 also showed an increase in sporulation at -4.0 MPa with respect to -0.1 MPa.

Generally lower levels of spore production at -4.0 MPa were associated with a low level of microcylic conidiation, regardless of site or isolate origin. From sporulation at -0.1 MPa, microcycle conidiation was either negligible (less than 0.1×10^4 spores ml^{-1}) or absent for all isolates; These data are shown in Table 3.10, although data for -0.1 MPa was too low to allow statistical analysis.

A t-test comparing the mean of pooled root isolate data and the mean of pooled stem base isolate data at each site, showed that there was no significant difference between root and stem base isolates at site T and B for biomass production and for site T and C and for sporulation, at both -0.1 MPa and -4.0 MPa. However, at site C, pooled stem base isolates produced significantly higher levels of biomass production at -4.0 MPa in comparison to pooled root isolates ($P = 0.05$). At site B, the t-test showed that the stem base isolates produced significantly higher levels of spores than the root isolate at -4.0 MPa ($P = 0.05$). There was no significant difference in microcyclic conidiation between root and stem base isolates at site T and C at either -0.1 MPa and -4.0 MPa, however, the stem base isolate from

site B showed a significantly higher level of microcycle conidiation at -4.0 MPa in comparison to the root isolate ($P = 0.05$).

3.4 Discussion

Within the soil, water potential is the sum of many components including matric, osmotic, pressure, gravitational and overburden potentials (Papendick & Campbell, 1981). In both soils and plant systems, matric and osmotic components contribute more significantly to the water potential than the others and exert a greater effect on water flow and availability for physiological processes (Papendick & Campbell, 1981).

Osmotic potential, due to the presence of solutes in the soil water, is likely to become significant in saline soils and in crop residues where soils have been amended with fertiliser or organic waste (Papendick & Campbell, 1981). Also, osmotic potential is likely to be relevant to growth within plant tissues; wheat tissues under dryland conditions in the Pacific north-west of the USA are commonly -2.5 to -3.5 MPa between tillering and heading stages (Papendick & Cook, 1974). Matric potential, which includes adsorption and capillary effects of the solid phase is the largest component in most unsaturated soils. Matric potential would be more relevant to growth in soil or on the root surface (Cook & Papendick, 1972), and also in dry crop residues (Papendick & Campbell, 1981).

Microbial tolerance to water stress varies widely and in general shows little relation to classical taxonomy (Harris, 1980). Many workers have tried to examine a range of micro-organisms for their water-stress tolerance *in vitro* (Sung & Cook, 1981; Howie *et al.*, 1987; McQuilken *et al.*, 1992), but there are often problems interpreting the effects of water potential *in vitro,* or to relate these to effects for growth in natural systems. In this study, two approaches were used to try and overcome these problems. Firstly the behaviour

of *I. bolleyi* was compared with that of two cereal pathogens that have been intensively studied for water-stress tolerance. In this respect, the results for *Fusarium culmorum* and *Gaeumannomyces graminis* var. *tritici* agree with many previous reports (e.g. Cook *et al.*, 1972); the former is tolerant of water-stress and the latter intolerant. Relative to the controls, we also observed an increase in growth of *F. culmorum*, but not of *G. graminis* var. *tritici* at low levels of water stress, as others have noted (Cook *et al.*, 1972; Griffin, 1972). Secondly, both KCl and Polyethylene glycol 8000 (PEG) was used to adjust the water potential. The salt acts by generating osmotic potential, although at high concentrations it might also have a directly inhibiting effect (Papendick & Mulla, 1986). In contrast PEG 8000 binds to water and thus generates matric potential; at water potentials below -1 MPa less than 10% is contributed by osmotic potential, the rest being matric potential (Steuter *et al.*, 1981). The cell walls and membranes of fungi are virtually impermeable to PEG 8000 (Harris, 1981), which permits physiological studies on the effects of matric potential on fungal growth without the complicating indirect physiological effects that might be encountered with the use of salts.

From the results in this study, it was noted that KCl often had a stimulatory effect on growth at osmotic potentials above the control, this being apparent in linear growth, biomass production and sporulation, although this effect was not observed for germination, and rarely observed for matric potential.

The use of salts and sugars for the study of growth response to osmotic potential can have complicated physiological effects and this must be considered when examining the results obtained. Specifically the choice of salt can result in growth responses due to the presence of ions. Generally potassium ions (K^+) are readily taken up by the cell compared to sodium ions (Na^+) and may have a stimulatory effect on cellular growth. Potassium is believed to possess specific functions as co-factors for certain enzymes, such as potassium-activated aldehyde dehydrogenase (Bostian & Betts, 1978). Uptake of the sodium ion does

not appear to be an essential requirement as even at very low concentrations *Neurospora crassa* showed maximal growth; in contrast *Saccharomyces cerevisiae* responded strongly to changes of external levels of the potassium ion in the range of 0.2 to 0.4 mM. The preference for the potassium ion to the sodium ion is not fully known, but it is believed that the heat absorbed by the addition of KCl to water may force the water molecules into a more active state, also the hydration interactions of potassium ions are significantly weaker than those of the sodium ions (Blomberg & Adler, 1993).

Differences in the growth on salts and sugars have also been shown, indicating that the sole use of either one or the other may not be an accurate estimation of a fungal tolerance to water potential. The highly tolerant yeast *Zygosaccharomyces rouxii*, shows high osmotolerance by growing on high concentrations of sugars, but growth becomes impaired on NaCl at a concentration of 150 mM (Rodriguéz-Navarro, 1971). The xerophillic filamentous fungi *Xeromyces bisporus* and *Chrysoporium fastidium*, normally found in high sugar environments, are intolerant of NaCl and KCl irrespective of pH (Pitt & Hocking, 1977; Luard & Griffin, 1981).

Although the majority of fungi do not have the physiological adaptation of xerophillic fungi, most have compensatory mechanisms for osmotic adjustment within their hyphae in order to maintain turgor pressure in saline environments (Blomberg & Adler, 1993). It is clear from the stimulation in hyphal growth of *I. bolleyi*, with an increased osmotic potential, that a compensatory mechanism exists. Although there may be a stimulatory effect from the potassium ion, there is also a water potential effect which is evident by a gradual decrease in growth with decreasing potential. Scott (1957) stated that the consequences of solute stress on microbial activity were largely related to the reduction in water availability whereas solute-specific effects were less important. Most fungi are able to compensate for external osmotic potentials by the accumulation of polyhydroxy alcohols (polyols) which lower the water potential within the hyphae and also act as compatible

solutes (Brown & Simpson, 1972; Pfyffer *et al.*, 1990). The nature of polyols within *I. bolleyi* have not been identified, however, from intensive study, the main polyols have been identified within in a range of yeast and in the hyphae of filamentous fungi. The predominant polyol found in both yeast and filamentous fungi appears to be the low molecular weight glycerol (mol. wt. 92.1) (Blomberg & Adler, 1993). Glycerol appears to be the primary osmotic stabiliser in growing cells and as a culture ages, other polyols including, mannitol (mol. wt. 182.2) erythritol (mol. wt. 122.1) and arabitol (mol. wt. 152.1) transiently increase in the hyphae, some of these acting as compatible solutes and mannitol often acting as a carbon source for respiration (Horikoshi *et al.*, 1965). These polyols generally do not provide the same degree of osmotic compensation as glycerol (Adler & Gustafsson, 1980; Hocking, 1993). Calculations of polyol concentration has shown that the intracellular osmotic pressure normally adds up to or exceeds that of the external osmotic pressure, thereby maintaining a turgid state (Jennings & Burke, 1990; Larsson *et al.*, 1990). At very low water potentials, the higher molecular weight polyols have been shown to inhibit yeast NADP-specific isocitrate dehydrogenase (Brown, 1976); this might explain why these polyols tend to decrease, as water potential decreases, in favour of the lower molecular weight polyols arabitol and glycerol. Glycerol is thought to act as a growth-associated compatible solute in fungi, and it may also have a functional role in maintaining enzyme activity by stabilising the conformation of protein (Brown, 1990). The result of this may be to enable increased metabolic activity during periods of water-stress. The other compatible solutes that appear to be constitutively present in the cells may serve to buffer cells growing at high water potentials (low salinity) against dehydration and sudden solute stress (Blomberg & Adler, 1993). Along with polyols and glycerol, trehalose is commonly found to accumulate in hyphae (Hallsworth & Magan, 1994abc). Trehalose acts as a compatible solute by actually replacing water in the membranes at reduced water potentials, its function then, is to serve as a protectant during dehydration (Crowe *et al*, 1984). Trehalose is also known to stabilise enzyme structure during desiccation (Carpenter & Crowe, 1988ab).

In this study, hyphal growth and sporulation were more drastically reduced by decreasing matric potential compared with osmotic potential. Hyphae do not have the same mechanisms for dealing with matric potential as they do with osmotic. A slight stimulatory effect was observed for matric potential with *F. culmorum* and with *I. bolleyi* strain AB1, however, this effect was not observed in sporulation for this strain, or indeed for the other two *I. bolleyi* strains. Experiments by Cook *et al.* (1972), found that *Fusarium culmorum* was stimulated in the first reductions of osmotic potential, but this was never found with matric potential. In view of these points, the results obtained at -1.0 MPa, may be an artefact. In agreement with previous literature (Cook *et al.*, 1972) however, *G. graminis* behaved similarly to matric potentials as to osmotic potentials. In general the decrease in growth and sporulation seen by *I. bolleyi* and the other comparative fungal species will obviously be related to a decrease in water availability, but there also may be an affect due to a corresponding decrease in nutrient availability. Nutrient availability was the limiting factor in soil of -0.1 to -1.5 MPa for the germination of oospores of *Pythium aphanidermatum* (Stanghellini & Burr, 1973). Germination of *Fusarium solani* f. sp. *pisi* chlamydospores occurred in soil of 10% water content, but not at 7% water content even though the water potential *per se* was not limiting, lack of nutrient diffusion was less in this soil than the more moist soil (Cook & Flentje, 1967).

As stated previously, no stimulatory effect was observed for germination whether in an osmotic solution, or a matric solution. In both cases an overall decrease was observed with increasing potential, however, matric potential had less of an effect on reducing germination than osmotic potential, indeed strain AB1 continued to have 100% germination down to -5 MPa.

With regard to matric potential, the ability of spores to germinate down to low potentials, as was specifically seen for *I. bolleyi* strain AB1 and some what for strain J10, may be related the small size of the spores. Large sclerotia, like plant seeds or roots, may

need large amounts of water to hydrate the propagule, however, small propagules require very small amounts of water which can even be satisfied by vapour diffusion (Cook & Duniway, 1980). The very small *I. bolleyi* spores are likely to require tiny amounts of available water to germinate, however, very low water potential is likely to restrict germination of *I. bolleyi* to some extent as was seen with strains J10 and T560. As stated earlier a nutrient effect might also play a role in decreased germination if internal food reserves are low.

Osmotic potential is likely to have a greater effect than matric potential on germination, as the internal osmotic potential must match or exceed that of the external environment. Cook and Dunniway (1980) suggested, particularly in the case of long-term survival spores, that spores may be self-sufficient in their internal osmotica if they have a good supply of concentrated food reserves, including carbohydrates, lipids and proteins. The spores may be able to maintain enough turgor for germination without the need for external osmotica unlike active hyphal growth, which relies to some extent on external solutes for its osmoregulation.

In the case of conidia large internal food reserves are unlikely to be present and so like hyphae they may need to rely on external solutes for their osmoregulation. Hallsworth and Magan (1994abc) found that polyols, including glycerol and trehalose, accumulate within the conidia of entomopathogenic fungi *Beauveria bassiana, Metarhizium anisopliae* and *Paecilomyces farinosusi*. Their findings showed that the type of polyol or the presence of trehalose accumulated within the conidia was dependant on the external carbohydrate source, indicating that the conidia may take up compounds from the external environment and use these as compatible solutes. In the case of an external source of trehalose, trehalose was seen to be the main compatible solute accumulating in two of the fungi. In the case of the fungus, *M. anisopliae*, (where mannitol and arabitol accumulated), and in the case of an external source of glucose or starch (where mannitol was the main

accumulating compatible solute), the external compound may have be degraded before or after uptake into the closest compatible solute to the external environment.

As with compatible solutes in hyphae, the main purpose of the compatible solutes within conidia, particularly trehalose, is to enhance desiccation tolerance and germination at low water potentials (van Laere *et al.*, 1987). Harman *et al.* (1991) found that the addition of PEG 8000 to a medium could increase the trehalose concentration in fungal mycelium and spores of the biological biocontrol agent *Trichoderma harzanium*, thus enhancing viability and desiccation tolerance.

The most noticeable feature of the *in vitro* results was that strains of *I. bolleyi* differed markedly from one another in tolerance of stress imposed by both osmotic and matric potential. The range of variation in this species was much greater than ever before reported for a single fungus. Indeed, one strain approached the water-stress tolerance of *F. culmorum* while another was almost as intolerant as *G. graminis* var. *tritici*. Their origins could not have been used to predict their stress tolerance because the most stress-tolerant strain (AB1) originated from diseased flax, where prolonged water-logging of the soil is thought to have predisposed the crop to attack by weak parasites (Black & Brown, 1986). It is possible that pro-longed laboratory culturing may have resulted in many physiological changes since its isolation from the field. Stetter & Leroul (1979) reported that strains of *I. bolleyi* vary in the effects on barley seedlings in laboratory assays. Strains from continuous cropping were mildly deleterious whereas strains from first barley crops had no deleterious effect or were mildly growth promoting. The deleterious effects of strains from continuous cropping was lost after laboratory culturing.

It is clear that *I. bolleyi* is a physiologically variable fungus, indeed the variability of *I. bolleyi* has also been observed between root and stem base isolates collected from three field sites. The field isolates tested for their water-stress tolerance were derived from single

spore cultures and were tested without prolonged laboratory culturing, thus are likely to be accurate representations of variability within the field. Isolates were seen to vary a great deal with regards to both biomass production and sporulation at -0.1 MPa and at -4.0 MPa. The majority of isolates produced more biomass at -4 MPa, than at -0.1 MPa and the reverse was true for sporulation, with this decreasing with decreasing osmotic potential. Despite the general trends, which indicate that *I. bolleyi* is a fairly osmotolerant fungus, it was the remarkable differences between isolates in the levels of biomass production and also sporulation that suggests a large degree of physiological variability. Some isolates actually went against the trend, and biomass production was largely reduced as a result of decreasing osmotic potential, likewise, some isolates produced more spores at the lower potential than at the control potential. With few exceptions high sporulation was related to a high level of microcyclic conidiation (the production of spores directly from spores), this factor also being highly variable between isolates. Microcycle conidiation was only really observed for growth at -4 MPa which suggests that it may act as way of increasing biomass when conditions are unsuitable for hyphal growth. Such dry conditions in the soil may limit the diffusion, of nutrients and hence its availability within the soil. Lascaris and Deacon (1994) found that the microcycle conidiation in *I. bolleyi* was influenced by nutrient levels, in particular increased microcycle conidiation was observed for low glucose levels. For the fungi *Phialophora asteris* (Burge & Isaac, 1977) and *Septoria tritici* (Jones & Lee, 1974) microcycle conidiation has been suggested to be an ecological adaption for rapid spread in liquid films or circulating fluids. The fact that the level of microcycle conidiation varied so much between isolates might suggest that those isolates with high microcycle conidiation may be showing a physiological adaptation to environmental stress. Sung & Cook (1981) reported isolate variation in the species *Fusarium* from two different geographical areas. The isolates responded differently to water potential with regard to the reproductive structures they produced. The differences observed at each location suggested the existence of ecotypes adapted to the conditions under which they occur.

Such strain variability in *I. bolleyi* has raised the question of whether strains might be selected for specific, stable, desirable attributes in biocontrol. One of these attributes could be water-stress tolerance that matches a specific pathogen. Ramakrishna *et al.*, (1993) showed that interactions of fungal species on stored cereal food could be predicted from the stress tolerance of the species *in vitro*. It has been argued that one major reason for the inconsistent performance of biocontrol inocula in field sites is that single colonial strains of biocontrol agents are expected to compete for resources with indigenous organisms in conditions that vary between sites and seasons (Deacon, 1994). This has led to the thought of using mixed strain inoculum to try and overcome this problem.

With regard to developing mixed strain inoculum, another remarkable finding from *in vitro* testing of field isolates was that isolates from the same plant differed in their response to osmotic stress. This finding showed that it is possible for more than one strain, with different levels of stress tolerance, to exist on the same plant, but whether stem base isolates are specifically more tolerant to water-stress than root isolates requires further analysis. Initial results showed that pooled data of stem base isolates against root isolates were not significantly different in their osmotolerance. However, the general variability between strains does indicate the possibility that strains could be selected for different degrees of water-stress tolerance for mixed strain inoculum.

Cook & Baker (1983) discussed how factors such as water potential could influence competition between non-pathogens and pathogens in the early stages on infection of plants. In view of this, *in vivo* experiments were done to test the ability of single and mixed strain inoculums of *I. bolleyi* to colonise straw soil at different water potentials. It was thought that some relation may be found with respect to the *in vitro* water potential experiments. The major observations drawn from this experiment were that *I. bolleyi* could colonise straw over all potentials, and the inoculum could remain active over a period of 6 weeks even at low water potentials. Straws colonised by the mixed strain inoculum appeared

to be as efficient as a single strain inoculum, and also both strains in the mixed inoculum could be reisolated from the straw showing that both strains were actively colonising the straw to a similar degree.

The *I bolleyi* strains (T560 and AB1) used in the *in vivo* experiment did not differ in their response to water stress to the same extent as was seen in the *in vitro* experiments. As stated earlier, there are often problems associating *in vitro* experimentation with *in vivo* work. Brownell & Schneider (1985), found that their measurements of water potential effects on *Fusarium oxysporum* in synthetic media adjusted osmotically with KCl and matrically with PEG 8000, did not accurately represent similar water potential experiments obtained in soil. It is likely that some of these discrepancies are related to the more complex nature of the soil. Griffin (1972) stated that the activity of a fungus in soil at a given matric suction depends not only upon the direct effect response of the fungus to the suction, but also on the ability of the fungus to compete with the rest of the microflora in colonisation. A number of fungal species other than *I. bolleyi* were isolated from the straws in the *in vivo* experiment suggesting a large amount of competition for straw colonisation. Patterns of colonisation emerged which were clearly related to water potential. Characteristically, *Fusarium* sp. was predominantly isolated at the lower potentials. Other fungi, including *Cephalosporium* sp, *Mucor* sp. and *Pythium oligandrum* were more commonly isolated at the higher potentials. In agreement with a similar study by Magan (1992), *Trichoderma* sp. was recovered more frequently in the wetter soil, however, in contrast to Magan's study, *Gliocladium* sp. was more commonly isolated in the drier soils rather than the wetter soils. For those fungal species including, *I. bolleyi, Fusarium* sp. and *Gliocladium* sp., which were commonly isolated at the lower potentials, the marked reduction in their recovery from straws buried in the wetter soils was likely to be as a direct result of increased competition. It is likely that most fungi would have been more active at the highest water potentials. Interestingly MacNish (1973) found that *Gaeumannomyces graminis* var. *tritici* could survive for 46 weeks as hyphae in straw in soil at -9.5 MPa, but

quickly lost viability, or was displaced in the straw by soil saprophytes when soil water potentials were near -0.1 MPa.

The recovery of the majority of fungi was found to increase over a period of four weeks suggesting that most fungi were actively competing for colonisation of the straw, however, the overall incidence of each fungal species did not differ. Parkinson & Pearson. (1965) found in a study of root colonisation, that once a stable root-surface population is established, the relative incidence of the different fungi does not change, although the amount of mycelium belonging to each species may alter with time.

Stenton (1958) stated that a young root presents to the soil in which it grows, 'a virgin ecological niche' for micro-organisms, however, Krauss & Deacon (1994a) showed that root turnover was also continuous and rapid for groundnut plants (*Arachis hypogaea*), and suggested that this was probably a normal feature of plants during their functional life. This rapid turnover could have important implications. The release of organic nutrients into the soil may support the rhizosphere microflora and even enable the build up of pathogens in the root zone. If pathogens can exploit the naturally senescing root tissues in advance of saprophytes, it may allow the build-up of pathogen inoculum from initially low levels (Kirk & Deacon, 1987b). Lascaris & Deacon (1991) have emphasised that successful biological control may rely on the early establishment of a seed applied inoculum biocontrol agent on the root system and that seed-applied inocula may be required to achieve this consistently. Lascaris & Deacon (1991) showed that wheat roots could be colonised by spores of *I. bolleyi* produced on the seed and which were washed down the rhizosphere in percolating water. This percolation of spores was likely to be responsible for the spread of the fungus down the root system. These results were obtained from sterile seeds in unsterile perlite, thus the ability of *I. bolleyi* proliferate on unsterile seed in competition with the resident seed and soil microflora was unknown.

Germinating seeds are known to exude a range of volatile organic substances into the surrounding environment (Catská, 1979). These compounds are likely to support fungal growth and have been shown to either stimulate or inhibit germination of fungal spores (Catská, 1979). Seed exudates, and also competition for seed colonisation, could have important influence on the success of *I. bolleyi* to establish itself on the seed. The results of this study showed that *I. bolleyi* could successfully compete with the resident microflora and increase its biomass on the seed in the form of spores. This was apparent by an initial drop in spore number, indicating germination of the original conidial inoculum, followed by an exponential increase in spore number. Spore number was higher on sterile seeds showing that competition was initially greater on unsterile seeds. For the sterile treatment, spore numbers was also higher on alginate coated seeds, the gel probably acting as a barrier to reduce competition from the soil microflora. *Idriella bolleyi* spore numbers dropped as the seed aged, probably as a result of conversion to chlamydospores in response to exhaustion of seed nutrients and increased competition on the decaying seed.

The ability of *I. bolleyi* to increase its biomass on the seed in a competitive situation improves its potential as a seed-applied biocontrol agent, although its colonisation of the root system in this situation still needs further assessment. The inter-strain variation observed in *I. bolleyi* could be used to produce mixed strain inocula, thus enhancing its performance under different conditions and perhaps improving colonisation of both the roots and stem base.

CHAPTER 4

PRODUCTION OF NITRATE NON-UTILISING MUTANTS AND THEIR COMPLEMENTATION IN *I. BOLLEYI*

4.1 Introduction

Idriella bolleyi has no known sexual stage in its life cycle, so for genetic exchange to occur it will be limited to the parasexual cycle (Sidhu, 1983) or some other form of genetic transfer (Kistler & Miao, 1992).

Asexual genetic exchange has been studied intensively in the genus *Fusarium* (Puhalla, 1985; Correll *et al.*, 1987), where no sexual stage has been found in some species (Leslie, 1990). Asexual genetic exchange can occur when two isolates form a heterokaryon, this formation being controlled by a complex system of loci involved in self and non-self recognition (Leslie, 1993). The formation of a heterokaryon between two isolates shows that they are vegetatively compatible, this results from complementation at the alleles at each compatibility locus (Pittenger, 1964). In *Fusarium* species, vegetative compatibility has been used to determine the genetic relatedness of isolates which belong to the same race or *forma specialis* and has since been used to try to subdivide the groups (vegetative compatibility groups; VCGs) within field populations (Leslie, 1990).

Identification of VCGs by screening for vegetative compatibility has relied on the laborious production of auxotrophic mutants by either UV mutation or the use of chemical mutagens (Fincham *et al.*, 1979). More recently a simple technique to produce a high frequency of nitrate non-utilising (*nit*) mutants has been successfully applied to a range of

fungal species including *Fusarium* spp. (Correll & Leslie, 1987; Klittch & Leslie, 1989), *Aspergillus nidulans* (Cove, 1976a,b) and *Neurospora crassa* (Tomsett & Garrett, 1980). *Nit* mutants are produced by growing cultures on medium supplemented with potassium chlorate. Spontaneous colony or sector growth on this toxic analogue of nitrate generally stems from the loss of ability to use nitrate as a source of nitrogen (Cove, 1976a,b). Mutation in nitrogen regulation can occur at several different loci, resulting in a range of phenotypes suitable for use in heterokaryon studies (Cove 1976a,b; Tomsett & Garrett, 1980; Klittch & Leslie, 1988). *Nit* mutant production has not only made simpler the process of producing a large number of mutants for heterokaryon studies, but also has been valuable in giving information regarding both the degree of genetic stability within each fungal species and aspects of their nitrogen metabolism (Marzluf, 1981).

4.2 Methods

Nitrate non-utilising (*nit*) mutants which could be grouped into different classes, were produced from different strains of *I. bolleyi* for use in vegetative compatibility tests.

4.2.1 Isolation of chlorate-resistant mutants

Discs (5 mm diameter) of *I. bolleyi* were removed from the colony margin of 14 day old PDA cultures using a cork borer, placed into the centre of Minimal Medium plates and incubated in the dark at 25°C. After 4 days, 2 mm x 2 mm blocks were removed from the colony margin and transferred onto Minimal Medium containing potassium chlorate ($KClO_3$) at 15g l^{-1} and incubated in the dark at 25°C for up to 14 days. Two hundred and sixty mycelial blocks were plated for each of five *I. bolleyi* strains - AB1, T560, T560-R2, J10 and J10-R2. Chlorate resistant sectors developing during the incubation period were

removed and transferred to Nitrate Medium (Section 2.1.2) and Asparagine Medium (Section 2.1.2) in order to determine their ability to utilise nitrate. Three replicate plates were used for each chlorate resistant sector for both the Nitrate Medium and the Asparagine Medium. Colonies resistant to chlorate and unable to utilise nitrate as sole nitrogen source were considered to be *nit* mutants.

4.2.2 Characterisation of nit *mutant phenotype*

Nitrate non-utilising mutants were examined for their mutational phenotype by growing them on media containing different nitrogen sources as follows: Nitrate Medium (basal medium as in Section 2.1.2, containing sodium nitrate, 2g l^{-1}); Nitrite Medium (basal medium containing sodium nitrite, 0.5g l^{-1}); Hypoxanthine Medium (basal medium containing hypoxanthine, 0.2g l^{-1}); Ammonium Medium (basal medium containing ammonium tartrate, 1.0g l^{-1}); Uric Acid Medium (basal medium containing uric acid, 0.2g l^{-1}).

Agar discs (5 mm) were removed from the edge of 7 day old Asparagine Medium and Nitrate Medium cultures, and placed onto the centre of each of the five media. The plates were incubated in the dark at 25°C for 7 days and growth was compared with respect to the wild-type.

4.2.3 Generation of nit *mutants on different nitrogen sources*

The generation of different types of *nit* mutant was examined by the method described above (Section 4.2.1), but by supplementing potassium chlorate medium with alternative nitrogen sources: sodium nitrite (0.5g l^{-1}), hypoxanthine (0.2g l^{-1}) or uric acid

(0.2 g l^{-1}). Two hundred and sixty mycelial blocks (2 mm x 2 mm) were plated on each medium for each of five *I. bolleyi* strains - AB1, T560, T560-R2, J10 and J10-R2. The plates were incubated at 25°C in the dark for up to 14 days. Chlorate resistant sectors developing during the incubation period were removed and transferred to Nitrate Medium and then their mutational phenotypes were determined as described in Section 4.2.2.

4.2.4 Complementation tests

Nit mutants were tested for their ability to complement other *nit* mutants, indicated by dense prototrophic growth at the colony junction of paired mutants.

Mycelial discs (5 mm) were removed from the edge of a 7 day culture of an *I. bolleyi* *nit* mutant on Nitrate Medium. The mycelial discs were placed in the centre of Minimal Medium plates. Mycelial discs of four other *nit* mutants from the same strain were placed around the central disc at a distance of 2 cm. All possible combinations of mutants were used for each strain, with each mutant being placed either in the centre of a plate surrounded by other mutants in one test, and also around a centred mutant in a second test. The plates were incubated in the dark at 25°C for up to 14 days and checked regularly for complementation which was indicated by dense prototrophic growth at the colony junctions. The growth was scored for complementation on the basis of the thickness of growth as follows: - = no complementation; + = slight complementation, seen as slightly denser growth at colony junction; ++ = moderate complementation; +++ strong complementation; ++++ = very strong complementation.

Nitrate non-utilising mutants of the different *I. bolleyi* strains were tested for their ability to complement one another using the same method as described above. All

combinations of mutants were tested between each strain and complementation was scored as described above.

4.2.5 Video microscopy

Observation chambers using Water Agar-coated coverslips were prepared as described in Section 2.1.10. The coverslips were inoculated with agar discs (5 mm diameter) of two *nit* mutants at a distance of 2 cm apart. The coverslips were incubated on the surface of Water Agar plates at 25°C for 5 to 7 days until the two growing colonies were seen to merge into one another. Paired mutants were examined from strains AB1, T560, T560-R2 and J10-R2 as follows: AB1-3 and AB1-7 (*nitM* x *nitM*); AB1-3 and AB1-6 (*nitM* x *nit3*); AB1-3 and AB1-13 (*nitM* x *nit1*); AB1-1 and AB1-14 (*nit3* x *nit 1*); AB1-16 x AB1-16 (*nit3* x *nit3*); AB1-14 x AB1-14 (*nit1* x *nit1*); T560-1 and T560-2 (*nitM* x *nit3*); T560-R2-1 and T560-R2-4 (*nit1* x *nit3*); J10-R2-1 and J10-R2-4 (*nit3* x *nit1*). The coverslips were removed from the agar plates and inverted onto a chamber consisting of a microscope slide (76 x 51 mm) to which a rectangle of glass capillary tubes, 2 mm high, had been glued. To prevent drying of the agar film on the coverslip, the capillary tubes were coated in Vaseline to create a seal. The back of the coverslip was wiped clean prior to observation. Observations using bright field microscopy, were done on a Leitz Orthoplan microscope with x70 oil-immersion objective as described in Section 2.2.10

Mutants of AB1 which showed complementation when paired on Minimal Medium were examined for the number of nuclei in anastomosing cells. The method was as above, but using strain AB1 mutant pairs as follows: AB1-3 and AB1-7 (*nitM* x *nitM*); AB1-3 and AB1-6 (*nitM* x *nit3*); and AB1-3 x AB1-14 (*nitM* x *nit1*). After incubation for 5 to 7 days, the growing colonies on the coverslips were stained with the nuclear binding stain, 4'6'-diamididino-2-phenyl indole dihydrochloride (DAPI; Sigma) at 10μg ml^{-1} by dropping a few

drops onto the coverslip. The slide was tipped to disperse the stain and left for 30 sec. The stain was gently rinsed off with distilled water while the coverslip was held at an angle to allow drainage. The coverslip was placed on an observation chamber as described above. Observations were made by fluorescence microscopy, with a x70 oil-immersion objective on a Leitz Orthoplan microscope equipped with a Ploemopak 2.1 fluorescence vertical illuminator giving UV light from a mercury vapour lamp. Filter block A (BP 330-385 nm exciting filter) was used, giving excitation in the ultra-violet range. A Panasonic VW CL-350 colour video camera was attached to the microscope.

4.3 Results

Five strains of *Idriella bolleyi* were used for the production of nitrate non-utilising (*nit*) mutants, by placing mycelial blocks onto media supplemented with 1.5% $KClO_3$. Colonies that grew on this medium were tested for their ability to use nitrate as sole source of nitrogen. The mutational phenotype of each nitrate non-utilising colony was determined. These *nit* mutants were subsequently paired on Minimal Medium plates, with nitrate as the sole nitrogen source, to test for complementation and thus presumptive heterokaryon formation (Section 4.2.4).

4.3.1 The recovery of nit *mutants*

All strains of *I. bolleyi* produced chlorate-resistant colonies from the mycelial blocks placed on chlorate media containing sodium nitrate, sodium nitrite, hypoxanthine and uric acid.

Table 4.1: Numbers of colonies that grew on chlorate-containing media with different nitrogen sources, and numbers of these colonies that grew sparsely when transferred to nitrate-containing medium in the absence of chlorate, indicating that they were nitrate non-utilising (*nit*) mutants.

I. bolleyi strain	Nitrogen source	Total number of mycelial blocks plated	Total number of colonies growing from mycelial blocks	Total number of *nit* mutants recovered from colonies
T560	Sodium nitrate	260	165	12
	Sodium nitrite	260	169	0
	Hypoxanthine	260	177	0
	Uric acid	260	86	0
T560-R2	Sodium nitrate	260	136	2
	Sodium nitrite	260	162	0
	Hypoxanthine	260	162	4
	Uric acid	260	161	0
AB1	Sodium nitrate	260	110	17
	sodium nitrite	260	220	0
	Hypoxanthine	260	188	0
	Uric acid	260	205	0
J10	Sodium nitrate	260	77	0
	Sodium nitrite	260	230	0
	Hypoxanthine	260	208	0
	Uric acid	260	145	0
J10-R2	Sodium nitrate	260	181	0
	Sodium nitrite	260	260	13
	Hypoxanthine	260	215	0
	Uric acid	260	221	0

With few exceptions, between 50 and 100% of the mycelial blocks plated gave rise to colonies able to grow on chlorate-supplemented medium containing each nitrogen source (Table 4.1). Overall, the incidence of growth on chlorate-containing media was highest with sodium nitrite as the nitrogen source, and lowest with sodium nitrate. Growth of the strains on chlorate media was reduced compared with that on non-chlorate media regardless of the nitrogen source. However the level of growth reduction was variable between strains (Table 4.2). Notably, the two strains that previously had been selected for tolerance of carbendazim T560-R2 and J10-R2) were much less tolerant of the presence of chlorate than were the wild-type parents, T560 and J10.

In all cases, when colonies growing on the chlorate plates were sub-cultured onto Nitrate Medium in the absence of chlorate, the mycelium extended at the same rate as the wild-type on this medium. However, the density of growth varied, from either sparse and thin to luxuriant (equivalent to the wild-type). Colonies that showed luxuriant growth were designated *crn* mutants, i.e. those that are resistant to chlorate but can also utilise nitrate as the sole nitrogen source. The thin sparse colonies were designated as nitrate non-utilisers or *nit* mutants. When the *nit* mutants were transferred to Asparagine Medium, they grew luxuriantly similar to the wild-type, but if these mutants were transferred back onto Nitrate Medium, their growth was again thin and sparse (Figure 4.1). The frequency of *nit* mutants obtained from chlorate resistant colonies was extremely low, at about 1% of the total colonies produced, the remainder being *crn* mutants.

The frequency of *nit* mutants varied considerably with nitrogen source used in the chlorate media and also with *I. bolleyi* strain (Table 4.1). Of the total 48 mutants obtained, 31 mutants were isolated on sodium nitrate, 13 were isolated on sodium nitrite, and 4 were isolated on hypoxanthine. No *nit* mutants were isolated on uric acid. Mutants were recovered from all strains of *I. bolleyi* except strain J10. All *nit* mutants of strain AB1 were produced on sodium nitrate, whereas the *nit* mutants of strain T560-R2 were isolated on

Table 4.2: Comparison of hyphal growth at 6 days, on Minimal Medium containing different nitrogen sources, supplemented with and without 1.5% $KClO_3$ (means ± SEM for 4 replicates).*

I. bolleyi strain	Colony diameter (mm) on Minimal Medium containing different nitrogen sources and 1.5% $KClO_3$				Colony diameter (mm) on Minimal Medium containing different nitrogen sources (without $KClO_3$)			
	NO_3	NO_2	HX	UA	NO_3	NO_2	HX	UA
AB1	18.5±0.2	18.4±0.0	19.4±0.3	13.4±3.3	33.0±0.7	32.4±0.7	33.0±0.0	34.7±0.3
T560	18.8±0.8	25.0±0.0	24.8±0.1	17.7±2.0	39.0±0.0	37.7±1.2	33.0±1.0	33.0±0.3
T560-R2	11.0±1.0	15.3±0.2	11.4±0.7	10.0±1.5	33.3±2.4	34.3±0.7	33.0±1.0	33.0±0.6
J10	5.4±0.7	16.7±0.3	19.1±0.1	18.8±0.1	33.3±0.0	34.0±1.0	29.3±0.3	33.7±0.7
J10-R2	5.4±0.7	7.0±0.1	5.8±0.4	6.1±0.1	32.0±0.0	31.3±0.7	32.0±0.5	25.6±0.3

* NO_3 = sodium nitrate; NO_2 = sodium nitrite; HX = hypoxanthine; UA = uric acid

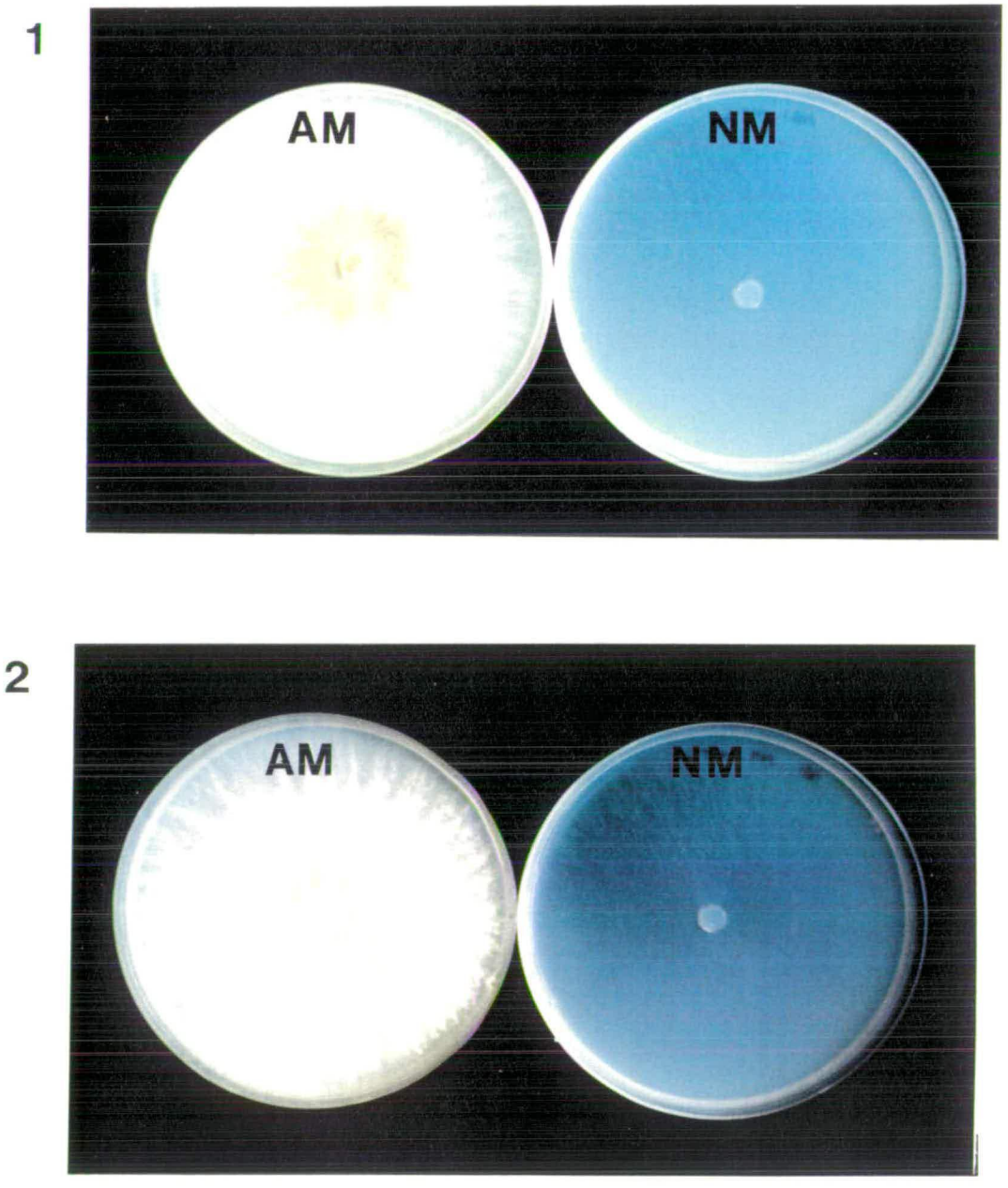
both nitrate and hypoxanthine media, and all *nit* mutants of strain J10-R2 were isolated on nitrite medium.

The number of *nit* mutants varied with *I. bolleyi* strain, as did the stability of the mutants. Seventeen mutants were isolated from strain AB1, from which no mutants reverted back to wild-type. Thirteen mutants were isolated from strain J10-R2 and none of these reverted. Twelve mutants were isolated from strain T560, but nine of them reverted. Six mutants were isolated from T560-R2, and two of these reverted. Eight of the T560 *nit* mutants reverted after the first sub-culturing and a further two mutants were stable for a long period in culture before sectors of wild-type growth developed; one of these mutants could be recovered by sub-culturing from the non-reverted areas, but for the remaining nine revertants this was not possible.

4.3.2 Nit *mutant phenotypes*

The phenotypes of the *nit* mutants were determined by plating the mutants onto media containing different nitrogen sources and comparing growth to that of the corresponding wild-type (Table 4.3). Mutants obtained from chlorate-resistant colonies were of three mutational classes (named according to the criteria for *Fusarium* spp; Klittch & Leslie, 1988). Mutants designated *nit1* were able to grow on ammonium, nitrite, hypoxanthine and uric acid but not on nitrate as sole source of nitrogen, indicating that the mutation is in the nitrate reductase structural locus. Mutants designated *nit3* were able to grow on ammonium, hypoxanthine and uric acid but not on nitrate and nitrite as sole source of nitrogen, indicating that the mutation is in the nitrate reductase regulatory pathway. Mutants designated *nitM* were able to grow on ammonium, nitrite, and uric acid but not on nitrate and hypoxanthine, indicating that the mutation is in one of a number of loci affecting the production of a molybdenum containing co-factor necessary for nitrate reductase

Figure 4.1: Growth of *nit* mutants (1) AB1-4 (*nitM*) (2) AB1-6 (*nit3*) on Asparagine Medium (AM) and Nitrate Medium (NM) as sole nitrogen source. Growth of the *nit* mutants on NM is sparse and thin compared to normal wild-type growth on AM.



activity, and for the activity of xanthine dehydrogenase. No mutants analogous to the major nitrate regulatory locus or the nitrite reductase locus of *Aspergillus nidulans* and *Neurospora crassa* were isolated.

The frequency of the types of *nit* mutant varied. Most mutants recovered were of the *nit3* phenotype, followed by *nit1* and then *nitM*. The numbers of mutants obtained were insufficient for statistical analysis, but there was an indication that the source of nitrogen may have influenced the type of mutation along the nitrate reduction pathway that was detected. In particular, mutants of all three types (*nit1*, *nit3* and *nitM*) were obtained from nitrate plates, whereas *nitM* mutants were not obtained from nitrite or hypoxanthine plates (Table 4.4). The distribution of the different mutant categories between strains is shown in Table 4.5.

4.3.3 Pairing of nit *mutants for heterokaryon formation*

Nitrate non-utilising mutants were plated onto Minimal Medium containing nitrate as sole nitrogen source, in all possible paired combinations to test for their ability to form heterokaryons. Mutants of the same strain were plated against each other, and also against mutants of other strains. The test for complementation was that a line of dense (prototrophic) growth developed at the junction of any two colonies in a pairing. The frequency of this presumed heterokaryon formation differed between strains and between mutants within the strains.

4.3.3.1 Complementation testing of paired *nit* mutants

Table 4.3: Identification the phenotypes of *nit* mutants obtained from five *I. bolleyi* strains. *Nit mutants* were grown on medium containing different nitrogen sources and density of growth was compared to that of the corresponding wild-type growth (which had normal dense growth on all nitrogen sources).

I. bolleyi strain	*Nit* mutant isolate	Growth of *nit mutant* on nitrogen sources(+ = dense growth; - = sparse colony growth; n/t = not tested, due to reversion)					Reversion of *nit* mutant to wild-type	*Nit* phenotype
		NH_4	NO_3	NO_2	HX	UA		
AB1	1	+	-	-	+	+	no	*nit3*
	2	+	-	-	+	+	no	*nit3*
	3	+	-	+	-	+	no	*nitM*
	4	+	-	-	+	+	no	*nit3*
	5	+	-	-	+	+	no	*nit3*
	6	+	-	-	+	+	no	*nit3*
	7	+	-	+	-	+	no	*nitM*
	8	+	-	-	+	+	no	*nit3*
	9	+	-	-	+	+	no	*nit3*
	10	+	-	-	+	+	no	*nit3*
	11	+	-	-	+	+	no	*nit3*
	12	+	-	-	+	+	no	*nit3*
	13	+	-	+	+	+	no	*nit1*
	14	+	-	+	+	+	no	*nit1*
	15	+	-	+	+	+	no	*nit1*
	16	+	-	-	+	+	no	*nit3*
	17	+	-	-	+	+	no	*nit3*
T560	1	+	-	+	-	+	no	*nitM*
	2	+	-	-	+	+	no	*nit3*
	3	+	-	-	+	+	yes	*nit3*
	4	n/t	-	n/t	n/t	n/t	yes	-
	5	n/t	-	n/t	n/t	n/t	yes	-
	6	+	-	-	+	+	no	*nit3*
	7	n/t	-	n/t	n/t	n/t	yes	-
	8	n/t	-	n/t	n/t	n/t	yes	-
	9	n/t	-	n/t	n/t	n/t	yes	-

Table 4.3 continued.....

	10	n/t	-	n/t	n/t	n/t	yes	-
	11	n/t	-	n/t	n/t	n/t	yes	-
	12	n/t	-	n/t	n/t	n/t	yes	-
T560-R2	1	+	-	+	+	+	no	*nit1*
	2	+	-	-	+	+	no	*nit3*
	3	+	-	-	+	+	no	*nit3*
	4	+	-	-	+	+	no	*nit3*
	5	+	-	+	+	+	yes	*nit1*
	6	+	-	+	-	+	yes	*nitM*
J10-R2	1	+	-	-	+	+	no	*nit3*
	2	+	-	+	+	+	no	*nit1*
	3	+	-	-	+	+	no	*nit3*
	4	+	-	+	+	+	no	*nit1*
	5	+	-	-	+	+	no	*nit3*
	6	+	-	-	+	+	no	*nit3*
	7	+	-	-	+	+	no	*nit3*
	8	+	-	-	+	+	no	*nit3*
	9	+	-	-	+	+	no	*nit3*
	10	+	-	-	+	+	no	*nit3*
	11	+	-	-	+	+	no	*nit3*
	12	+	-	-	+	+	no	*nit3*
	13	+	-	-	+	+	no	*nit3*

* NO_2 sodium nitrite, NO_3 sodium nitrate, NH_4 ammonium nitrate, HX = hypoxanthine, UA = uric acid.

No complementation was observed between mutant pairs within the strains T560, T560-R2 and J10-R2. However complementation was observed between pairings of AB1 mutants (Table 4.6). This complementation was of four types: (1) For all pairings involving the *nitM* mutants AB1-3 and AB1-7, a zone of dense or very dense growth developed rapidly at the junction with *nit1* mutants AB1-13, AB1-14 and AB1-15 (Figures 4.2(i) and (ii)). This growth progressively extended across the colonies of both strains in each pairing (Figure 4.3(iii)). The same *nitM* mutants gave rise to enhanced growth at the junction with all *nit3* mutants, but in these cases the growth at the colony junctions was usually less dense than with *nit1* mutants and it extended across the existing colonies after only a long (>21 days) incubation time. The two *nitM* mutants complemented one another, leading to dense growth at the colony junction and rapid spread of this dense growth across the existing sparse colonies (Figure 4.3).

(2) Pairings of *nit1* mutants with *nit3* mutants gave rise to enhanced growth that was slight to moderate when complementation occurred, but the prototrophic growth did not spread across the existing sparse colonies even after a prolonged period of incubation (>21 days). (Figure 4.4(I)). Mutant AB1-13 (*nit 1*) complemented all the *nit3* mutants, with the exception of AB1-2 and AB1-4. *Nit1* mutant AB1-14 complemented five *nit3* mutants, AB1-1, AB1-2, AB1-5, AB1-11 and AB1-12. *Nit1* mutant 15 complemented only the *nit3* mutant, AB1-1. These same *nit1* mutants complemented one another when paired to produce a slight increase in growth density at the colony junction (Figure 4.4 (ii)).

(3) Pairings between *nit3* mutants did not give rise to enhanced growth at the colony junction indicating that there was no complementation between mutants (Figure 4.5).

(4) No complementation was observed when each mutant was paired against itself. Hyphae were seen to merge together at the colony junction with no increase in growth density (Figure 4.6(i)). Mutant AB1-7 *nitM* was an exception, when paired against itself

Table 4.4: Number of nitrate non-utilising (*nit*) mutants of *I. bolleyi* (pooled for strains T560, T560-R2, AB1, J10-R2 and J10) of different phenotypes that had been obtained from chlorate-containing agar plates containing different nitrogen sources (sodium nitrate, 2g l^{-1}); sodium nitrite, 0.5g l^{-1}; hypoxanthine, 0.2g l^{-1}; uric acid, 0.2g l^{-1}.

		Number of mutants		
Nitrogen source	**Total number of *nit* mutants**	***Nit* 1**	***Nit* 3**	***Nit* M**
Sodium nitrate*	31	3	15	4
Sodium nitrite	13	2	11	0
Hypoxanthine	4	2	2	0
Uric acid	0	0	0	0

* Missing data are due to reversion of mutants

Phenotypic classes of *nit* mutants are thought to reflect mutations in the nitrate reduction pathway as follows: *nit1* = structural gene for nitrate reductase; *nit3* = pathway specific regulatory gene; *nitM* = genes controlling the production of a molybdenum-containing cofactor necessary for nitrate reductase activity, and for the activity of xanthine dehydrogenase.

Table 4.5: Number of different types of nitrate non-utilising (*nit)* mutants recovered for each *I. bolleyi* strain; data pooled for mutants selected on chlorate containing plates with different nitrogen sources (sodium nitrate, 2g l^{-1}; sodium nitrite, 0.5g^{-1}; hypoxanthine, 0.2g l^{-1}; uric acid 0.2g l^{-1}).

		Number of mutants		
***I. bolleyi* Strain**	**Total number of *nit* mutants**	***Nit* 1**	***Nit* 3**	***Nit* M**
T560*	12	0	3	1
T560 R2	6	2	3	1
AB1	17	3	12	2
J10	0	0	0	0
J10 R2	13	2	11	0

* Missing data are due to reversion of mutants

Phenotypic classes of *nit* mutants are thought to reflect mutations in the nitrate utilizational pathway as follows: *nit* 1 = structural gene for nitrate reductase; *nit* 3 = pathway specific regulatory gene; *nit* M = genes controlling production of a molybdenum-containing cofactor necessary for nitrate reductase activity, and for the activity of xanthine dehydrogenase.

Table 4.6: Complementation between pairings of nitrate non-utilising mutants of *I. bolleyi* strain AB1(numbers refer to AB1 *nit* isolate number in Table 4.3).*

	Nit 3 mutants												*NitM* mutants		*Nit1* mutants		
	1	2	4	5	6	8	9	10	11	12	16	17	3	7	13	14	15
1	-	2															
2	-	-	4														
4	-	-	-	5													
5	-	-	-	-	6												
6	-	-	-	-	-	8											
8	-	-	-	-	-	-	9										
9	-	-	-	-	-	-	-	10									
10	-	-	-	-	-	-	-	-	11								
11	-	-	-	-	-	-	-	-	-	12							
12	-	-	-	-	-	-	-	-	-	-	16						
16	-	-	-	-	-	-	-	-	-	-	-	17					
17	-	-	-	-	-	-	-	-	-	-	-	-	3				
3	+++	+++	++++	++++	++++	++++	++++	++++	++++	++++	++++	+++	-	7			
7	+	+++	+++	++	+++	++	++	++	+++	+++	+++	++	++++	-	13		
13	++	-	-	-	+	++	+	+	-	++	-	-	++++	++++	-	14	
14	+	+	-	+	-	-	-	-	+	+	-	-	++++	+++	+++	-	15
15	++	-	-	-	-	-	-	-	-	-	-	-	++++	+++	++	+	-

* - = no complementation; + = slight complementation, seen as slightly denser growth at junction of colonies; ++ = moderate complementation; +++ = strong complementation; ++++ = very strong complementation.

the hyphae did not merge together as was seen for the other mutants, instead a zone where no or few hyphae grew was apparent between the paired colonies. (Figure 4.6(ii)).

No complementation was observed between pairings of mutants from different strains. Quite often a zone where no or few hyphae grew was observed at the colony junction between mutants of T560 and T560-R2 and between mutants of AB1 and J10-R2. Pairings of AB1 with T560 and T560-R2 generally resulted in merging of the hyphae at the colony junction with no increase in growth density.

4.3.3.2 Microscopic examination of paired mutants

Microscopic examination of pairings of mutants from any single strain always led to hyphal anastomosis in the regions of hyphal contact (Figures 4.7 to 4.8). The same degree of anastomosis was observed for all mutant types, regardless of their pairing, and whether or not the mutants were able to complement one another. In pairings of mutants from different strains, anastomosis was less frequently observed than between mutants from the same parental strain; hyphal bridges between touching hyphae were rarely observed in these cases and often hyphal branches grew past the touching hyphae without attempting to anastomose.

When mutants from the same strain complemented one another, conidium production occurred rapidly at the colony junction and germinating conidia were noted to be anastomosing with other germinating conidia (Figure 4.9), and with nearby hyphae. Hyphae further back in the individual *nit* mutant colonies were seen to form anastomosis bridges with nearby hyphae in the same colony.

Figure 4.2: Complementation (seen as dense growth at colony junctions) between *nit* mutants on Minimal Medium; **(i)** Strong complementation between *nitM* mutant AB1-7 (a=centre) and *nit1* mutants AB1-14 (b), AB1-15 (c), and moderate complementation with *nit3* mutant AB1-17 (d). **(ii)** Strong complementation between *nitM* mutant AB1-3 (a=centre) and *nit1* mutant AB1-13 (b) and moderate complementation with *nit3* mutants AB1-9 (c), AB1-12 (d) and AB1-16 (e). **(iii)** Strong complementation between *nitM* AB1-3 (a) and *nit1* AB1-14 (b). The dense prototrophic growth has grown over the original colonies. Photographed 14 days after initial contact of colonies.

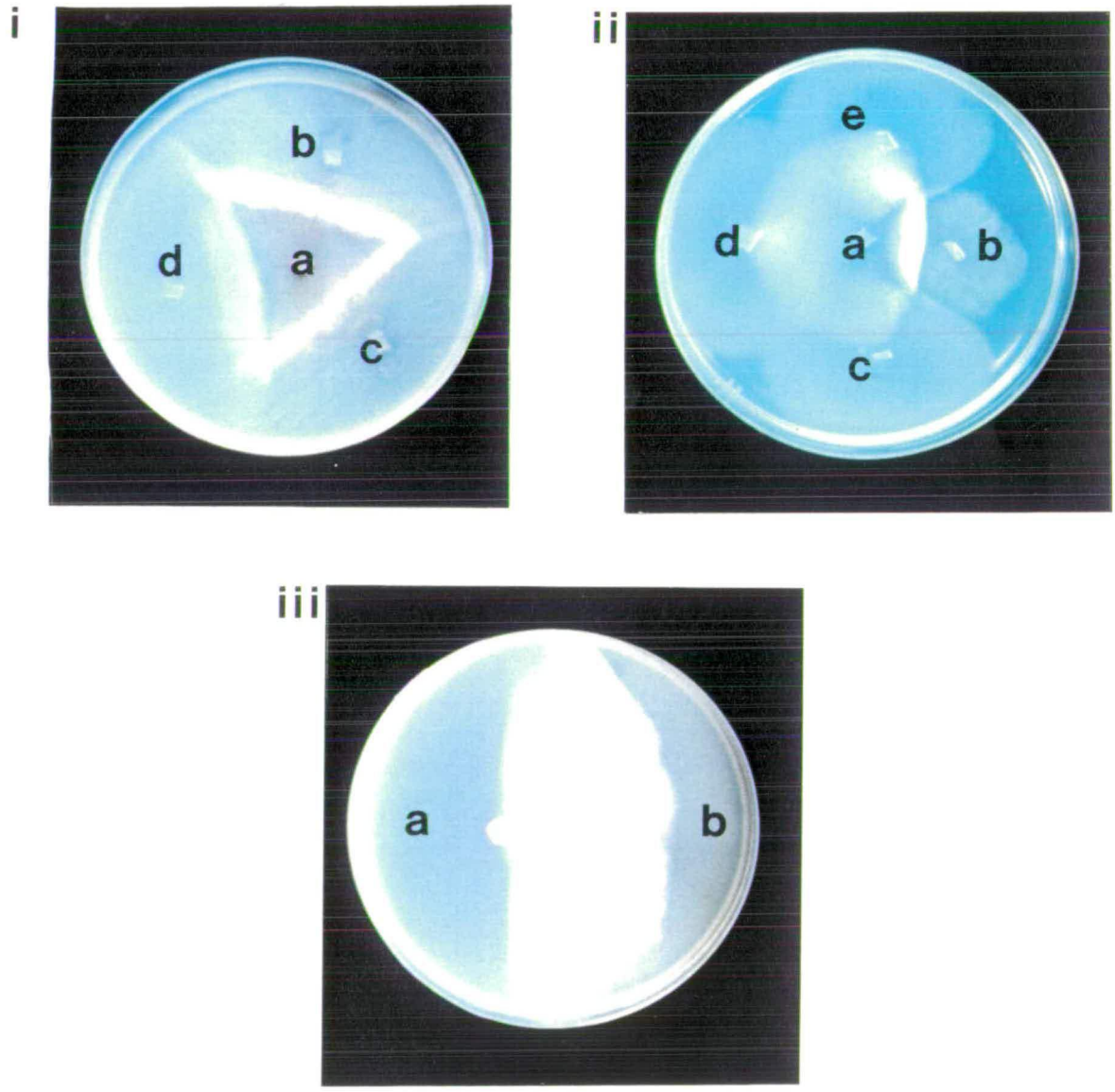


Figure 4.3: Strong complementation between the *nitM* mutants AB1-3 (a) and AB1-7 (b) at 7 days (left) and 14 days (right). The dense prototrophic growth extends rapidly across the original colonies after their initial contact.

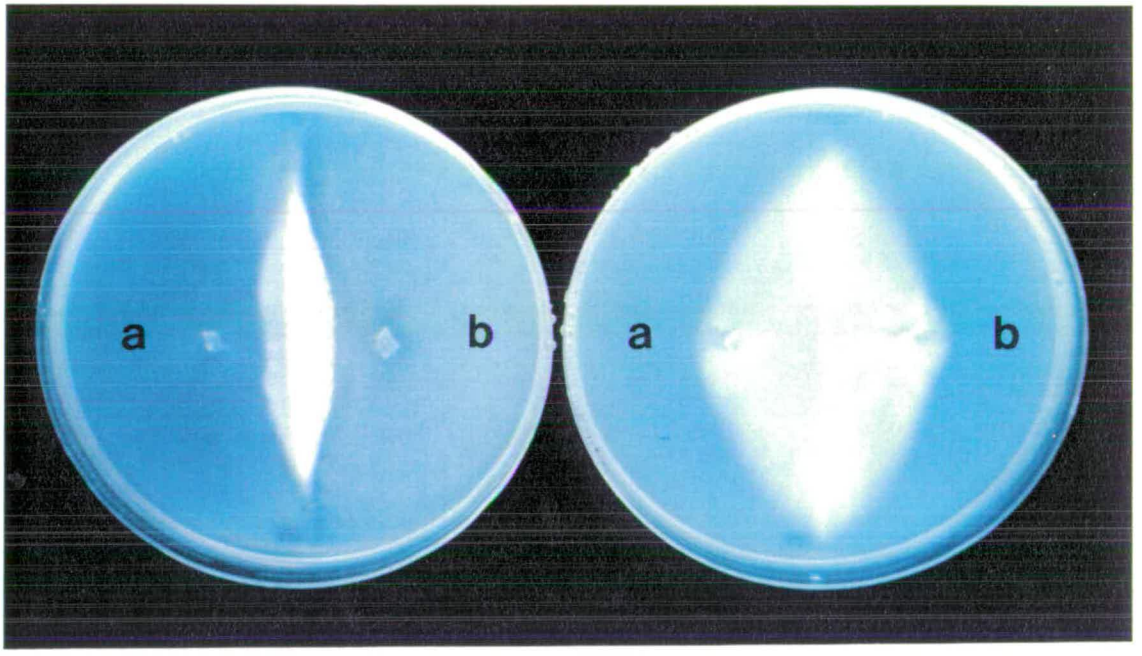


Figure 4.4: (i) Slight complementation between *nit1* mutant AB1-13 (a=centre) and *nit3* mutants AB1-8 (b), AB1-9 (c), AB1-16 (d) and AB1-17 (e), seen as an increase in growth density at the colony junctions. **(ii)** Slight complementation between *nit1* mutants AB1-14 (a) and AB1-15 (b) observed as an increase in growth density at colony junctions.

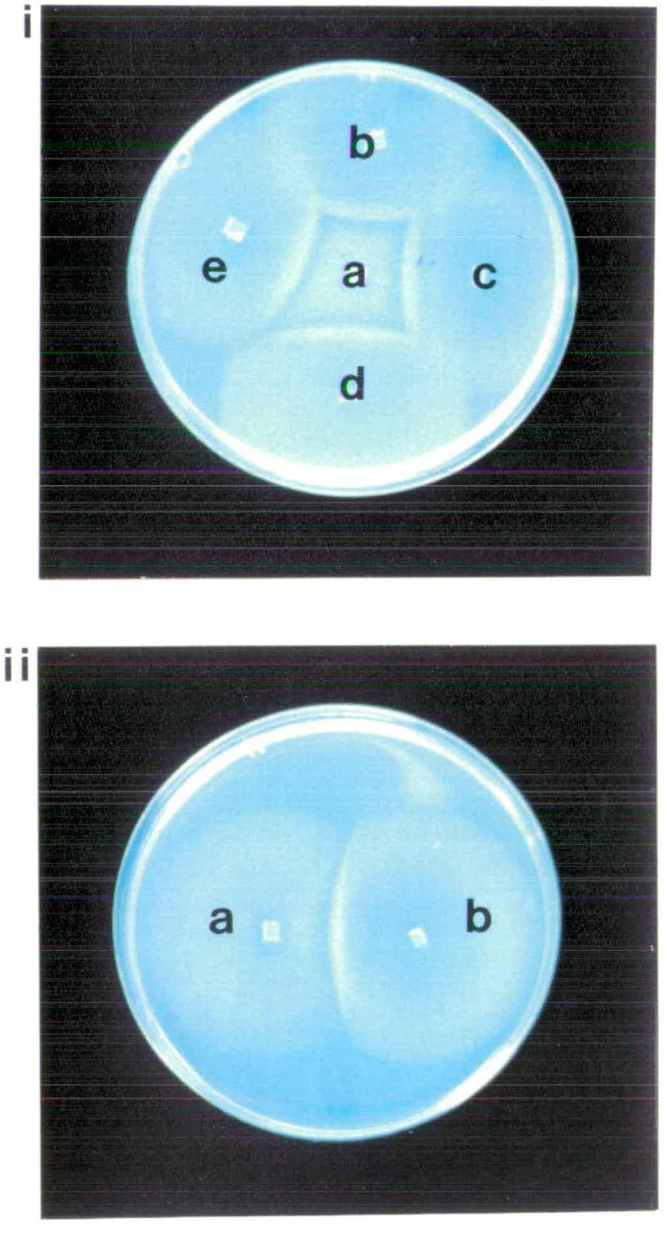


Figure 4.5: Pairing of *nit3* mutants AB1-6 (a) and AB1-17 (b). The hyphae of the two mutants can be seen merging together at the colony junction with no increase in growth density suggesting that no complementation occurred between these mutants.

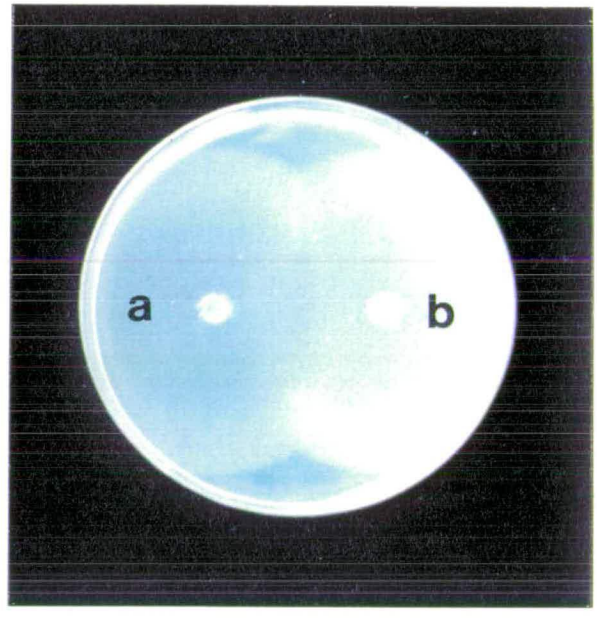
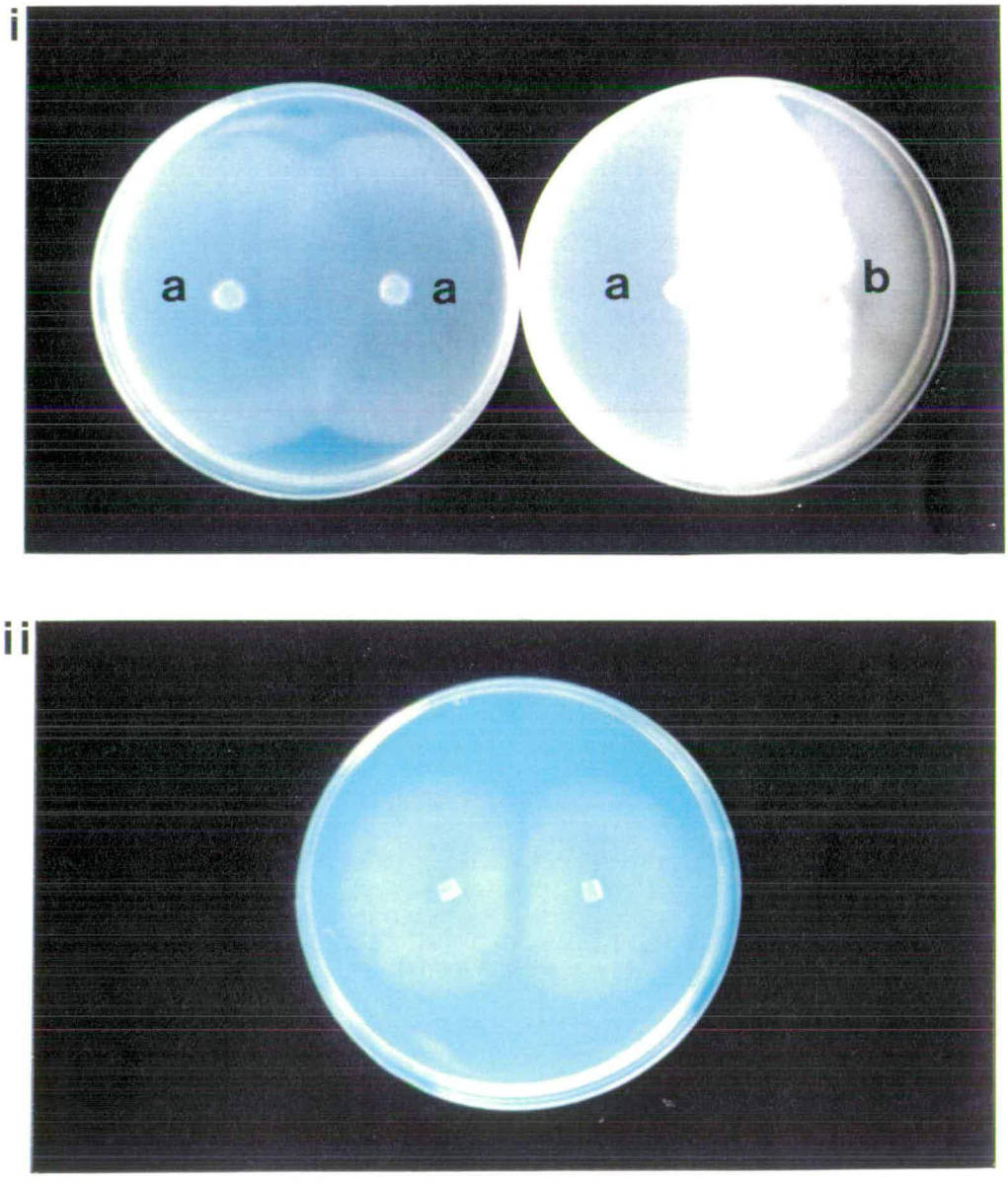


Figure 4.6: (i) Comparison of the growth of *nitM* mutant AB1-3 (a) when paired against itself (left) and against *nit1* mutant AB1-14 (b) (right). Complementation between the *nitM* mutant and *nit1* mutant is apparent as an increase in growth density, however when the *nitM* mutant is paired against itself, the colonies merge suggesting no complementation. **(ii)** Pairing of *nitM* mutant against itself. Unlike *nitM* mutant AB1-3, the hyphae were not seen to merge together, instead a zone where no or few hyphae grew was apparent.



When anastomosis events were observed in colonies stained with DAPI nuclei were rarely observed in the hyphal bridges. Occasionally nuclei were seen to be in close proximity to the bridges. Cells in the hyphae were always observed as being uninucleate.

4.4 Discussion

Nitrate non-utilising mutants have been obtained from a number of fungal species including *Fusarium* spp. (Correll *et al.*, 1987), *Aspergillus nidulans* (Cove, 1976b), *Neurospora crassa* (Tomsett & Garrett, 1980) and *Septoria nodorum* (Newton *et al.*, 1988), using chlorate resistance for selection of mutations along the nitrate reduction pathway. *Nit* mutant phenotypes have been used in genetical and nutritional studies, providing information on the regulation of nitrogen metabolism and gene expression. This regulation is by no means straightforward and anomalies have been found between fungal species, pointing to some possible differences in regulation.

Nit mutants of *I. bolleyi* could be obtained from growth on chlorate media but this growth was always as suppressed colonies rather than as fast growing sectors, as has been recorded for other fungi (Correll *et al.*, 1987; Klittch & Leslie, 1988; Carla *et al.*, 1989). *Nit* mutants have been recovered from *Pseudocercosporella herpotrichoides* where growth was recorded as being virtually similar to that of the wild-type except for a slight colour change in parts of the colony (M. Hocart, personal communication). The high frequency of chlorate resistant colonies obtained suggests that *I. bolleyi* may be highly genetically unstable.

The benefits of genetic plasticity are apparent for plant pathogens, giving them a greater chance of survival by overcoming host resistance and generating fungicide resistance.

Figure 4.7: Hyphal anastomosis between the vegetatively compatible *nitM* mutants AB1-3 (a) and AB1-7 (b). (Bridges between hyphae are indicated by arrows).

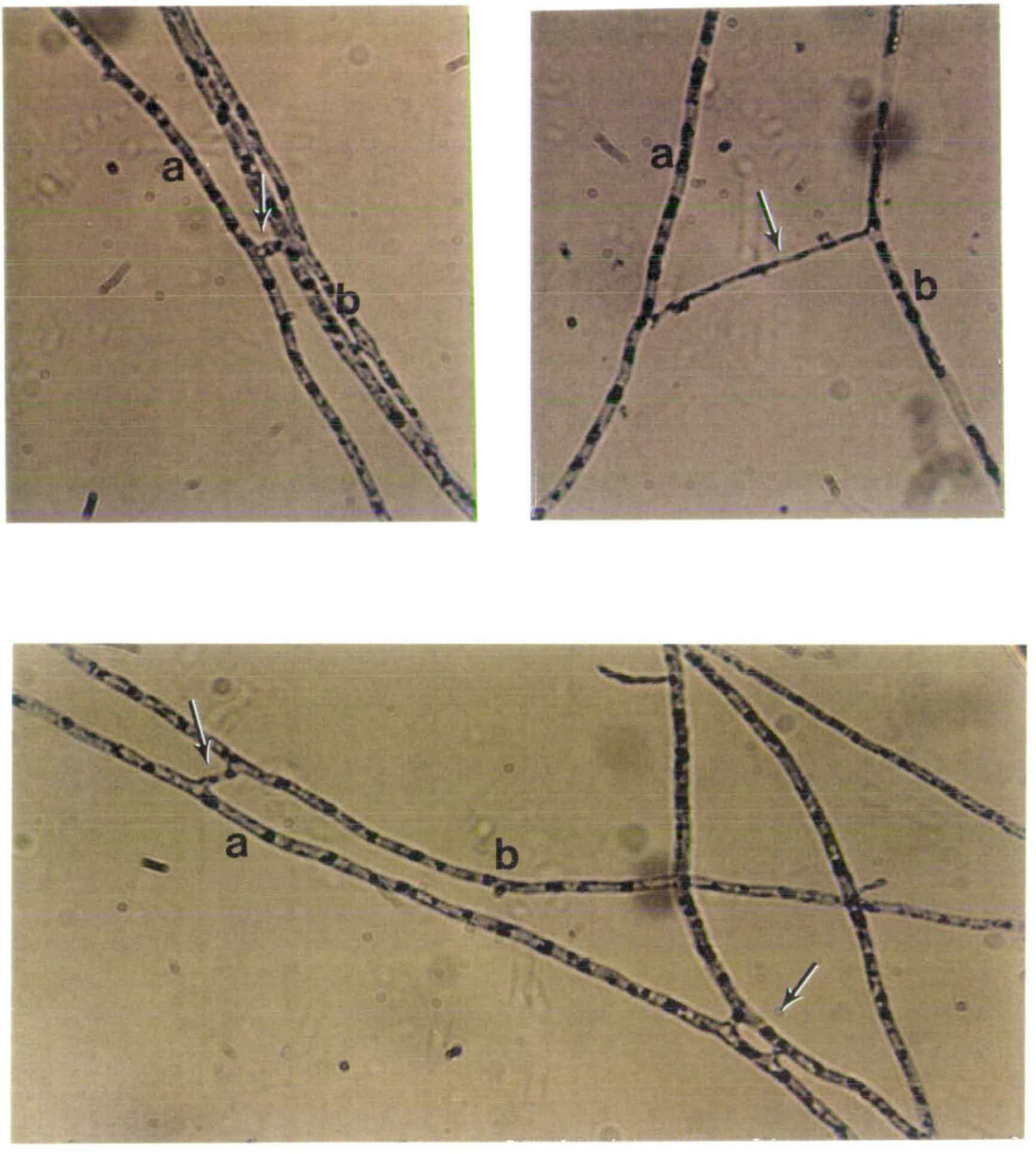
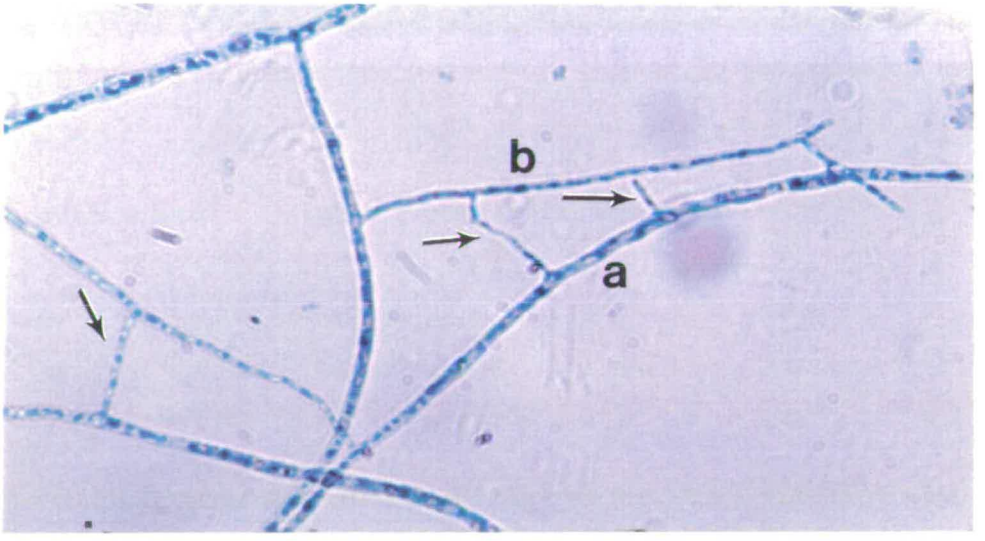


Figure 4.8: Hyphal anastomosis between the incompatible *mtA1* mutant T560-1(a) and *mt3* mutant T560-2 (b). (Bridges between hyphae are indicated by arrows).



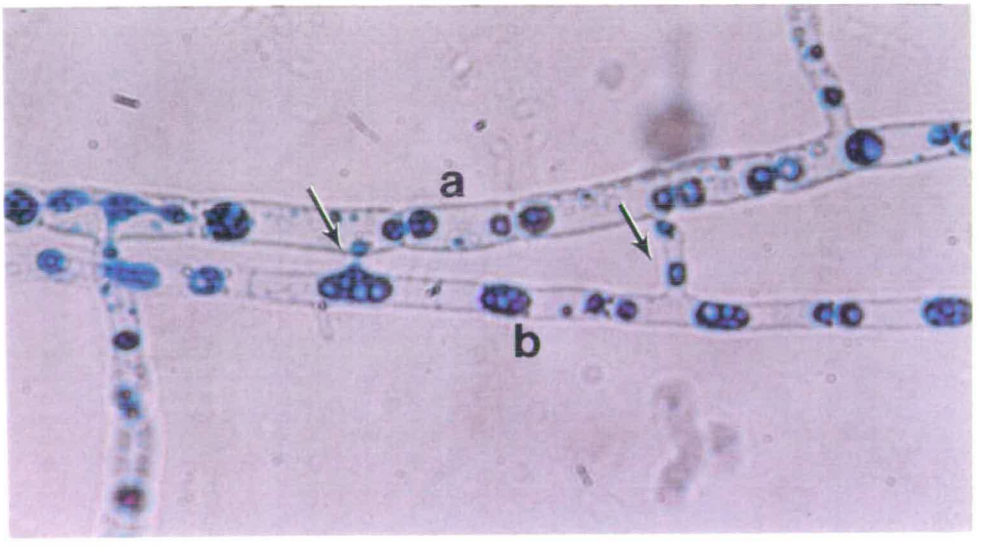
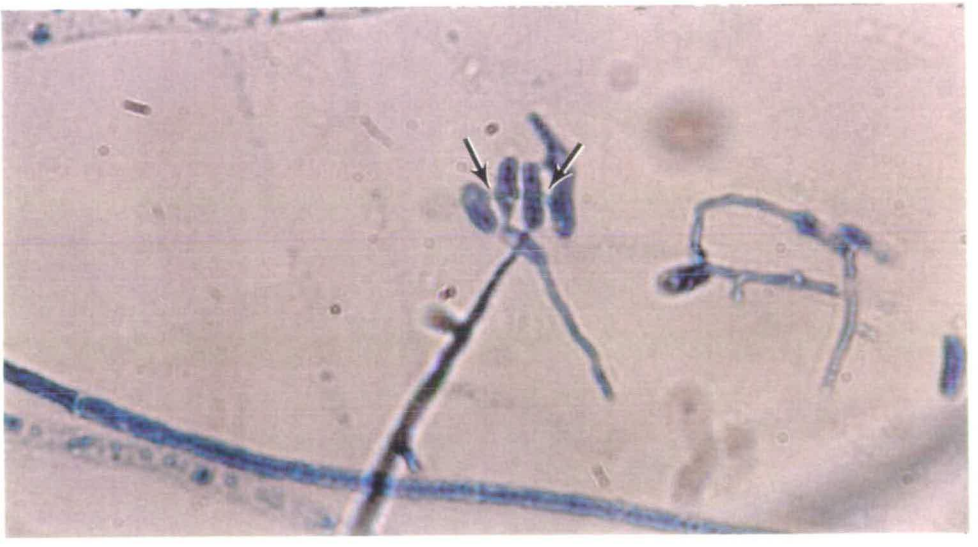


Figure 4.9: Nitrate non-utilising (*nit*) mutant AB1-3 showing anastomosis between germinating conidia by the formation of bridges (indicated by arrow). Germtubes often went on to anastomose with nearby hyphae.



However, survival is a fact of life for all fungal species in the face of environmental change, and Klittich & Leslie (1988) have suggested that a high mutation rate may be an important means of developing variability where sexual or parasexual recombination is rare, as would seem to be the case for *I. bolleyi*.

Suggestions have been put forward for the cause of genetic instability and these include environmental stress which may influence mutation. In the production of *nit* mutants, the frequency of spontaneous sectoring varies from species to species and from strain to strain within species (Leslie, 1990), but sectoring is also influenced by temperature, nutrient levels, and selection pressures on toxic media (Brown, 1926; Correll & Leslie, 1987). Klittich & Leslie (1988) found that in *Fusarium moniliforme*, less than 5% of uninucleate conidia carry a *nit* mutation prior to culture on chlorate medium, so chlorate resistant growth is unlikely to be as a result of an inherently high frequency of chlorate resistant nuclei in the parental culture. Spontaneous chlorate resistant growth occurred on the medium, so that the medium *per se* may or may not be the direct cause of mutation. The slow irregular growth of *I. bolleyi* on chlorate media and the high production of chlorate resistant colonies suggests that mutation could well result from environmental stress due to the toxic effects of the medium.

A factor that may influence mutation is the presence of a transposable element. Klittich *et al.*, (1988) found that sectoring was strain dependent and classed strains into three categories: high frequency, moderate and infrequent for sectoring. On crossing of combinations of strains, the frequencies of progeny were distributed around the mean of the parental frequencies, and segregants with higher and lower frequencies than those of the parents were obtained in all crosses. The distribution of progeny was statistically consistent with a model of additive polygenic inheritance, indicating that sectoring is controlled by many genes. Through other reciprocal crosses, they also showed that genetic control of sectoring is nuclear rather than cytoplasmic.

Tests of field isolates of *F. moniliforme* showed differences in sectoring frequency (Klittich *et al.*, 1988). A high frequency of sectoring could be similar to that recorded in other eukaryotes including yeast (Roeder *et al.*, 1980) and Drosophila (Engels, 1983), where the frequency of mutation is a measure of the movement of transposable elements, and thus those isolates showing the most sectoring may have active transposable elements allowing them to respond to selection pressure more rapidly than others (Klittich *et al.*, 1988).

In a recent study, Kistler & Miao (1994) have suggested that transposons may be a common feature of the fungal genome and they noted that repetitive DNA sequences in some species have been identified as transposable elements.

In *I. bolleyi* the involvement of a transposable element in mutation is a possibility worthy of examination. The frequency of mutation of *I.* bolleyi was strain dependent, because no mutants were recovered from strain J10. This could indicate that strain J10 has few transposable elements or they are inactive. In normal storage of cultures, it has been noticed that strain AB1 frequently sectors spontaneously whereas strain T560 is highly stable. The most *nit* mutants were recovered from strain AB1 and these mutations appeared to be stable in comparison with *nit* mutants from both the T560 and T560-R2 strains. It is not known why the strain J10-R2 differed from strain J10 in production of *nit* mutants.

A range of *nit* phenotypes were recovered for *I. bolleyi* and these reflect mutations at different sites along the nitrate reduction pathway. This pathway has been characterised in *A. nidulans*, *N. crassa* and *Fusarium* spp. (Pateman & Kinghorn, 1976) and the loci involved have been genetically mapped. For these well-characterised fungi, reduction of nitrate to ammonium requires only two enzymes: nitrate reductase, which converts nitrate to nitrite, and nitrite reductase, which converts nitrite to ammonium (Garraway & Evans, 1984). The number of genes involved in this assimilation process varies between fungal

species. In *A. nidulans* at least 11 genes are involved, in *N. crassa* there are at least 8 and in *Fusarium* spp. there are at least 7 (Cove, 1976b; Tomsett & Garrett, 1980; Correll & Leslie, 1987). In each organism, the nitrate reductase enzyme is encoded by a single structural locus, as is the pathway-specific regulatory locus. The production of the nitrate reductase enzyme involves a molybdenum containing cofactor, this cofactor being a functional constituent of nitrate reductase (Kinsry & McElroy, 1985). The production of this cofactor is under the control of multiple loci in all three organisms. Tomsett & Garrett (1980) have mapped 4 loci in *N. crassa* and 8 in *A. nidulans*. In the production of the molybdenum cofactor, one locus in all three organisms is believed to be complex and homologous across the three species; these loci are termed the *nit*-9 locus of *N. crassa*, the *nit-4* locus of *F. moniliforme* and the *cnxABC* locus of *A. nidulans*. Through heterokaryon studies, these loci are known to contain up to three complementation groups (Marzluf, 1981).

Other loci involved in the pathway include the major nitrogen regulatory locus corresponding to the *nit-2* locus in *N. crassa* and the *areA* locus in *A. nidulans*, and the nitrite reductase locus corresponding to the *nit-6* locus in *N. crassa* and the *niiA* locus in *A. nidulans* (Marzluf, 1981). Major nitrogen regulatory mutants have been regularly found to constitute between 12 to 15% of *nit* mutants in *N. crassa* (Marzluf, 1981; Perrine & Marzluf, 1986). In *A. nidulans* these mutants have been isolated, but less frequently than for *N. crassa* (Arst & Cove, 1973). Mutants of the nitrite reductase locus have also been isolated, but less commonly, and these mutants can be distinguished from the *nit* phenotype for the pathway regulatory gene by the excretion of nitrite into the medium (Pateman & Kinghorn, 1976).

I. bolleyi appeared to be similar to *Fusarium* spp. in that no mutants of the major nitrogen regulatory locus were isolated. In *A. nidulans*, where the nitrite reduction pathway has been intensely studied, the major regulatory gene, *areA*, is believed to be involved in

ammonium regulation along with a complex of other genes (Pateman & Kinghorn, 1976). In low external levels of ammonium, the *areA* gene determines a regulatory product that allows the synthesis of a number of enzymes of nitrogen metabolism including nitrate reductase. *A. nidulans* shows low levels of nitrate reductase when the *areA* gene is mutated, and, due to the inactivity or absence of the regulatory protein, low enzyme and uptake levels render the mutant resistant to a toxic analogue (Pateman & Kinghorn, 1976). In *Fusarium* spp., the absence of any mutants in this locus is believed to be due to the fact that this mutation is either lethal or results in chlorate sensitive mutants (Klittich & Leslie, 1988). A *nnu* mutant of *Gibberella zeae* was recovered following UV irradiation and subsequent growth in a liquid medium containing nitrate as a sole source of nitrogen. This mutant was unable to grow on ammonium as sole source of nitrogen and also did not grow on any inorganic nitrogen source used by its wild-type parent. This mutant was sensitive to chlorate and the mutation was thought to be a lesion in the major regulatory locus (Leslie, 1987). This *nnu* mutant is significantly different from those of *N. crassa* and *A. nidulans* in their response to chlorate sensitivity, suggesting that the *Fusarium* spp. could differ in their overall regulation of nitrogen metabolism (Correll *et al.*, 1987). The absence of a major regulatory mutant for *I. bolleyi* suggests that nitrogen regulation may be similar to that observed for *Fusarium* spp. However, mutant selection on a greater range of nitrogen sources is required before a strong conclusion can be drawn on this part.

In *I. bolleyi*, the loci involved in the nitrate reduction pathway seemed to differ in their susceptibility to mutation. *Nit3* mutants were most frequently isolated followed by *nit1* and *nitM*. These frequencies for *I. bolleyi* seem to differ in comparison with other fungal species. *Fusarium* spp., *A. nidulans* and *N. crassa* all mutated most commonly in the nitrate reductase structural locus. Less frequent, but also common, is mutation in the nitrate regulatory locus of *A. nidulans* and *Fusarium* spp., but the equivalent mutational type was infrequently found in *N. crassa* (Tomsett & Garrett, 1980). Mutants of the molybdenum cofactor synthesis were isolated in all three fungi, but less commonly than the two loci

involved in the production and regulation of nitrate reductase (Cove, 1976b; Tomsett & Garrett, 1980; Puhalla, 1985; Klittich & Leslie, 1988).

Hypotheses put forward to explain the differing susceptibility of loci to mutation include differences of gene size. It is thought that a larger gene may represent a larger target, or alternatively the presence of 'hot spots' in certain sequences within loci render them more susceptible to mutation (Klittich & Leslie, 1988). There may be difficulties in accurately establishing the susceptibility of some genes to mutation, particularly where those mutations result in a lethal effect such as that thought to occur in the major nitrogen regulatory gene of *Fusarium* spp. Lethal mutations will be impossible or extremely difficult to recover unless they are conditional, but it is possible that lethal mutation is more common than the non-lethal types.

Most *nit* mutants of *I. bolleyi* were recovered from the Nitrate Medium and no mutants were recovered on uric acid, so it seems that nitrogen source used had an influence on mutant selection. There is no apparent reason for the lack of *nit* mutants isolated on uric acid. If mutations in the major nitrogen regulatory gene did occur, this would make it impossible for these mutants to grow on uric acid. However, *nit1* and *nit3* mutants should be able to grow on this medium. All mutant types of *N. crassa* were successfully isolated on uric acid as the sole source of nitrogen (Tomsett & Garrett, 1980) and a number of different mutants of *A. nidulans* were also isolated on this medium (Cove, 1976b).

Cove (1976b) found that under various circumstances, the nitrogen source used in the medium affects the relative proportion of mutants. A similar biasing of *nit* mutant frequency on different nitrogen sources was also observed for *F. oxysporum*, but this was strain dependent (Correll & Leslie, 1987). The reason for nitrogen source influencing which locus is mutated is not fully understood, but it is believed to be related to the overall roles

of genes involved in the complex process of nitrogen metabolism (Pateman & Kinghorn, 1976).

In *I. bolleyi*, only 1% of mutants recovered were actually *nit* mutants; the remainder were chlorate-resistant, nitrate-utilising mutants which have been classed as *crn* mutants. *Crn* mutants are regularly obtained from other fungi, but usually the frequency of *crn* mutants is much lower than that recorded for *I. bolleyi*. For *Fusarium* spp., *A. nidulans* and *N. crassa, crn* mutants usually constituted between 10 to 20 % of total mutants (Cove, 1976b; Tomsett & Garrett, 1980; Correll & Leslie, 1987). The mechanism which allows some mutants to utilise nitrate but also to be resistant to chlorate is complex. Klittich & Leslie (1989) found that *crn* mutants are inherited through meiosis and are stable. The main mechanism involved in chlorate resistance is avoidance of chlorate toxicity. Cove (1976a) has suggested that mutants of the *crn* loci might have altered nitrate reductase activity, allowing enough nitrate to be reduced as a nitrogen source, but not enough to poison the cell by a build up of chlorite. Young mycelium of *crn* mutants of *A. nidulans* was found to have normal levels of nitrite reductase, but decreased levels of nitrate reductase, which seemed to confer chlorate resistance (Cove, 1976a).

Five unlinked loci have been mapped in *F. moniliforme*: *crn1, crn2, crn3, crn4* and *crn5* (Klittich & Leslie, 1989). It is thought that the *crn* loci could be closely linked to some of the *nit* loci. The findings of Klittich & Leslie (1989) in their study of *crn* mutants of *F. moniliforme* were as follows. Mutants of the *crn2* locus had higher nitrate reductase levels than the wild-type progenitors and this mutation was believed to be closely linked to the *nit5* locus. It was considered unlikely that *crn2* is allelic with *nit5* locus because *nit5* mutants have undetectable nitrate reductase activity, and inactivation of the *nit5* locus should decrease rather than enhance nitrate reduction. It is believed that the *crn2* locus could be analogous to the *crnA* locus of *A. nidulans* which also shows high nitrate reductase activity

and is linked to other nitrate reductase loci. Mutations at the *crnA* locus decreased nitrate uptake and are also likely to decrease chlorate uptake (Brownlee & Arst, 1983).

The *crn1* locus is linked and may be allelic to the *nit3* locus, which has regulatory control of nitrate reductase (Klittich & Leslie, 1988). *Crn1* mutants have low levels of nitrate reductase.

The *crn5* locus may be closely linked to the *nit1* locus - the structural locus for nitrate reductase. *Crn5* mutants have a low level of nitrate reductase and so allowing some nitrate reduction for survival but not enough to accumulate high levels of chlorite.

The *crn3* and the *crn4* are not believed to be linked to another *nit* or *crn* locus. *Crn3* mutants show a low level of nitrate reductase and *crn4* mutants have a moderate level (Klittich & Leslie, 1989).

In relation to the recovery of both *crn* and *nit* mutants, Cove (1976a) has challenged the overall belief that chlorate toxicity is due to the conversion of chlorate to chlorite by nitrate reductase. Cove (1976a) found that both-chlorate resistant and chlorate sensitive mutants lacked nitrate reductase, and that the chlorite toxicity of wild-type strains can vary with the nitrogen source used. He also found that with nitrogen sources also containing carbon sources, the carbon sources were less catabolically repressing than 1% D-glucose, where the alleviation of carbon catabolite repression can also alleviate chlorate toxicity. Thirdly he also found that *cnx* and *niaD* mutants, which abolish nitrate reductase activity, bring about chlorate resistance. The interactions between the *nirA* mutant and the *niaD* or the *cnx* mutants were not simple and no pattern of epistasy was found. Cove (1976a) concluded that both the *niaD* gene product and the nitrate reductase holo-enzyme (the molybdenum cofactor specified by the five genes) are directly involved in the mediation of toxicity.

Using *nit* mutants as auxotrophic markers, it was possible to force stable prototrophic growth between *I. bolleyi* mutants of the same strain. It was found that mutants in the loci governing the production of the molybdenum cofactor could readily complement when paired with both the *nit1* and *nit3* mutants. *Nit1* and *nit3* mutants could also complement each other, but complementation was generally quite weak. When mutants were paired against isolates of the same mutational designation, both *nitM* and *nit1* mutant strains could complement one another. In *I. bolleyi nitM* and *nit1* mutants could complement other isolates of the same mutational designation when paired. Complementation between isolates of the same mutation has been observed in *Fusarium* spp. Complementation between different *nit1* mutants is believed to be typical of intragenic complementation (ie within the same gene) rather than intergenic (ie between different genes) (Fincham, 1966). It is possible for two *nit1* mutants to complement if they alter the gene product in different ways, but the degree of complementation is likely to be variable (Fincham, 1966).

Complementation between *nitM* mutants of *I. bolleyi* was rapid, with dense growth indicating possible heterokaryosis. Such findings have also been observed in *F. oxysporum* where six or seven strains with two to six complementation groups were identified among *nitM* mutants (Correll *et al.*, 1987). Because of this pattern of complementation and because of the number of genes involved in the production of the molybdenum cofactor, it is understandable that this reaction could be one of intergenic complementation.

Strains capable of forming a heterokaryon are termed vegetatively compatible, and those compatible with one another are grouped together to form vegetative compatibility groups (VCGs). Within a population those strains in the same VCG are more similar than those in different VCGs (Leslie, 1993).

In an extensive study with plant pathogenic strains of *F. oxysporum*, 21 strains of *F. oxsporum* were found to belong to 16 different VCG groups and there was a correlation between VCG and *forma specialis* (Puhalla, 1985).

The loci governing heterokaryon incompatibility are termed *het* or *vic* genes and they are believed to act as part of a self/non-self recognition system (Leslie, 1990). In *A. nidulans* at least 8 *het* loci are known (Croft, 1977), in *N. crassa* at least 10 are known (Perkins *et al.*, 1982), in *Cryphonectria parasitica* 5 to 7 are known (Kuhlman & Bhattacharyya, 1984) and in *Fusarium* spp. 8 are known.

Only two alleles have been found at each locus and if strains have the same alleles at each incompatibility locus then they are vegetatively compatible. Sexual crossing of imcompatible strains is possible, so vegetative incompatibility is known to be independent of the factors that govern sexual reproduction (Puhalla, 1985).

Incompatibility due to differences at the *het* or *vic* loci will effectively limit the non-sexual exchange of genetic information to strains that belong to the same VCG. Recombination would be via a parasexual cycle, if such a cycle exists in nature. Each VCG will thus form a genetically isolated subpopulation. Within a population the fitness of the members is likely to determine their domination within a population (Leslie, 1993).

Complementation of *I. bolleyi nit* mutants within the AB1 strain showed that this is capable of possible heterokaryon formation. From the results of pairings between different strains however, no complementation was observed. This might be because the origins of the strains were quite distinct, but in any case it demonstrates the possible existence of different VCGs within *I. bolleyi*. If sexual reproduction occurs more often than selection or genetic drift can eliminate the different VCGs types from a population, then a sexually reproducing population will always have a high level of diversity (Leslie, 1993). Asexual populations, such as those of *I. bolleyi* which have no known sexual stage, will

have limited genetic exchange because of the isolated subpopulations. The subpopulations will be subject to genetic drift via selection forces. Crossings between VCG groups may have to rely on mutations that enable such crossing. Studies on the stability of VCGs have shown that most are stable (Leslie, 1993). However UV induced mutants of *C. parasitica* were able to change their VCG phenotype (Rizwanna & Powell, 1992); whether the instability was a result of mutations in the alleles at one or more *vic* loci is not known (Puhalla., 1985). Instability of VCG in *N. crassa* appeared to be associated with gross genomic reorganisation rather than allelic mutation (Pittenger, 1964).

Pairings of mutants between the strains T560, T560-R2, J10-R2 and AB1 produced no complementation. Pairings of *nit1* and *nit3* mutants from the same strain produced no complementation in strains T560, T560-R2 and J10-R2. In strains T560-R2 and T560 where a *nitM* mutant was available, no complementation was observed when the nit mutants were paired with the *nit1* and *nit3* mutants of the same strain.

Lack of complementation between strains indicates that they are vegetatively incompatible. Other fungi in which self-incompatibility has been observed include *Rhizoctonia solani* (Hyakumuchi & Ui, 1987) and *Verticillium* spp. (Puhalla & Hummel, 1983; Correll *et al.*, 1987).

Genetic and biochemical mechanisms are believed to control vegetative incompatibility. Genetical mechanisms include allelic and non-allelic systems. Vegetative incompatibility is triggered when two or more strains that contain different alleles at one or more *het* or *vic* loci fuse (Glass & Kuldau, 1992).

Hyphae that fuse to form incompatible reactions have been observed to undergo a barrage reaction. This can occur at the point where two incompatible strains meet. The barrage region consists of a central region that contains dead or dying cells often surrounded by perithecia or other sexual structures. This formation of perithecia has been observed with

Cyphonectria parasitica (Leslie, 1993). It is thought that in such reactions a proteinaceous cytoplasmic factor is involved in cell death (Glass & Kuldau, 1992). Studies of allelic interactions of *N. crassa* have involved injecting a cytoplasm from cell death reactions into recipient cells; these cells subsequently died. The cytoplasmic extracts could be inactivated by heating, indicating the involvement of a protein.

Incompatible reactions where no cell death occurs can be overcome by protoplast fusion (Dales & Croft, 1977). It has been suggested that with such reactions, the cell wall plays a role in vegetative self/non-self recognition (Glass & Kuldau, 1992). With *I. bolleyi*, the rare anastomosis events between the mutants of different strains did not reveal cell death, prehaps because no cytoplasmic continuity was established.

Where no complementation occurs between mutants of the same strain, these strains are referred to as being heterokaryon self-incompatible (HSI). It is possible that strains T560 and J10 could be heterokaryon self-incompatible, as no complementation was observed between their mutants.

From twelve strains, one vegetatively self-incompatible mutant was observed for *F. moniliforme* (Correll *et al.*, 1987; Klittich & Leslie, 1988). Lack of complementation was also observed for some mutants of *F. oxysporum* where results indicated that hyphae of these mutants rarely anastomosed (Jacobson & Gorden, 1988). Correll *et al.* (1987) found that self-incompatibility was a heritable trait and is likely to be controlled by a single nuclear gene.

The mechanisms controlling HSI are not fully known, but in some cases of reduced anastomosis, the HSI strains have a reduced number of fimbriae which play an important role in the formation of the conjugation tubes, normally observed between adjacent hyphae in the HSC strains (Leslie, 1990).

The real importance of vegetative incompatibility in nature is not known, but it has been thought that it may help to prevent the spread of viruses and other extrachromasomal genes throughout a population (Glass & Kuldau, 1992). The question of whether the loci involved in compatibility reactions evolved to limit heterokaryon formation remains unanswered.

Microscopic examination of *I. bolleyi nit* mutants that were able to complement one another showed that in hyphae stained with the nuclear binding stain DAPI, the cells remained uninucleate despite the presence of anatomosing hyphae. No cells were observed to be multiinucleate, but nuclei were occasionally observed in close proximity to the anastomosing bridges. The ability of *nit* mutants to complement one another despite the lack of an observable heterokaryon is unusual. The presence of uninucleate cells implies that if a heterokaryon is formed (indicated by complementation) then *I. bolleyi* is unable to maintain the heterokaryotic state or the heterokaryotic state is limited. Hocart & Peberdy (1989) stated that fungal species which are uninucleate may undergo heterokaryosis as a result of hyphal anastomosis, and nuclear migration may occur along the bridge to yield isolated heterokaryotic cells. In *Verticillium dahliae* each hyphal cell contains one nucleus and following anastomosis heterokaryotic cells are formed, but the remaining cells remain uninucleate. The result is that heterokaryosis is restricted to the anatomosed cells giving a less stable mosaic of homokaryotic growth and isolated heterokaryotic cells (Hastie, 1981). In *Fusarium moniliforme* heterokaryons are limited to the cells that participated in heterokaryosis, but prototrophic growth occurred as there were enough heterokaryotic cells to allow cross-feeding with the rest of the thallus (Burnett, 1975). As *I. bolleyi* did not show a mosaic pattern of prototrophic growth, it is possible that the uninucleate thallus was cross feeding with a few cells that were in a heterokaryotic state. In some species a sustainable heterokaryotic state does not occur. Elander (1974) was unable to isolate heterokaryons in *Cephalosporium acremonium* after pairing mutants for complementation

but recombinant progeny could be obtained following protoplast fusion. It is possible that a heterokaryotic state in *I. bolleyi* is highly unstable and nuclear fusions do not occur. Plating the conidia of *I. bolleyi* from a prototrophic region of growth would show whether karyogamy has occurred. Spores from the region of prototrophic growth were not tested for their phenotype so this requires further experimentation.

If a heterokaryotic state in *I. bolleyi* is limited then this suggests that genetic exchange by this mechanism is likely to be limited and insignificant in the field. Also, if heterokaryosis is limited, then it is unlikely that the parasexual cycle operates for *I. bolleyi* and thus genetic diversity must be maintained by alternative means.

CHAPTER 5

THE PRODUCTION AND FUSION OF PROTOPLASTS OF *IDRIELLA BOLLEYI*

5.1 Introduction

Protoplast technology has been used extensively for genetic manipulation of plants, but this technology has only more recently been successfully applied to fungi where it has had a major impact on fungal genetics (Peberdy, 1987).

For successful use of fungal protoplasts, it is important that a number of protoplasts are generated and that these protoplasts can revert back to normal growth after this process (Peberdy, 1979). For all fungal species, finding the optimal conditions for protoplast production is empirical (Peberdy, 1976).

Protoplasts can have many uses in fungal genetics. The removal of the cell wall has many advantages for genetically manipulating these organisms through transformation, where uptake of DNA provides a means of introducing a selective gene (Tilburn *et al.*, 1983). Alternatively, protoplasts can be fused to allow exchange of genetic material (Ferenczy *et al.*, 1976) and generation of novel hybrids (Peberdy, 1987). The use of protoplast fusion has also led to an understanding of heterokaryon formation (Phillips, 1993) and vegetative incompatibility (Di San Lio *et al.*, 1994).

Protoplasts can be used to fuse fungi involved in biological control of plant disease, in an attempt to obtain superior recombinant strains (Harman & Stasz, 1991). The use of

specific biocontrol agents has become an important priority in plant disease control, rather than trying to modify an environment to enhance the levels of naturally present biocontrol organisms (Cook & Baker, 1983). Genetic alteration is becoming a feasible option to improve strains in order to give them a greater degree of environmental adaptation and competitive ability (Harman & Stasz, 1991). Improvement of these organisms has generally relied in the use on UV irradiation or chemical mutation and such methods have drawbacks in that deleterious mutation may occur, resulting in undesired genetic changes (Hocart & Peberdy, 1989). The production of protoplasts and their application in fusion can allow whole genomes to undergo recombination (Harman & Stasz, 1991). This may be particularly important in asexual fungi where heterokaryosis is rare or does not exist (Typas, 1983), or where vegetative incompatibility barriers must be overcome (Di San Lio *et al.*, 1994).

Improvement of biocontrol agents is still in its early stages mainly because the advances in this technology have been made only recently. One biocontrol agent has been successfully improved through protoplast fusion. Harman & Stasz (1991) fused different auxotrophic mutants of *Trichoderma harzanium* and produced progeny that showed increased growth rate and bio-protection for plants against a range of plant pathogens, including *Pythium* spp., *Alternaria* spp. and *Rhizoctonia* spp., when compared to the parental strains. This success offers much hope for the improvement of other biocontrol fungi.

This part of the study aimed to examine the conditions required to produce high numbers of viable protoplasts from *I. bolleyi,* and assess their use for protoplast fusion and potential improvement of the biocontrol abilities of *I. bolleyi.*

5.2 Methods

Commercial lysing enzymes were used to see if protoplasts could be produced for *I. bolleyi*. On successful protoplast production, the protocol was refined by determining the optimum conditions required. The following Section 5.2.1, describes the standard protocol for optimal production of *I. bolleyi* protoplasts and Sections 5.2.1.1 to 5.2.1.6 describe the experiments used to determine these optimal conditions.

Idriella bolleyi protoplasts were used to evaluate the potential of protoplast fusion to cross strains of *I. bolleyi* following treatment with polyethylene glycol 8000 (PEG). Protoplasts were obtained from auxotrophic mutants of *I. bolleyi*, these mutants being unable to utilise nitrate as sole source of nitrogen because of mutation along the nitrogen reduction pathway (Section 4.3.1). These *nit* mutants were previously tested for vegetative compatibility by pairing mutants on Minimal Medium. Complementation, observed as a thickening of growth at the colony junctions, indicated vegetative compatibility between the paired mutants and possible heterokaryon formation (Section 4.3.3). Pairings of *nit* mutants from strain AB1 often complemented one another, whereas pairings of mutants from strain AB1 with mutants of T560 showed no complementation, implying vegetative incompatibility. Fusion crosses were made with two complementing *nit* mutants, AB1-4 and AB1-31, and also between incompatible *nit* mutants of different strains, AB1-4 with T560-2 and AB1-11 with T560-2. The standard protocol for the fusion experiments is described in Section 5.2.2.

5.2.1 Production of protoplasts from I. bolleyi

Spore suspensions were prepared from 14 day old PDA cultures of *I. bolleyi* (Section 2.2.3) and 0.5 ml aliquots of the suspension were spread onto PDA plates with a sterile glass spreader. The plates were incubated in the dark at 25°C for 4 days then the plates were flooded with sterile distilled water and spore suspensions were again prepared.

The spore concentration was assessed using a haemocytometer and diluted to a standard concentration with sterile distilled water.

Conical flasks (200 ml) containing 25 ml of Standard Liquid Medium (Section 2.1.2) were inoculated with 1 ml of the spore suspension to give a final concentration of c. 5×10^4 spores ml^{-1}. The flasks were incubated on a controlled environment incubator shaker (New Brunswick Scientific Co.) at 170 rpm for 18 h, then the flasks contents were transferred to sterile centrifuge tubes and centrifuged at 18 877 x *g* on a Sorvall RB-5B refrigerated super-speed centrifuge using a Sorvall GSA rotor, for 15 min. The supernatant was removed and the pellet was resuspended in 5 ml of 0.4 M $MgCl_2$ solution (Section 2.1.3) then re-centrifuged at 18 877 *g* for 15 min. The supernatant was removed and (unless stated) 60 mg (fresh weight) of mycelium was resuspended in 2 ml of lysing enzyme solution comprising lysing enzyme (Sigma) (10µg ml^{-1}) and ß-glucuronidase (Sigma) (10µg ml^{-1}) dissolved in 50 mM sodium maleate buffer (pH 5.8) containing 0.4 M $MgCl_2$ as an osmotic stabilizer. The enzyme-mycelial mixture was transferred to sterile 10 ml conical flasks which were plugged, wrapped in foil and shaken gently on Denley orbital shaker at 25°C in the dark for 4 h.

Protoplasts were collected in a 250 ml conical flask after gently pipetting the flask contents through a sintered glass filter (porosity 1) with a Pasteur pipette. The filtered protoplasts were transferred to sterile centrifuge tubes and centrifuged slowly at 180 x *g* on a MSE Mistral 1000 bench centrifuge. The supernatant was removed and the protoplasts were resuspended in 1 ml of 0.4 M $MgCl_2$ solution. The number of protoplasts was determined using a haemocytometer and appropriate dilutions of the protoplasts were prepared in 0.4 M $MgCl_2$ solution. Aliquots (100 µl) of the dilutions were plated onto Complete Regeneration Medium (PDA containing 0.6 M sucrose as an osmoticum) (Section 2.1.2) and spread with a sterile glass spreader. Controls were used to determine the percentage of colonies that developed from conidia present in the protoplast suspension.

For this, protoplast suspensions were diluted in sterile distilled water and left at room temperature for 45 min so that the protoplasts would lyse before the suspension was plated onto Complete Regeneration Medium as above. Six replicate plates were used for both the protoplasts diluted in 0.4 M $MgCl_2$ solution and for the control. The plates were incubated in the dark at 25°C for 3 to 4 days after which the colony numbers were counted. The number of protoplasts regenerated on the plates was calculated by subtracting the number of conidium-derived colonies obtained on the control plates from the number of colonies obtained on the protoplast plates. The percentage protoplast regeneration could then be calculated from the actual number of protoplasts plated (this number was calculated from the original protoplast count in the undiluted 0.4 M $MgCl_2$ solution), and from the known number of regenerating protoplasts on the plates.

5.2.1.1 Lysing enzymes

A range of lysing enzymes were used to produce protoplasts from *I. bolleyi* so that the enzyme preparation producing the highest numbers of protoplasts could be determined.

Commercial lysing enzymes (Table 5.1) were prepared in 2 ml of 50 mM sodium maleate buffer (pH 5.8) containing 0.4 M $MgCl_2$ as an osmotic stabilizer (Section 2.1.3).

(1) A mixture of Driselase (Sigma), 10mg ml^{-1}, Rhozyme HP150 (Pollock & Poole Ltd), 10mg ml^{-1} and Cellulase CP (John & Sturge, Ltd), 10mg ml^{-1}.

(2) Lysing enzyme (Sigma), 10mg ml^{-1}.

(3) ß-glucuronidase (Sigma), 10µl ml^{-1}.

(4) Snail Szeged (Attila Jozsef University, Szeged, Hungary) 10mg ml^{-1}.

Strain AB1 of *I. bolleyi* was used, with a standard 4 h digestion of 10 mg (fresh weight) of mycelium in 2 ml of enzyme solution as described in Section 5.2.1. Three replicate flasks were used for each lysing enzyme solution. Protoplast numbers in each case were determined using a haemocytometer, and the protoplasts and controls were plated onto Complete Regeneration Medium to determine protoplast regeneration frequency (Section 5.2.1). Using six replicate plates for protoplasts and for a corresponding control.

5.2.1.2 Determination of optimal mycelial age for the production of protoplasts

Shake-flask cultures of *I. bolleyi* strain AB1 and strain T560 were prepared in Standard Liquid Medium as described in Section 5.2.1. Also, shake-flask cultures of a nitrate non-utilising (*nit*) mutant, AB1-4, from strain AB1 (Section 4.2.2) were prepared in Standard Liquid Medium supplemented with asparagine at 1.6g l^{-1}. The flasks were removed from the shaker at 14 h then at hourly intervals until 26 h. Three replicate flasks were used for each strain, including the *nit* mutant, at each sampling time. The mycelium was collected by centrifugation as described (Section 5.2.1) and 60 mg of the mycelium was placed in 10 ml conical flasks containing 2 ml of mixed lysing enzyme solution (Lysing enzyme, 10mg ml^{-1} and ß-glucuronidase, 10µl ml^{1} dissolved in 50 mM sodium maleate buffer containing 0.4 M $MgCl_2$. The flasks were plugged, wrapped in foil and shaken gently on an orbital shaker at 25°C in the dark for 4 h. Protoplast numbers were determined using a haemocytometer.

5.2.1.3 Determination of optimal time for enzymatic digestion of mycelium

The mycelium from 18 h shake-flask cultures of *I. bolleyi* strain AB1 was collected and 20 mg (fresh weight) of mycelium was shaken as above in 10 ml conical flasks

containing 2 ml lysing enzyme solution comprising Lysing enzyme (10mg ml^{-1}) and ß-glucuronidase (10µl ml^{-1}) dissolved in the 50 mM sodium maleate buffer containing 0.4 M $MgCl_2$. Aliquots (20µl) of the flask contents were removed at 1 h then at hourly intervals

Table 5.1: Commercial lysing enzymes used in the production of protoplasts from *I. bolleyi*

Enzyme Preparation	Source Organism	Manufacturer/Supplier*
Cellulase CP	*Penicillium funiculosum*	John Sturge Ltd., Selby, North Yorkshire.
Driselase	*Irpex lacteus*	Kyowa Hakko Kiogo Co. Ltd., Ohtemachi Building. Ohtemachi, Chiyoda-Ku Tokyo, Japan. *Sigma Chemical Co.
Lysing Enzyme	*Trichoderma harzanium*	*Sigma Chemical Co.
β-glucuronidase	*Helix Pomatia*	*Sigma Chemical Co.
Rhozyme HP150	Unknown	Rohm & Haas Co., Independence Mall West, Philadelphia, PA 19105. * Pollock & Poole Ltd. Ladbroke Close, Woodley, Reading, RG5 4DX.
Snail Szeged	*Helix Pomatia*	* Attila Jozsef University, Szeged, Hungary.

until 6 h and protoplast number was assessed with a haemocytometer. Three replicates were used at each sampling time.

5.2.1.4 Microscopic examination of protoplast appearance.

The mycelia from 18 h shake-flask cultures of *I. bolleyi* strains AB1 and T560 were collected as described in Section 5.2.1 and 20 mg (fresh weight) was incubated as above in 10 ml conical flasks containing 2 ml lysing enzyme solution (Lysing enzyme, 10mg ml^{-1}, and ß-glucuronidase, 10µl ml^{-1}). Aliquots (20µl) were removed at 1 h then at hourly intervals until 6 h and the protoplasts were measured using a calibrated eye piece, with twenty protoplasts for each strain at each sampling time. The appearance of the protoplasts was also noted. The flasks were then incubated overnight on an orbital shaker at 25°C, then the appearance and size of protoplasts (20 per sample) was again recorded.

5.2.1.5 Assessment of nuclear number in protoplast production

Six flasks of protoplasts were prepared as above and the flasks were harvested at hourly intervals from 1 h until 6 h. Protoplasts from each flask were collected in a 250 ml conical flask after filtration through a sintered glass filter, and the collected protoplasts were transferred to sterile centrifuge tubes and slowly centrifuged at 180 x *g* on a MSE Mistral 1000 bench centrifuge. The protoplasts from each flask were resuspended in 0.5 ml of 0.4 M $MgCl_2$ solution containing 4'6'-diamididino-2-phenyl indole dihydrochloride (DAPI; Sigma) at 10µg ml^{-1}. Twenty µl of the protoplast suspension was pipetted onto a slide and a coverslip was placed on top. The slide was observed on a Leitz Orthoplan microscope using a x70 oil immersion objective with a Ploemopak 2.1 fluorescence vertical illuminator supplying UV light from an HBO-200 mercury vapour lamp, a Filter block A, incorporating

a BP 330-385 nm exciting filter (ultraviolet), TK400 dichroic beam splitter mirror and K400 suppression filter. DAPI is a nuclear binding stain that gives a blue fluorescence under UV irradiation. Nuclei stained with DAPI were clearly seen within the protoplast on each of three replicate slides. Two hundred protoplasts were assessed at each sampling time.

5.2.1.6 Assessment of protoplast viability

Three flasks of *I. bolleyi* protoplasts, strain AB1, were produced and collected as described in Section 5.2.1. Freshly harvested protoplasts from one flask were resuspended in 0.5ml of 0.4 M $MgCl_2$ solution containing fluorescein diacetate (FDA; Sigma) at 10µg ml^{-1}. Twenty µl of the protoplast suspension was pipetted onto a slide and a coverslip was placed on top. The slide was observed on a Leitz Orthoplan microscope using a x 70 oil immersion objective, as previously described, except that Filter block H was used, incorporating a BP 390-490 nm exciting filter (violet and blue), TK455 dichroic beam splitter mirror and K460 suppression filter. Harvested protoplasts from the two remaining flasks were left to stand in 0.5 ml of 0.4 M $MgCl_2$ solution at room temperature. FDA was added at 10µg ml^{-1} to one lot of protoplasts at 30 min and to the second lot at 1 h. On addition of FDA to each protoplast suspension, 20µl of the suspension was immediately pipetted onto a slide with a coverslip and examined by fluorescence microscopy. Fluorescein diacetate is a vital stain which is hydrolysed within living cells to release fluorescein. The fluorescein which accumulates in the cytoplasm produces a bright green fluorescence under blue light. Three replicate counts of 200 protoplasts were made for the freshly harvested protoplasts and for the protoplasts left to stand for 30 min and 1 h. The percentage of protoplasts fluorescing green was determined.

5.2.2 Protoplast fusion

Nitrate non-utilising (*nit*) mutants of *I. bolleyi* designated as AB1-4, AB1-11, AB1-31 and T560-2 (Section 4.2.2) were used for fusion experiments. Fusions were attempted between the vegetatively compatible *nit* mutants AB1-4 and AB1-31; and between the vegetatively incompatible *nit* mutants AB1-4 and T560-2, and AB1-11 and T560-2. Protoplasts of each *nit* mutant were produced from 800 mg (fresh weight) of 18 h mycelium using the standard protoplast protocol described in Section 5.2.1. The number of protoplasts from each mutant was determined using a haemocytometer and 100 µl of the protoplast suspensions were removed and diluted appropriately in 0.4 M $MgCl_2$ solution according to the protoplast count. Aliquots (100 µl) of the diluted protoplasts were spread onto Complete Regeneration Medium using a sterile glass spreader. Ten replicate plates were used for each mutant. These plates served as controls to assess percentage regeneration and further controls were prepared to determine the number of colonies that derived from conidia (Section 5.2.1).

Protoplasts (10^5 to 10^7) of each mutant (in each fusion experiment) were mixed by placing into a centrifuge tube and pelleted by centrifugation at 180 x *g* on a MSE Mistral 1000 bench centrifuge for 10 min. The supernatant was removed and the protoplasts were resuspended in 500 µl polyethylene glycol solution (30% PEG 8000; 50 mM glycine; 10 mM $CaCl_2$; pH 7.5). After 10 min at room temperature, a small sample of the suspension was examined under a microscope to determine whether the protoplasts were aggregated. The remaining suspension was diluted with 3 ml of 0.4 M $MgCl_2$ and the protoplasts were centrifuged as before. After removal of the supernatant the protoplasts were resuspended in 1 ml of 0.4 M $MgCl_2$ and the number was determined using a haemocytometer. To determine the percentage of protoplasts regenerating from the fusion mixture, a 100 µl aliquot of the fusion mixture was diluted appropriately in 0.4 M $MgCl_2$ according to the count, and spread onto Complete Regeneration Medium. Ten replicate plates were used. Controls were prepared as described above to assess the percentage of colonies derived from conidia, and the protoplast plates and the control plates were incubated for 3 to 4 days

in the dark at 25°C. The colonies on the plates were counted and the percentage protoplast regeneration was calculated. Aliquots (50 µl) of the remaining protoplast fusion mixture was spread undiluted onto Minimal Regeneration Medium (containing 0.6 M sucrose as an osmoticum) using a sterile glass spreader. Sixteen replicate plates were used. The plates were incubated in the dark, at 25°C for 3 to 4 days, then any possible fusion products were identified as prototrophic colonies of wild-type appearance against a background of thin sparse growth of the *nit* mutants (Section 4.2.1). The number of prototrophic colonies were counted on each plate and the percentage fusion frequency could be calculated from the known number of protoplasts in the original undiluted solution. The fusion products were removed from the plates by cutting a 2 x 2 mm square of agar in the centre of the prototrophic colonies and transferring these to fresh Minimal Medium plates. The plates were incubated in the dark at 25°C to assess for continued prototrophic growth and colony appearance.

5.3 Results

5.3.1 Comparison of the effects of commercial lysing enzymes

Strain AB1 of *I. bolleyi* was used with a standard 4 h incubation of mycelium in a number of different commercial lysing enzymes, used either as a single enzyme preparation, or in combination (Section 5.2.1.1).

Protoplast yield, as determined with a haemocytometer, varied for each lysing enzyme solution (Table 5.2). The highest protoplast yield of 10^5 ml^{-1} was recorded for the single enzyme preparation Lysing enzyme followed by the enzyme solution containing Cellulase CP, Driselase and Rhozyome HP150 (C.D.R.) which gave a yield of 9×10^4 ml^{-1}.

Low protoplast yields of 1 x 10^4 ml^{-1} and 3 x 10^4 ml^{-1} were recorded for single preparation enzymes, β-glucuronidase and Snail Szeged respectively.

Protoplasts plated onto Complete Regeneration Medium as described in Section 5.2.1 regenerated rapidly, with colonies appearing on the medium after only 2 to 3 days. The percentage of regeneration was influenced by the preparation of commercial lysing enzyme used to digest the mycelium (Table 5.2). The highest level of protoplast regeneration was obtained from protoplasts produced by Lysing enzyme. Low regeneration was observed from protoplasts produced by ß-glucuronidase and also Snail Szeged.

The percentage of colonies deriving from conidia in the protoplasts preparations was found to be between 0.24 and 0.83% for all regeneration plates, regardless of which enzyme was used to produce protoplasts.

5.3.2 Effect of mycelial age on protoplast production

The optimal culture age for protoplast production was determined by growing *I. bolleyi* cultures in Standard Liquid Medium as described in Section 5.2.1. The mycelium was harvested at hourly intervals from 14 h until 26 h and digested for a standard time of 4 h in a lysing enzyme solution comprising Lysing enzyme (10μg ml^{-1}) and ß-glucuronidase (10μl ml^{-1}). Protoplast numbers at each age were assessed using a haemocytometer.

Mycelial age was found to be important in obtaining the maximum protoplast yield (Figure 5.1). Optimal mycelial age for *I. bolleyi* strains AB1 and T560 was found to be 4 h after conidial germination (18 h old culture). Protoplast yield recorded from 60 mg fresh weight of mycelium showed that very young germlings of 14 h produced a low protoplast number of 17.1 x 10^4 ml^{-1}, 16.8 x 10^4 ml^{-1} and 17.9 x 10^4 ml^{-1} for the AB1 *nit* mutant AB1-

4, strain T560 and strain AB1 respectively. The yield slowly increased as the germlings became older reaching a peak for 18 h cultures. The protoplast number at 18 h was 43 x 10^4 ml^{-1}, 109.6 x 10^4 ml^{-1} and 121.3 x 10^4 ml^{-1} for the AB1 *nit* mutant AB1-4, strain T560 and strain AB1 respectively. After 18 h, protoplast number rapidly declined, and at 24 h the protoplast yield was less than that recorded at 14 h. No protoplasts were produced from cultures older than 24 h.

Table 5.2: Protoplast yields (x 10^4 ml^{-1}) from *I. bolleyi* strain AB1 using commercial lysing enzymes dissolved in 50 mM sodium maleate buffer (pH 5.8) containing 0.4 M $MgCl_2$ as an osmotic stabiliser (Means ± SEM for 3 replicates), and protoplast regeneration on Complete Regeneration Medium (PDA medium supplemented with 0.6 M sucrose as an osmoticum), shown as percentage based on 18 replicate plates.

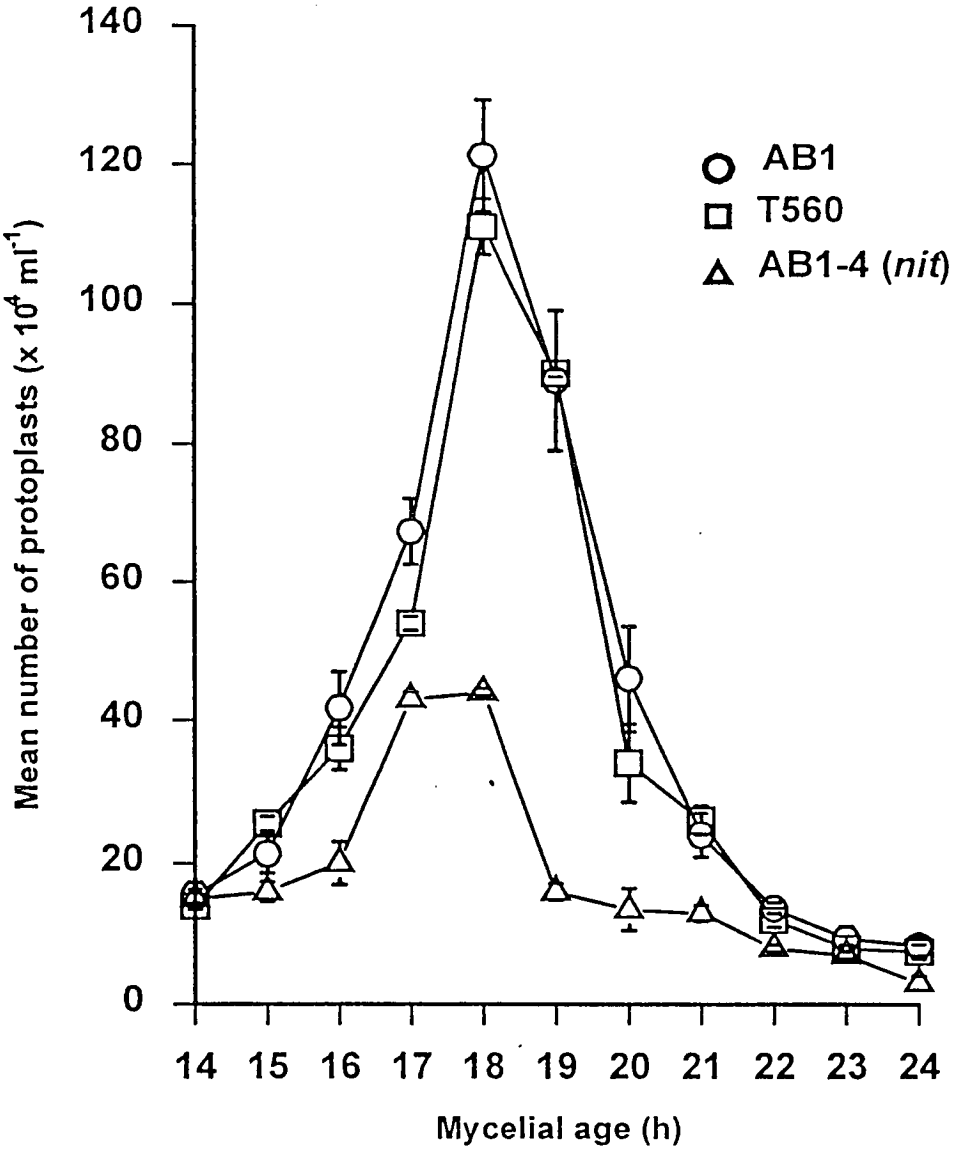
Commercial lysing enzyme	**Protoplast yield (x 10^4 ml^{-1})**	**% Protoplast regeneration on Complete Regeneration Medium**
Lysing enzyme	10 ± 1.0	15.85
C.D.R*	9 ± 2.6	6.35
Snail Szeged	3 ± 0.0	1.57
ß-glucuronidase	1 ± 0.5	0.04

* C.D.R. = Cellulase CP + Driselase + Rhozyome HP150

5.3.3 Assessment of optimal enzymatic digestion time

Mycelium (18 h) of *I. bolleyi* strain AB1 was grown in Standard Liquid Medium as described in Section 5.2.1. Protoplast numbers were assessed with a haemocytometer at

Figure 5.1: The effect of mycelial age on protoplast yield (x 10^4 ml^{-1}) from *I. bolleyi* strains AB1, T560 and a nitrate non-utilising (*nit*) mutant AB1-4 (means ± SEM for 3 replicates).



1 h and then at hourly intervals for 6 h, during enzymatic digestion of the mycelium in a lysing enzyme solution comprising Lysing enzyme (10µg ml^{-1}) and ß-glucuronidase (10µl ml^{-1}). Optimal enzymatic digestion time for 20 mg fresh weight of 18 h old mycelium of *I. bolleyi* strain AB1 was found to be at 5 h (Table 5.3). Protoplast yield recorded at hourly intervals showed low protoplast numbers of 4.25 x 10^4 ml^{-1} at 1 h; the protoplast number increased gradually and at 5 h the number recorded was 45.73 x 10^4 ml^{-1}. At 6 h protoplast number had not increased and was recorded at 44.23 x 10^4 ml^{-1}.

Table 5.3: Optimal time for protoplast release from 18 h mycelium of *I. bolleyi* (strain AB1) in lysing enzyme solution comprising Lysing enzyme (10µg ml^{-1}) and ß-glucuronidase (10µl ml^{-1}). Protoplast numbers were recorded at 1 h then at hourly intervals until 6 h. (Mean ± SEM for 3 replicates).

Time (h)	Number of protoplasts (x 10^4 ml^{-1})
1	4.3 ± 1.3
2	9.3 ± 1.0
3	23.8 ± 3.2
4	42.8 ± 7.5
5	45.7 ± 6.3
6	44.2 ± 6.8

5.3.4 Protoplast appearance during production

During the enzymatic digestion of 18 h *I. bolleyi* mycelium (strains AB1 and T560), samples of protoplasts were removed from an enzyme lysing solution comprising of Lysing

enzyme (10µg ml^{-1}) and ß-glucuronidase (10µl ml^{-1}), at 1 h then at hourly intervals until 6 h, and then after overnight incubation of the mycelium in the lysing enzyme solution. The protoplasts were examined microscopically for their appearance and measured with a calibrated microscope eyepiece.

Protoplast appearance was observed to change during mycelial digestion, and the protoplasts showed much heterogeneity in size. Protoplasts of strain AB1, produced during the first hour were generally of a small size, varying between 2 and 3 µm diameter. Protoplasts produced between 2 and 6 h of mycelial digestion were slightly larger varying between 2 and 6 µm diameter. Protoplasts of strain AB1 produced between 1 and 6 hours of mycelial digestion were generally granular in appearance and often contained large vacuoles. Cell organelles could often be seen moving within the cytoplasm of the protoplasts. Overnight digested mycelium, from strain AB1, produced protoplasts that were considerably larger than those produced between 1 and 6 h digestion time, the protoplast size ranging from 9 to 12 µm diameter. These large protoplasts did not have a granular appearance, instead, had a 'ghost-like' faint-grey appearance. Protoplasts produced from strain T560 between 1 and 6 h varied in size between 2 and 6 µm diameter, and the protoplasts had a granular appearance similar to the protoplasts observed for strain AB1. Overnight digestion of T560 did not produce larger protoplasts as was seen for strain AB1, indeed the protoplast number was reduced by about 90% of the number obtained at 6 h, indicating that protoplasts may have lysed during extended incubation.

5.3.5 Assessment of protoplasts containing nuclei during enzymatic digestion of mycelium

Mycelium (18 h) of *I. bolleyi* strain AB1 was digested in a lysing enzyme solution comprising Lysing enzyme (10µg ml^{-1}) and ß-glucuronidase (10µl ml^{-1}). The protoplasts were harvested at 1 h then at hourly intervals until 6 h and suspended in a 0.4 M $MgCl_2$

solution containing the fluorescent nuclear stain, DAPI. The stained protoplasts were examined under UV light for the presence of nuclei (Section 5.2.1.5).

The number of nuclei in the protoplasts increased with digestion time of the mycelium. (Table 5.4). Nuclei were not seen in the protoplasts until after 2 h of enzymatic mycelial digestion. After 3 h the number of nucleate protoplasts had risen considerably, with 26% of protoplasts containing a nucleus. Numbers of protoplasts containing nuclei continued to rise gradually during enzymatic digestion of the mycelium, reaching 82% of total protoplast number at 6 h. Less than 1% of the protoplasts contained more than one nucleus.

Table 5.4: The number of *I. bolleyi* (strain AB1) protoplasts containing nuclei. Protoplasts were harvested at hourly intervals from 1 h until 6 h, from 18 h mycelium digested in a lysing enzyme solution (Lysing enzyme, 10µg ml^{-1} and ß-glucuronidase, 10µl ml^{-1}) and stained with the nuclear binding stain DAPI. Data are means ± SEM for 3 replicate samples of 200 protoplasts each.

Mycelial digestion in lysing enzyme solution (h)	**Number of protoplasts containing nuclei (max. 200)**
1	0
2	60.3 ± 17.8
3	52.4 ± 12.6
4	96.2 ± 30.6
5	154.0 ± 44.8
6	164.0 ± 27.2

5.3.6 Determination of protoplast viability

Protoplasts either freshly harvested or left to stand for 30 min or 1 hour were examined by fluorescence microscopy after the addition of the vital stain Fluorescein diacetate (FDA) (Table 5.5). The green fluorescence seen under blue light indicates protoplast viability because FDA must be cleaved by esterases in the protoplasts before it can fluoresce. Of the freshly harvested protoplasts, 99% (200 counted, 3 replicate counts) showed viability as indicated by an intense green fluorescence with FDA (Figure 5.2). Thirty minutes after harvesting, 83% of protoplasts stained with FDA, produced a green fluorescence and the value decreased to 57% when protoplasts were incubated for 1 h before FDA was applied. This results suggest that protoplasts of *I. bolleyi* rapidly lose viability during storage.

Table 5.5: The number of *I. bolleyi* (strain AB1) protoplasts (out of 200 counted ± SEM for 3 replicate counts) that produced a green fluorescence in the presence of fluorescein diacetate when this compound was added to protoplasts that were freshly harvested or stored in 0.4 M $MgCl_2$ for 30 min or 1 h before FDA was added.

Time after protoplast harvesting (min)	**Mean number of fluorescing (viable) protoplasts (max. 200)**
(Freshly harvested)	197.2 ± 8.2
30	166.4 ± 14.6
60	114.0 ± 13.5

5.3.7 Protoplast fusion

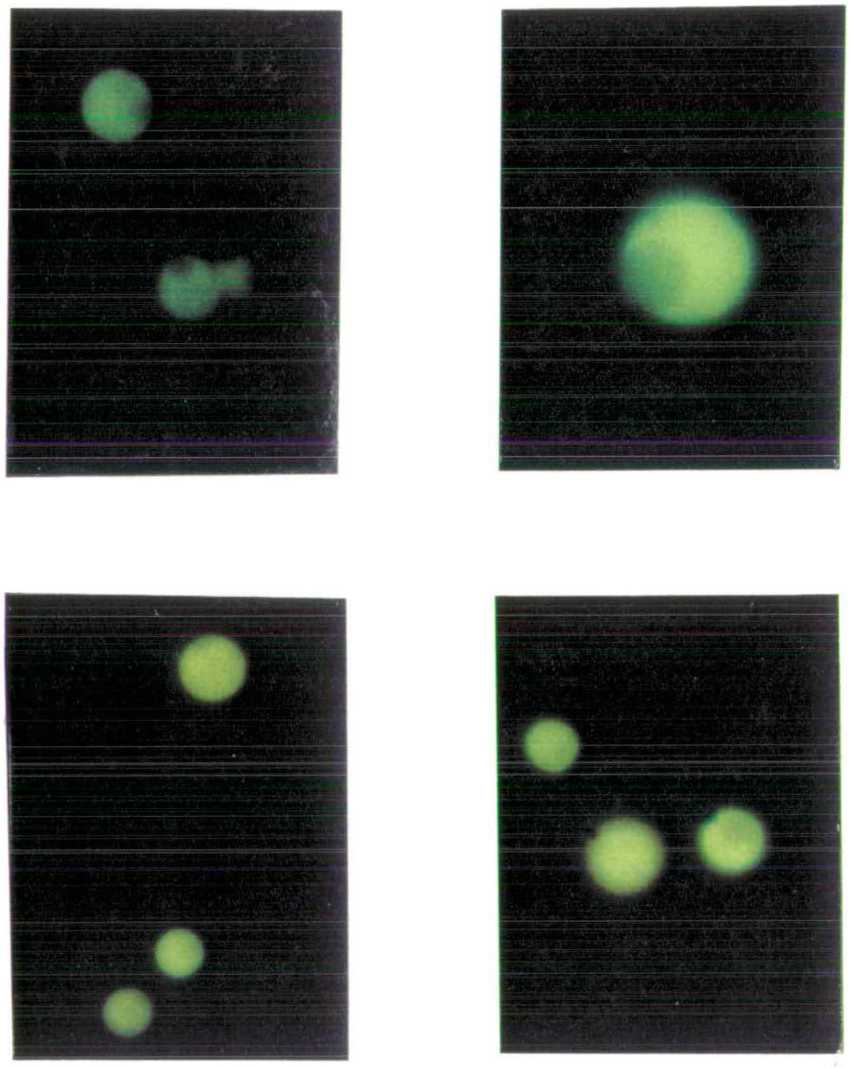
Protoplast fusion products were successfully obtained in three crosses of *I. bolleyi* nitrate non-utilising (*nit*) mutants; these crosses were between the vegetatively compatible

nit mutants AB1-4 and AB1-31 and also between the vegetatively incompatible *nit* mutants, AB1-4 and T560-2, and AB1-11 and T560-2. A mycelial fresh weight of 800 mg gave high protoplast numbers of 10^5 to $10^7 ml^{-1}$. To increase the effective concentration of protoplasts in the mixture during fusion, a low volume (500µl) of PEG solution was used. Upon addition of the PEG solution, microscopic examination of the protoplasts showed that the protoplasts were aggregating and often looked misshapen, probably due to shrinkage as a result of the osmotic pressure generated by PEG 8000.

The number of protoplasts in a suspension seemed to influence the efficiency of protoplast regeneration of 'parental' mutant strains (Table 5.6). Protoplast numbers of mutant T560-2 were high at about $9 \times 10^7 ml^{-1}$. and Table 5.5 shows that 25% or more of these protoplasts regenerated to form colonies. In contrast, a much lower percentage regeneration was found for all the mutant strains of AB1, which had protoplast numbers ranging from about 2 to $4 \times 10^7 ml^{-1}$.

Complete Regeneration Medium was used to plate protoplasts in the non-mixed treatments. The same medium was also used to plate the protoplast fusion mixtures. The regeneration frequency of each separate parent in cross 1 was 6.9% and 8.7 % for AB1- 4 and AB1-31 respectively. For cross 2 the regeneration of the separate parental strains was 4% and 39% for AB1-4 and T560-2 respectively. For cross 3 the regeneration of the separate parental strains was 6% and 25% for AB1-11 and T560-2 respectively. Protoplast fusion products were recovered by plating undiluted protoplasts onto Minimal Regeneration Medium. Possible fusion products were recognised as darkly pigmented, irregular, prototrophic colonies growing on a background of thin sparse growth normally observed for *nit* mutants (Figures 5.3 to 5.4). Assuming the prototrophic colonies were fusion products, the actual frequency of fusion was low for all three crosses but the level of protoplast regeneration from the mixture on Complete Regeneration Medium seemed to

Figure 5.2: Viable protoplasts of *I. bolleyi* stained with the vital stain Fluorescein Diacetate (FDA). FDA is hydrolysed within living cells to produce a green fluorescence under blue light.



influence the fusion frequency. The highest percentage of fusion was recorded at 0.02% for the cross between AB1-4 and AB1-31. The fusion frequency for cross 3, between AB1-11 and T560-2, was recorded at 0.01%. Cross 2, between AB1-4 and T560-2 gave a fusion frequency of 0.006%.

The prototrophic colonies from each cross were transferred onto fresh Minimal Medium and growth was assessed for morphology, sectoring and nitrate utilisation. All prototrophic colonies from the cross between AB1-4 and AB1-31 continued to show wild-type growth after sub-culturing to fresh Minimal Medium. Morphology was similar for all colonies and no sectoring was observed. For the two crosses of *nit* mutants, AB1-4 x T560-2 and AB1-11 x T560-2, some prototrophic colonies differed slightly in their morphological appearance and the majority of colonies seemed unstable and showed sectoring after about 4 to 5 days of growth following sub-culturing (Figure 5.5). From the colonies sub-cultured, 15 from the AB1 x T560-2 cross, and 26 from the AB1 11 x T560 cross reverted back to thin sparse growth that was associated with the parental *nit* mutants immediately upon sub-culturing, or after only 2 to 3 days of prototrophic growth on Minimal Medium (Figure 5.6). The conidia from the prototrophic colonies were not tested for their phenotype, therefore it is not known which genetic event, such as heterokaryosis, hybridisation or any, had occurred to produce this type of growth.

5.4 Discussion

Protocols for the production of protoplasts have been developed in a number of fungi including *Rhizoctonia solani* (Hashiba & Yamada, 1982). *Psuedocercosporella herpotrichoides* (Hocart *et al.*, 1987) and *fulvia fulva* (Harling *et al.*, 1988). Generally lysing enzymes have been used most commonly for protoplast release from fungal mycelium.

Table 5.6: Protoplast regeneration and fusion between vegetatively compatible and incompatible nitrate non-utilising (*nit*) mutants of *I. bolleyi* strains AB1 and T560

Cross number	Parental strain	Vegetative compatibility	Protoplast numbers of parental strains x 10^7 ml^{-1}	% Regeneration of parental strains	% Regeneration of fused protoplasts	% fusion frequency
1	AB1-4		2.26	6.9		
	AB1-31	Yes	4.13	8.7	26.0	0.02
2	AB1-4		2.26	4.0		
	T560-2	no	9.07	39.0	8.7	0.006
3	AB1-11		3.05	6.0		
	T560-2	no	9.07	25.0	3.3	0.01

Figure 5.3: Fusion between the *nit* mutants AB1-4 and AB1-31 resulted in the growth of irregular prototrophic colonies (indicated by arrow) seen against a background of thin sparse growth normally observed for *nit* mutants, suggesting the possible formation of fusion products. Prototrophic colonies developed on selective Minimal Regeneration Medium. (photographed approximately 10 days after plating the protoplasts onto the medium).

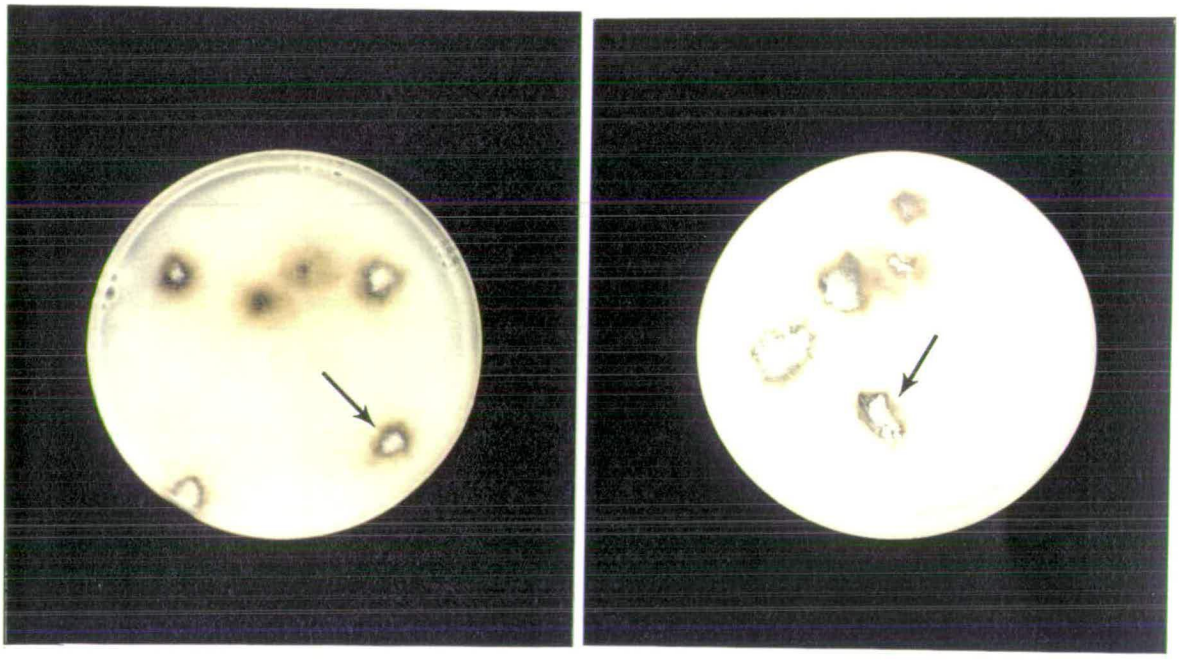


Figure 5.4: Fusion between the *nit* mutants AB1-4 and T560-2 resulted in the growth of irregular, dark prototrophic colonies (indicated by arrow) seen against a background of thin sparse growth normally observed for *nit* mutants, suggesting the possible formation of fusion products. Prototrophic colonies developed on selective Minimal Regeneration Medium. (photographed approximately 10 days after plating the protoplasts onto the medium).

Figure 5.5: After plating fused protoplasts of the *nit* mutants AB1-4 and T560-2 on Minimal Regeneration Medium, prototrophic colonies were sub-cultured onto fresh Minimal Medium. Many colonies continued to show wild-type growth, but growth appeared unstable and sectoring was observed (indicated by arrows) 2 to 3 days after their transfer to fresh medium. (Photographed 4 days after sub-culturing onto fresh Minimal Medium).

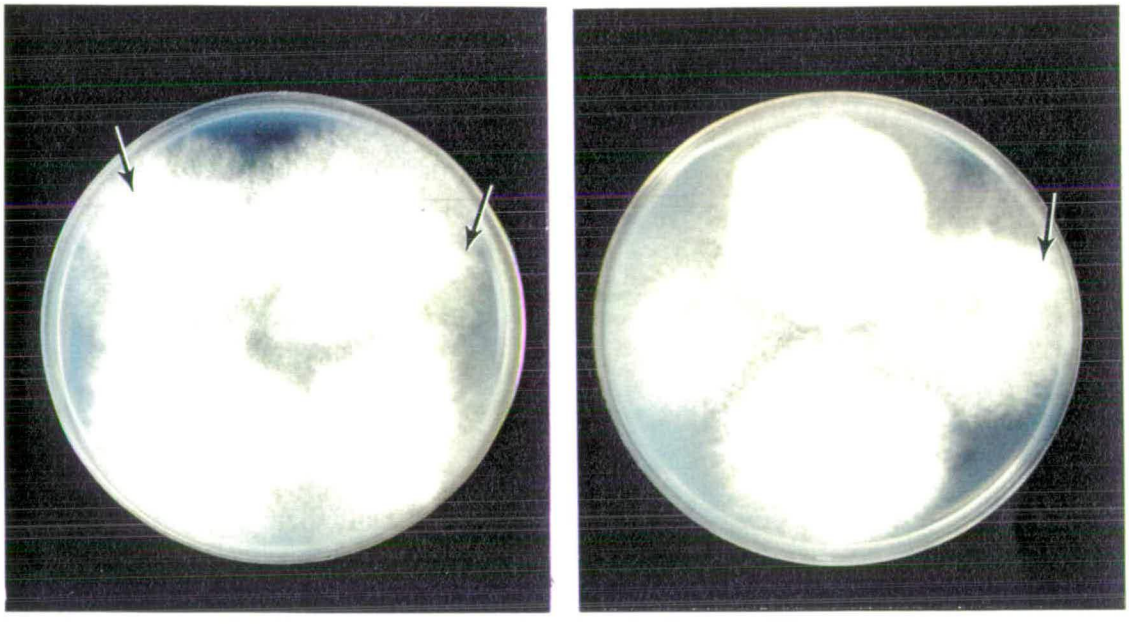
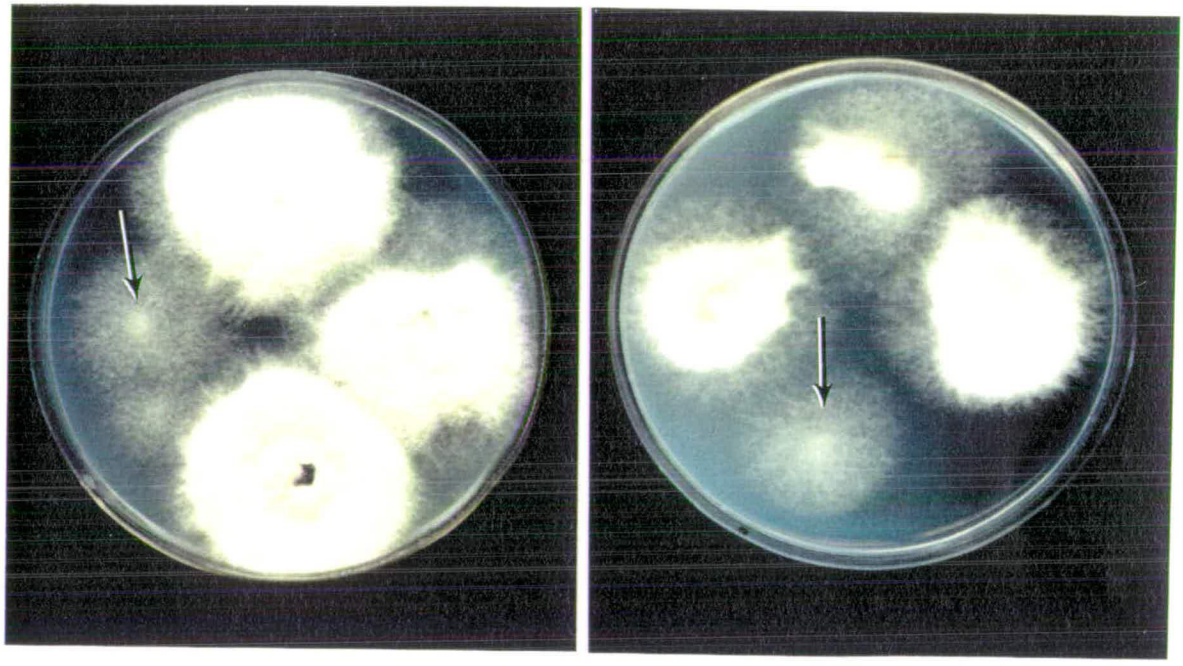


Figure 5.6: After plating fused protoplasts of the *nit* mutants AB1-4 and T560-2 on Minimal Regeneration Medium, prototrophic colonies were sub-cultured onto fresh Minimal Medium. Upon sub-culturing many colonies resumed growth as thin sparse colonies (indicated by arrows) normally observed for *nit* mutants. WT = colonies continuing to show prototrophic wild-type growth after sub-culturing. (Photographed 4 days after sub-culturing onto fresh Minimal Medium).



The type of commercial enzyme used for each fungi species varies considerably and often the use of a combination of enzymes is more effective than one used on its own; for each fungal species, this merits empirical investigation. For most enzymes, the specific activity is not known, but a range of enzymes will have different specific activities. As the diversity of cell wall composition in fungi is great (Bartnicki-Garcia, 1968), it is understandable that the enzyme or combination of enzymes will differ considerably for each fungal species. Some major components of enzymes include chitinase, cellulase, glusulase, ß-glucuronidase and protease (Peberdy, 1979). Glusulase and chitinase have been found to be effective against *Aspergillus niger* and *Aspergillus fumigatus* (Peberdy, 1979). For *P. herpotrichoides,* a combination of Cellulase CP, Driselase and Rhozyme HP150 (C. D. R.) was found to be

most suitable for the release of protoplasts (Hocart *et al.,* 1987). Rhozyme is an enzyme generally used for plant protoplast production rather than for fungal protoplast production (Davey, 1983). For protoplast production from *P. herpotrichoides,* ß-D-glucanase and chitinase activity are believed to be important in the enzyme mixture described above, as it is present in all three components. Hocart *et al.,* (1987) believed that Rhozyme HP150 plays a role in the removal of a mucilage layer known to surround the hyphae of *P. herpotrichoides* (Riess, 1971), allowing the enzymes more accessibility to the cell wall. Eveleigh *et al.,* (1968) showed that cellulase and laminarase were essential for protoplast release from *Pythium* spp., thus highlighting the major difference in cell wall composition between this and other fungal species.

A C.D.R. mixture was found to be successful in releasing protoplasts from the cell walls of *I. bolleyi*, although a lysing enzyme mixture from *Trichoderma harzanium* was found to be even more effective for protoplast release. The lysing enzyme from *T. harzanium* has been found necessary for protoplast release in *Schizophyllum commune,* and was found to be a complex containing S-glucanase (α -1,3,-glucanase), and R-glucanase (ß-1,3,-, ß-1,6-glucanase) and also chitinase (de Vries & Wessel, 1973). Although the cell wall

composition of *I. bolleyi* has not been defined, successful breakdown of the cell wall by commercial enzymes suggests the wall is composed of chitin-glucan, also, Bartnicki-Garcia (1968) stated that the walls of Deuteromycetes may be chitin-glucan ß-1,3 and ß-1,6 linked.

With regards to most enzymes, Hamlyn *et al.*, 1981 stressed that unspecified side activities are important in determining the effectiveness of a particular enzyme.

One of the most important factors stated in the literature as influencing protoplast yield is mycelial age. *I. bolleyi* grows quickly in liquid culture and germination occurs within 14 h. Sporulation within the liquid culture can occur within 48 h (Lascaris & Deacon, 1991). From growth in liquid culture, *I. bolleyi* germlings of 18 h were found to produce the highest yield of protoplasts, and before or after this time yield was decreased. For other slower growing fungi, an older culture age is required. *Fulvia fulva* required cultures aged between 24 to 48 h (Harling *et al.*, 1988), whereas *P. herpotrichoides* required a 44 h culture for optimal protoplast production (Hocart & McNaughton, 1994). For *Rhizoctonia solani*, a culture age of around 20 h was found most suitable for high protoplast yield and mycelium aged at 3 to 4 days produced very low protoplast numbers (Hashiba & Yamada, 1982).

Optimal mycelial age has been associated with a culture in the exponential phase of growth. This has been confirmed with protoplast yields from *A. flavus* (Peberdy *et al.*, 1976). In *Penicillium chrysogenum*, a second optimal peak in mycelial age was at the stationary phase of growth (Anné *et al.*, 1974). Yeast cells have been found to have optimal culture age for protoplast release, with cells in exponential culture giving highest yields. It was found also that yeasts cells in the stationary phase were resistant to lysis and this development of resistance is associated with RNA and protein synthesis (Deutch & Parry, 1974).

Mycelium in the exponential growth is believed to be at the stage of maximum branching and cell wall formation (Peberdy, 1979). A region close to the hyphal tip is most susceptible to lysis, this area being the thinnest and actively undergoing cell wall synthesis (Hocart & Peberdy, 1989). Many workers have added compounds that alter the cell wall physically and this has been found to improve protoplast yields. For most yeasts and filamentous fungi, thio compounds have been added to the lytic mixture to alter the disulphide bonds in the wall proteins. The result is the opening up of molecules in the cell wall thus favouring the passage of lytic enzymes (Anderson & Millbank, 1966). Triton-X-100 has been used to remove the lipid layer of *Pythium* spp., thus improving protoplast yields (Sietsma & Boer, 1973).

Protoplasts are released through small pores in the wall from cells closest to the hyphal tip (Hocart & Peberdy, 1989). A large degree of heterogeneity was observed for *I. bolleyi* protoplasts and it was noted that with strain AB1 in particular, longer incubation in the lysing enzyme solution resulted in extremely large protoplasts. The reason for this is not known, but may be associated with a larger pore size in the hyphae developed as a result of increased cell wall digestion. Alternatively regions of the hyphae other than that near to the tip may have been lysed, or complete parts of the hyphae may have been lysed to form protoplasts. Peberdy (1979) stated that uncommonly, whole parts of the hyphae can be turned into protoplasts. Hocart & Peberdy (1989) also stated that protoplasts are likely to vary in size and also in cell organelles. Peberdy & Gibson (1971) found that with *A. nidulans*, during the initial hours of hyphal digestion, small non-vascular protoplasts were produced from the hyphal tips which had a high density of ribosomes, mitochondria, nuclei and endoplasmic reticulum. After 3 h, larger protoplasts of up to 13 μm diameter were present and these had large vacuoles and less granular cytoplasm. *Idriella bolleyi* protoplast appearance indicated that there may have been different cell components released in the protoplasts with increasing digestion. The presence of large 'ghost-like', ungranular protoplasts after overnight incubation does suggest that these protoplasts were released

from areas other than the hyphal tip. The early released protoplasts, showed a high level of vacuolation and a granular appearance and may well have been released mainly from the hyphal tip region. Further evidence for the release of protoplasts from different regions of the hyphae comes from the increasing number of protoplasts containing nuclei with increasing digestion time. Similar results of differing nuclei number was also found in a study with *P. chrysogenum*, where early released small non-vacuolate protoplasts had on average 2 to 3 nuclei, whereas later released protoplasts only had about 1 or 2 nuclei (Anné *et al.*, 1974). A more detailed study of organelle release would be valuable for *I. bolleyi*, as this may lead to improvement of protoplast regeneration frequency and also fungal transformation.

Protoplast release from conidia of *I. bolleyi* was not attempted. Isolation from the spores of other fungi have been achieved, resulting in more homogeneous sized protoplasts than was obtained from fungal mycelium. Protoplasts have been obtained from the conidia of *Trichoderma reesi* with the use of *Trichoderma viride* lysing enzymes. Generally protoplasts produced from spores require extended periods of lytic digestion as the conidial walls are more resistant than hyphal walls (Toyama *et al.*, 1984).

After finding the optimal mycelial age for protoplast release, their subsequent handling and regeneration are important to maintain protoplast numbers and viability. Generally inorganic salts such as potassium, sodium and magnesium have been used as osmotica for filamentous fungi, whereas sugars and sugar alcohols have more commonly been used for yeasts (Peberdy, 1979). For *Penicillium* spp., *Cephalosporium* spp. and *Aspergillus* spp., 0.7 M NaCl was found to be a suitable stabiliser (Anné & Peberdy, 1976). $MgCl_2$ is a commonly used stabiliser for *P. herpotrichoides* (Hocart & McNaughton, 1994) and 0.6 M mannitol was found most suitable to maintain the viability of *Rhizoctonia solani* even up to four days. For the production of *I. bolleyi* protoplasts, it is possible that the 0.4 M $MgCl_2$ osmotic stabiliser, used to prevent lysis, was not optimal. This statement can be

made in the knowledge that no other stabiliser was tested. Protoplast numbers did not appear to be reduced as a result of lysis in $MgCl_2$, however a large number of protoplasts were lost in the collection phase after filtration to remove mycelial debris. The reason for the reduction in number is likely to be due to poor pelleting upon centrifugation. The use of $MgSO_4$ as an osmotic stabiliser for *A. nidulans* caused the protoplasts to become boyant as a result of the presence of large vacuoles. This vacuolation was not found when other magnesium salts or sulphates were used. The mycelium of *Schizophyllum commune* fragmented early during enzymatic digestion with $MgSO_4$ as a stabilizer, resulting in the majority of protoplasts with large vacuoles, these could be more easily separated from the mycelium due to the fact that they floated at the surface of the centrifuged mixture (DeVries & Wessels, 1973). It is possible that although $MgCl_2$ did not affect *A. nidulans*, it may have caused a high degree of vacuolation in *I. bolleyi*. As previously mentioned, many *I. bolleyi* protoplasts did contain large vacuoles, thus it is possible that many protoplasts may have floated to the top of the centrifuged mixture and were consequently removed in the supernatant. It is possible that $MgCl_2$ could be used advantageously for *I. bolleyi*. Problems of protoplast contamination from mycelial fragments and conidia were evident and this contamination could be reduced if better separation of the protoplasts could be obtained through floatation. *Rhizoctonia solani* protoplasts require a liquid-liquid, two-phase method of separation. The protoplast suspension was purified by centrifuging in a sucrose-mannitol solution, and the protoplasts were collected at the interface of the two solutions, free of mycelium (Hashiba & yamada, 1982).

Regeneration on 0.6 M sucrose was generally found to vary for *I. bolleyi*. Lower regeneration frequency may have been related to high conidial contamination levels, or it is possible that 0.6 M sucrose is not optimal for regeneration. Further investigation into other suitable regeneration osmotica needs to be examined. For *Fusarium culmorum*, carbon source in the medium influenced the level of regeneration considerably with levels varying from 5 to 82% (Moore & Peberdy, 1976).

As well as unsuitable medium, low regeneration and reversion has been associated with a number of factors, including the lack of a nucleus (Kopecka *et al.*, 1974), and also the origin of the protoplast in relation to hyphal organisation. Peberdy (1979) stated that protoplasts from distal regions may be lacking the capacity for reversion, but the reason why is not fully understood.

Optimal conditions for more protoplast production and high regeneration is critical if protoplasts are to be used in fusion experiments and also for fungal transformation.

Protoplast fusion was successfully achieved with *Geotrichum candidum* by physical compaction of protoplasts following centrifugation (Peberdy, 1987). Modern methods of fusion rely almost entirely on polyethylene glycol. Through extensive testing, Anné & Peberdy (1975) defined the optimal conditions for protoplast fusion which seem to be suitable for most fungal species. Results from fusion experiments with *I. bolleyi* show that such conditions were adequate to obtain fusion products in this species. Generally, PEG is used at a mol. wt of 4000 to 8000 at a concentration of 30%. Below this concentration, PEG loses its stabilising properties and much above this, the osmotic pressure of PEG is prone to lyse protoplasts. A pH of 7 to 9 is essential along with the presence of calcium ions. (10 to 100 mM). Ferenczy *et al.*, (1976) found the presence of calcium ions was highly significant in stimulating fusion over a range from 10 to 100 mM, but in its absence, fusion was negligible.

Upon the addition of PEG solution, the aggregation and distortion observed for *I. bolleyi* protoplasts is a common occurrence during fusion. Anné & Peberdy (1976) observed that protoplasts of various *Penicillium* spp. become dehydrated resulting in shrinkage and protoplast aggregation. PEG is known to dehydrate the cells causing them to draw together and those with touching membranes then become distorted. When close membrane to membrane contact is established it is thought that cytoplasmic bridges form

as a result of contact localised reorganisation of the membrane proteins, these bridges enlarge when two protoplasts fuse (Ferenczy, 1981). Anné & Peberdy (1976) noted the reshapement of the protoplasts after dilution of the fusion mixture and also the presence of larger bodies which were believed to be fused protoplasts. After fusing, the resulting protoplasts may be various aggregates of protoplasts made up of either the same parental strain or a mixture of two parental strains (Peberdy, 1987).

On plating *I. bolleyi* protoplasts after fusion, differences in regeneration were observed for all crosses. In crosses 2 (AB1-4 x T560-2) and 3 (AB1-11 x T560-2), regeneration levels were higher than that obtained with the AB1 parental strain but much lower than the regeneration level recorded for the parent strain T560. These regeneration levels are not unusual. Fusion levels are often lower than that obtained for parental strains because protoplast aggregation can decrease the total number of protoplasts, or many protoplasts may lyse in the PEG solution (Anné & Peberdy, 1975). In cross 1 (AB1- 4 and AB1-31), a regeneration level of 26% was recorded, a 3-fold increase in regeneration than was obtained for the individual parental strains. This increase in regeneration was quite unusual and the reason for it is not known. It may be that the stabilisers, 0.4 M $MgCl_2$ and 0.6 M sucrose, are not optimal for protoplast regeneration and that the protoplasts became more stable in the higher osmotic potential generated by the addition of PEG solution, thus improving regeneration and reversion.

Although protoplast fusion products were recovered from all three crosses of *I. bolleyi nit* mutants, the actual fusion frequency appeared to be very low at less than 1%. Low frequency of fusion in *I. bolleyi* may be related to unsatisfactory fusion conditions. Ferenczy *et al.* (1976) found that protoplast age could influence fusion along with the presence of mycelial debris and the number of protoplasts of each parental strain. Ferenczy *et al.* (1976) found that protoplasts should be as young as possible, pure and protoplast numbers should be about 10^7 and at a ratio 1:1 of the parental strains. *Idriella bolleyi*

protoplasts stained with the vital stain FDA, showed that within 30 min protoplast viability had decreased. In view of this, fusion was carried out as rapidly as possible after protoplast harvesting, thus it is unlikely that viability would have affected the fusion frequency to a significant level. The presence of mycelial debris and conidia in protoplasts suspensions was always a problem for *I. bolleyi,* thus the fusion frequency in all crosses may have been affected by the impurity of the mixture. Protoplast number was always around 10^7, but the ratio of parents was not 1:1 in crosses 2 and 3, thus this may have reduced the fusion level. In cross one, parental protoplasts numbers were about even and regeneration and fusion frequency was higher. It is possible that for *I. bolleyi*, there may be some other conditional factors that may need modification, or else physiological factors such as vegetative incompatibility may be influencing the fusion success. In crosses with *I. bolleyi nit* mutants, lower fusion frequency was recorded between the incompatible AB1 and T560 mutants, than between the compatible AB1 mutants.

The recovery of prototrophic colonies, albeit low as they were, show that protoplast fusion can be used to overcome vegetative incompatibility between *I. bolleyi* strains. When protoplast fusion can overcome incompatibility, this often indicates that incompatibility results from interactions at the level of the cell wall. Where incompatibility is not successful by fusion, it is likely to be related to cytoplasmic or nuclear mechanisms of control, this suggests that incompatibility is a complex process controlled by a number of mechanisms (Peberdy, 1987).

It is unlikely that prototrophic colonies developed as a result of reversion of mutants to *nit*$^+$ phenotype as all mutants seemed stable in culture. Prototrophs from the cross of strain AB1 with strain T560 are unlikely to be produced as a result of cross-feeding. Cross-feeding did not occur when mutants of these strains were paired on Minimal Medium to test for complementation. Cross-feeding may have occurred between fused regenerating protoplasts of the AB1 *nit* mutants on Minimal Medium due to the large number of

protoplasts plated. Hocart & McNaughton (1994) found that cross-feeding occurred when high concentrations of protoplasts of *P. herpotrichoides* and *P. anguioides* were plated. Also it is possible that some of the prototrophic colonies may have developed as a result of heterokaryon formation. The number of prototrophic colonies was low for each plate, so more prototrophic growth would have been expected, if a high level of cross-feeding was occurring.

Prototrophic products following fusion are classically believed to fall into two categories; progeny developed as a result of heterokaryosis or from recombination within a single nucleus (Harman & Stasz, 1991). Sub-culturing of prototrophs from *I. bolleyi* onto fresh Minimal Medium enabled the morphology and growth to be compared. Fusion between AB1 mutants resulted in prototrophic colonies that were similar in morphology and which were relatively stable showing little or no sectoring. Crosses between AB1 and T560 mutants however, produced progeny that were unstable, sectored frequently and some colonies rapidly reverted back to the thin sparse growth normally associated with *nit* mutants. The progeny from all crosses were not tested for heterokaryosis or recombination by plating conidia, so the exact genetic-make up of these prototrophs is not known. It is believed that heterokaryosis, resulting in the recovery of auxotrophic progeny of either parent, can indicate that the classical parasexual cycle operates. The recovery of parental phenotypes will be either equal, if the nuclear types present are balanced, or unequal if the they are unbalanced (Harman & Stasz, 1991). If karyogamy occurs, the addition a haploidising agent such as fluorphenylalanine or benomyl induces the formation of haploids or aneuploids, and may result in the recovery of novel genotypes (Hastie, 1970).

In *I. bolleyi*, complementation indicates the possibility of heterokaryosis. Prototrophs recovered from crosses between mutants of the same strains and mutants of different strains, produce results similar to that obtained for *Trichoderma harzanium* in a study by Harman & Stasz (1991). Parasexuality is not believed to exist in this fungus, and

Harman & Stasz (1991) challenge whether the classical parasexual cycle operates at all. In their study of protoplast fusion between inter-strain and intra-strain crosses of *T. harzanium*, prototrophs obtained in intra-strain crosses were stable and had similar morphology to the wild-type. Genetic analysis revealed that these were balanced heterokaryons containing an equal number of auxotrophic nuclei. Those crosses between different strains produced prototrophic colonies that were unstable and produced many sectors. The results obtained from the inter-strain crosses were interpreted as a possible integration of one nucleus to another (transformation) during fusion, resulting in a series of complex of genetic events and expression of parental nuclei. Harman & Stasz (1991) did not find evidence of heterokaryosis and the results obtained were inconsistent with the classical model of parasexuality. The prototrophic progeny obtained were stable through conidiation and genotypically identical to one another or to either of the parental strains, but were tremendously different in morphotype.

These results indicate that genetic events following fusion may not be clear cut as previously thought, particularly in asexual fungi where heterokaryosis may be absent or rare. Alternative forms of genetic exchange seem feasible.

As two strains of *I. bolleyi* appeared to successfully fuse using protoplasts, there is a possibility that novel phenotypes could be produced which show improved biocontrol abilities such as faster growth rate, greater competitive ability and greater adaptation to environmental conditions. Stable prototrophs need to be compared to the wild-type for their growth and tolerance to different environmental conditions. This may establish to some degree whether the phenotype of the prototrophs has been improved or made less competitive as a result of crossing strains. To date, the only superior biocontrol strain has been produced from *Trichoderma harzanium.* It is thought that its production was a result of the type of genetic exchange described above. The parasexual cycle was not believed to have played a role (Harman *et al.*, 1989). A greater understanding of the genetic events

following protoplast fusion of *I. bolleyi* strains may greatly help to improve production and selection of superior biocontrol agents.

CHAPTER 6

TRANSFORMATION OF *I. BOLLEYI* WITH A β-GLUCURONIDASE (GUS) GENE

6.1 Introduction

The study of fungal populations to date has relied heavily on isolation techniques and measurements of colony forming units (Singh, 1979). In the case of biocontrol organisms added to soil, it has been common to use fungicide or antibiotic-resistant mutants (Lascaris & Deacon, 1991) so that the population levels of introduced strains can be detected by plating onto selective media and thereby distinguished from the resident populations of the same or other organisms. Recent developments with genetic manipulation of filamentous fungi has offered a new approach to tracking organisms added to soil or to seeds. The GUS (ß-glucuronidase) reporter gene system is rapidly becoming a popular tool for use in industrial and phytopathogenic fungi, mainly because GUS is robust and the assay methods are simple (Roberts *et al.*, 1989). One key advantage in using the GUS system is that natural GUS levels in most fungi are negligible, so even small levels of GUS activity can be measured in cells containing this reporter gene (Jefferson, 1989). The GUS reporter gene opens up a range of applications in plant and fungal interactions, including fungal population monitoring and the study of mycorrhizal associations within the soil (Jefferson, 1989). Specifically, it enables the detection of GUS-containing organisms *in situ* on plants roots. As an aid to future work on *I. bolleyi*, it was

decided to try to introduced a GUS reporter gene into this fungus. The work in this chapter describes these efforts.

6.2 Methods

Idriella bolleyi was transformed with the pGUS5 plasmid, containing the GUS gene (GUS A) and the hygromycin resistance gene (hph), both under the control of the GDP-1 promoter of *Cochliobolus heterostrophus* (Mönke & Schäfer, 1993), (Figure 6.1). Transformants were selected for resistance to hygromycin B and then tested for GUS activity both fluorimetrically and in X-gluc solution. Wheat seedling roots were inoculated with the transformants and tested for GUS activity in order to assess their value for use in ecological studies.

6.2.1 Transformation of I. bolleyi

The transformation procedure was done using the method of Liljeroth *et al.* (1993). A quantity of plasmid DNA was obtained by transformation of *Escherichia coli* with the pGUS5 plasmid, and subsequent plasmid extraction (Section 2.2.6 to 2.2.7). In order to check that the plasmid extracted from *E. coli* was pGUS5, the plasmid uncut and cut with *Xba* I restriction enzyme, was run on a 1% agarose gel (Section 2.2.8 to 2.2.9) in order to determine the plasmid size. For comparison, an original sample of the plasmid was also run on the same gel along with a bacteriophage lambda DNA cut with *Hind* III as a size marker. The band produced from the original pGUS5 sample was found to be 7.4 kb when compared with the size marker. The uncut DNA produced a single band which was the same size (7.4 kb) as the original plasmid sample. The two bands from the cut DNA were found to be around 2.4 kb and 5 kb (Figure 6.2). It was concluded from these results that

Figure 6.1: The pGUS5 plasmid containing the GUS gene (GUS A) and the hygromycin-resistance gene (hph), both under the control of the constitutive promoter gene, glyceraldehyde 3-phosphate dehydrogenase (GPD-1).

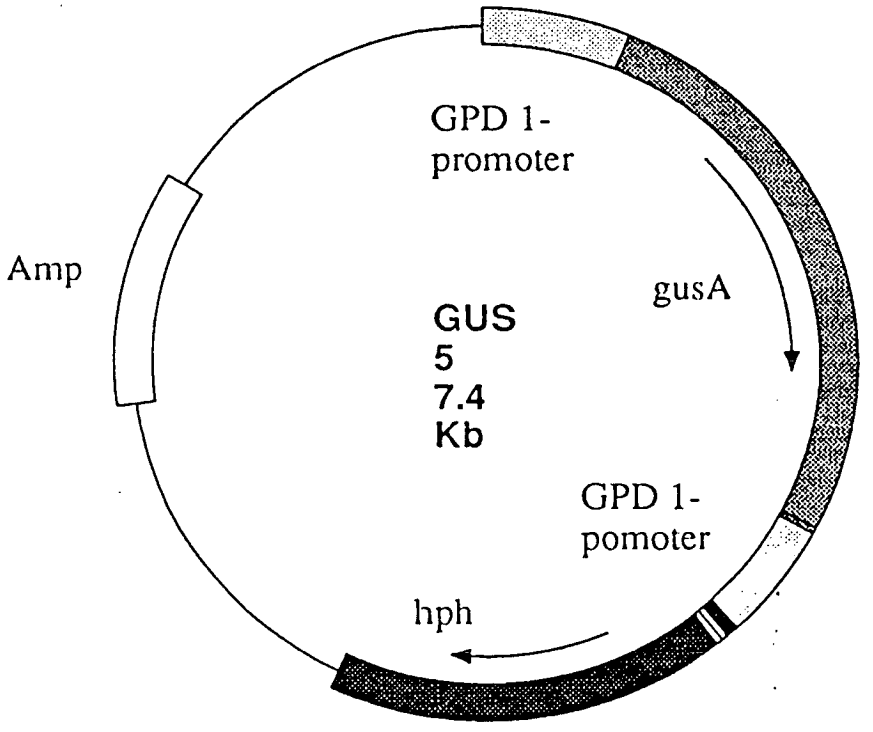
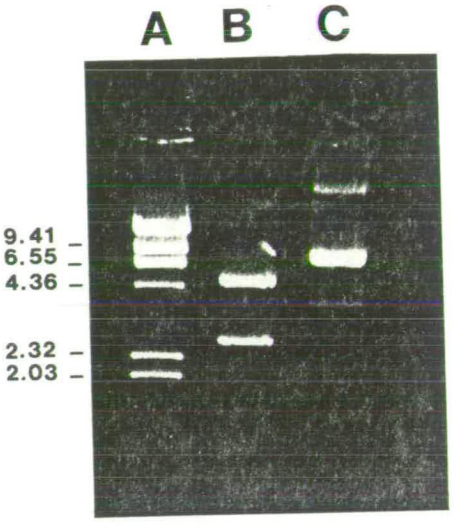


Figure 6.2: A 1% agarose gel showing plasmid pGUS5 (7.4 kb) extracted from transformed *Escherichia coli*, cut (lane B) showing two bands at 2.4 and 5.0 kb, and uncut (lane C) showing one band at 7.4 kb. Bacteriophage lambda DNA cut with *Hind* III (lane A) was used as a size marker.



the DNA extracted from *E. coli* was the pGUS5 plasmid.About 10^7 pelleted protoplasts of *I. bolleyi*, strain AB1 (prepared as in Section 5.2.1), were suspended in 100µl of STC solution (1.2 M sorbitol, 10 mM Tris, pH 7.0; 50 mM $CaCl_2$), and 15µg of plasmid DNA was added. Two volumes of PEG solution (60% Polyethylene glycol 8000, 50 mM $CaCl_2$, 10 mM Tris, pH 7.0) was added. STC solution (1 ml) was added to increase the total volume, then aliquots (100µl) of the solution were plated directly onto Complete Regeneration Medium (Section 2.1.2) containing hygromycin B (200µg ml^{-1}; Sigma). The plates were incubated in the dark at 25°C for up to 14 days and colonies that grew fast compared to the surrounding colonies were removed and transferred to fresh Complete Regeneration Medium containing hygromycin B (200µg ml^{-1}) and these colonies were observed for their ability to grow on this medium. The ability to grow rapidly on this medium was evidence of hygromycin resistance and thus possible transformation by the plasmid DNA because wild-type strains grew poorly in the presence of hygromycin B.

6.2.2 The test for GUS activity.

Possible transformants (indicated by fast growth on the hygromycin B plates compared to wild-type growth) were tested for the presence of the GUS gene. Sabouraud Dextrose Broth (Section 2.1.2) (200µl) was added to sterile Eppendorf tubes and each tube was inoculated with a 3 x 3 mm block of a possible transformant taken from the margin of a three day old PDA culture containing 200µg ml^{-1} of hygromycin B. Three replicate tubes were used for each transformant. The tubes were incubated in the dark at 25°C for 48 h. Following incubation, 100µl of X-gluc solution (0.5% Triton X-100, 50 mM sodium phosphate buffer (pH 7.5); 5% *N,N*-dimethyl-formamide) was added and the tubes were reincubated overnight in the dark at 25°C. The tubes were then examined for a blue colouration of the

solution indicating a GUS-positive isolate. Control tubes (3 replicates) were set up using wild-type (non-transformed) *I. bolleyi* strain AB1.

6.2.3 Radial growth rate and sporulation of transformants compared with the wild-type

GUS-positive isolates (indicated by a blue colouration in the X-gluc solution) were compared with the wild-type *I. bolleyi* strain AB1 for their radial growth rate and sporulation.

PDA plates (without hygromycin B) were centrally inoculated with a mycelial plug (using a 5 mm diameter cork borer) of a transformant taken from the margin of a four day old PDA culture containing hygromycin B (200μg ml^{-1}). Three replicates were used for each transformant and for the wild-type strain AB1 (obtained from a PDA culture without hygromycin B). The plates were incubated in the dark at 25°C for 7 days and the radial growth was measured.

Czapek-Dox Liquid Medium (without hygromycin B) was added in 10 ml amounts to sterile Universal bottles and each bottle was inoculated with a mycelial plug (5 mm) of a transformant taken from the margin of a four day old PDA culture containing hygromycin B at 200μg ml^{-1}. Three replicates were used for each transformant and for the wild-type strain AB1 (obtained from a 4 day old PDA culture without hygromycin B). The bottles were incubated on a Denley orbital mixer at 200 rpm, in the dark at 25°C for 72 h. At 72 h the spore number in each bottle was counted with a haemocytometer.

6.2.4 Fluorimetric determination of GUS activity in pure cultures of I. bolleyi *transformants*

Czapek-Dox Liquid Medium (10ml), was placed into sterile Universal bottles and each bottle was inoculated as above with a mycelial plug (5 mm) of a transformant taken from the margin of a four day old PDA culture containing hygromycin B at 200μg ml^{-1}. Three replicates were used for each transformant. Wild-type strain AB1 was used as a control. The bottles were incubated on a Denley orbital mixer at 200 rpm for 72 h. At 72 h, the mycelium was harvested by centrifugation at 630 x *g* for 10 min using a Mistral 1000 bench centrifuge. GUS activity was measured fluorimetrically using the method described in Section 2.2.5.

6.2.5 Test for mitotic stability

The *I. bolleyi* transformants were tested for integration of the GUS gene into the fungal genome, and for its mitotic stability if integrated.

Single spore cultures were obtained by harvesting the spores of each transformant (Section 2.2.3) from the hygromycin-supplemented PDA plates on which the transformants grew after they had been sub-cultured from the Complete Regeneration Medium. The spore suspension was streaked onto fresh PDA plates without hygromycin B using a sterile inoculating loop. The plates were incubated for 2 to 3 days in the dark at 25°C and examined for the growth of colonies. A single colony believed to have derived from a single spore, was removed from each of the transformant plates using a sterile scalpel and transferred into the centre of a fresh PDA plate without hygromycin B. The colony was allowed to grow in the dark, at 25° C for 7 to 14 days, until sporulation was observed. The

spores were then streaked again onto PDA plates as before, and this entire procedure was repeated through six conidiation cycles. After the six conidiation cycles, three 5 mm diameter agar discs were removed from the margin of a 4 day old PDA culture and added to Universal bottles containing 10 ml Czapek-Dox Liquid Medium without hygromycin B. Wild-type strain AB1 (obtained from a 4 day culture without hygromycin B) was used for comparison. The bottles were incubated on an orbital mixer at 200 rpm for 72 h, then the mycelium was harvested by centrifugation at 630 x *g* for 10 min. GUS activity was measured fluorimetrically using the method described in Section 2.2.5.

6.2.6 GUS-activity on wheat seeds and wheat seedling roots.

Plastic drinking cups with perforated bases for drainage, were three-quarters filled with unsterile perlite and the perlite was then saturated with distilled water. Mycelial plugs (5 mm) of each transformant were removed from the margins of PDA colonies containing hygromycin B (200μg ml^{-1}), and placed centrally on the perlite surface in the cup. A single ungerminated wheat seed was placed on top of each mycelial plug, then the cups were filled with moist perlite. Fifteen replicate cups were used for each transformant and fifteen control cups were set up in the same way for the wild-type strain AB1. The cups were stood in trays containing a 2 cm depth of distilled water, then the trays were placed in polythene bags to prevent moisture loss, and incubated between 20 and 25°C under a bank of fluorescent lights (Thorne 40 W) supplying light in a 12h light/12h dark cycle.

At 14 days, 5 replicate cups of each transformant, and controls, were sampled. The seedlings were gently removed from the perlite and the perlite was carefully washed off the roots under a slowly running tap. Any mycelial plugs still attached to the seeds were removed. The aerial parts of each seedling were cut off at the stem base, and the roots and

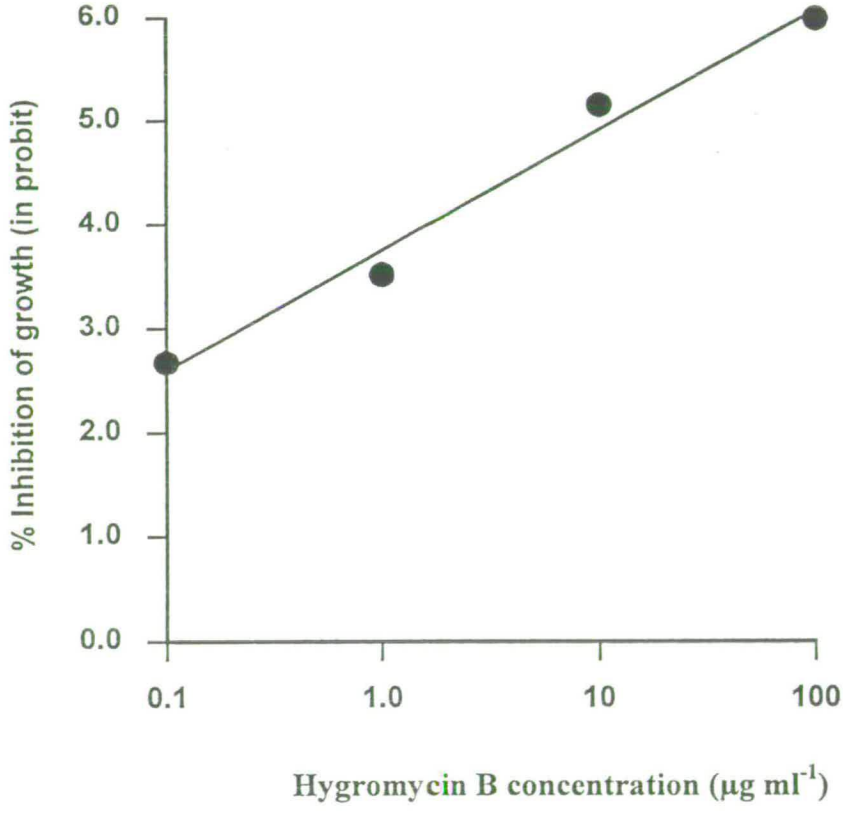
seed of each seedling were totally immersed into 5 ml of X-gluc solution and incubated in the dark at 25°C for 48 h. In X-gluc solution, a blue precipitation is produced on the tissue at the site of ß-glucuronidase activity, so the extent of tissue colonisation can be visually assessed. The seed and roots of each replicate were removed from the X-gluc solution, blotted dry on filter paper and examined for a blue colouration.

At 21 days the remaining 10 replicates for each transformant and controls were sampled. The seedlings were washed as before, but then the 10 replicate root systems with attached seeds were pooled for each transformant and the GUS activity of the inoculated plant tissue was measured fluorimetrically as described in Section 2.2.5. The natural GUS activity of inoculated wheat seedling roots and seeds was determined using the same method.

6.3 Results

Four transformants of *I. bolleyi* were obtained. These transformants showed both hygromycin B resistance and GUS activity at a higher level than was recorded for the wild-type strain. The GUS gene appeared to be integrated into the fungal genome as was shown by the retention of GUS activity after 6 conidiation cycles on media without hygromycin B (Section 6.2.5); however this was not confirmed by southern analysis. GUS activity could be visually observed on seeds and on seedling roots by a blue colouration following incubation in X-gluc solution (Section 6.2.6). However, fluorimetric analysis of seeds and seedling roots inoculated with the transformants, produced higher levels than the equivalent wild-type inoculated seedlings but lower levels of GUS activity than was obtained from the same transformants in pure culture (Table 6.2).

Figure 6.3: Percentage reduction of linear growth rate (expressed as probit of *Idriella bolleyi* strain AB1 against logarithm of hygromycin B concentration). The equation of the straight line was $y=1.6x + 3.8$; $r = -0.99$ $P<0.001$. From this the ED_{50} (estimated dose for 50% reduction of growth was calculated at 20µg ml^{-1}.



6.3.1 Transformation of I. bolleyi

The success of transforming *I. bolleyi* was found to be highly dependent on experimental conditions. Initial transformation experiments used conditions that varied from the method stated in Section 6.2.1. The variation in the conditions included using *I. bolleyi* strain AB1 protoplasts at a lower concentration of (c. 10^5) than the later standard concentration of 10^7, and using 3µl (rather than 6µl) of plasmid DNA preparation in 1 ml of STC solution. The rest of the transformation protocol was done as described in Section 6.2.1 except that after transformation, *I. bolleyi* protoplasts were plated onto Complete Regeneration Medium (Section 2.1.2) supplemented with hygromycin B at 100µg ml^{-1}. Prior to transformation, the wild-type strain AB1 had been examined for natural resistance to hygromycin B (Figure 6.3). The ED_{50} (estimated dose for 50% reduction in growth) was calculated as 20µg ml^{-1} for the wild-type strain on Hygromycin-supplemented medium. A concentration of 100µg ml^{-1} suppressed growth considerably, and this suppression of growth was considered sufficient to enable the recovery of hygromycin resistant colonies after transformation. However, it was found that the high concentration of protoplasts plated onto the Complete Regeneration Medium still resulted in complete cover of the agar by fungal growth so that fast growing colonies were difficult to recover and were mainly isolated around the colony margins.

Four separate transformation experiments were done using this initial method (with 100 µg of hygromycin ml^{-1} in the recovery medium) involving a total of 200 regeneration plates. Thirty four colonies showing hygromycin resistance were recovered from experiment one, and 3, 6 and 9 colonies were recovered from experiments two, three and four respectively. The protoplast regeneration rates for these experiments were 5.4%, 3.1%, 3.3% and 4.9% respectively. Out of the total of 34 fast-growing colonies sub-cultured onto fresh PDA plates containing hygromycin B (100 µg ml^{-1}), only 6 sub-cultures

continued to grow uninhibited; The remaining 28 were inhibited within a few days after sub-culturing. All the 34 fast growing colonies recovered from the Complete Regeneration plates tested negative for GUS activity when grown in X-gluc solution for up to 8 days, so it is unlikely that they had been transformed with the pGUS5 plasmid. Alternatively, the 6 sub-cultures showing hygromycin resistance may have been transformed with the pGUS5 plasmid, but only expressed the hph gene for hygromycin resistance, and not the GUS A gene for GUS activity. The first possibility is the most likely, because hygromycin-resistant colonies were also found on the control (untransformed) regeneration plates at about the same frequency as the transformed plates. The numbers of fast growing colonies on the control plates were- 3, 5, 11 and 4 for experiments one, two, three and four respectively. This indicates that enhanced hygromycin resistance may have occurred by spontaneous mutation in *I. bolleyi.*

These findings prompted the change of experimental conditions, mentioned earlier, involving selection for growth on plates containing hygromycin at 200μg ml^{-1}. Following transformation, *I. bolleyi* colonies appeared on the regeneration plates 2 to 3 days after plating. The same degree of *I. bolleyi* growth was observed on control plates with untransformed protoplasts. The percentage of regeneration was 8.9% from control protoplasts and 6.2% from plasmid-treated protoplasts. Faster growing colonies began to appear after 7 days on the regeneration plates bearing plasmid-treated protoplasts, whereas no fast growing colonies were observed on the control plates. Twenty-one colonies were obtained from a total of 100 regeneration plates, but only 10 of these colonies continued to grow after sub-culturing onto fresh PDA medium containing 200μg ml^{-1} of hygromycin B. Four of the 10 colonies gave a positive GUS reaction after growth in X-gluc solution (Section 6.2.2), (Figure 6.4(I)), the remaining six colonies were GUS negative even after 7 days incubation in X-gluc solution. The GUS-transformation frequency was found to be 0.02% of regenerating protoplasts.

6.3.2. Radial growth and sporulation of transformants

Transformants were compared to the wild-type *I. bolleyi* strain AB1 for their radial growth and sporulation as described in Section 6.2.3.

Radial extension rate and the degree of sporulation were seen to vary between transformants and between the transformants and wild-type (Table 6.1). Transformant T3 showed a much faster growth rate (5.4 mm 24 h^{-1}) compared to the wild-type (3.1 mm 24 h^{-1}). The transformants T1 and T4 showed a growth rate similar to that of the wild-type, whereas transformant T2 showed a somewhat slower growth rate. No sporulation was observed for two of the transformants, T1 and T2, whereas transformant T3 showed a similar level of sporulation to that of the wild-type and transformant T4 showed less sporulation, but not significantly so. It was noted that despite a good level of sporulation, the spores of transformant T3 were 'lemon-shaped' rather than the 'ovoid-shaped' conidia normally observed for *I. bolleyi.* The 'lemon-shaped' spores also appeared to be highly vacuolated, unlike the normal wild-type spores of *I. bolleyi.*

6.3.3 Fluorimetric analysis of GUS activity of transformants in pure culture

Pure cultures of each of the transformants and of the wild-type *I. bolleyi* strain AB1, were grown in Czapek-Dox Liquid Medium as described in Section 6.2.4. The GUS activities of each transformant were then determined fluorimetrically as described in Section 2.2.5.

Figure 6.4: Transformants of *Idriella bolleyi* were compared to the wild-type strain for GUS activity. **(I)** Mycelial blocks of *Idriella bolleyi* transformants - T1, T2, T3 and T4 turned X-gluc solution blue following incubation. A blue colouration of the solution indicated a GUS-positive reaction. No blue colouration was observed after incubation of the wild-type strain (WT) in X-gluc solution. **(ii)** Histochemical localisation of GUS activity on wheat seeds and roots following inoculation with *Idriella bolleyi* transformants. A blue colour was produced on the seeds and roots following incubation in X-gluc solution, indicating a high level of colonisation by the transformants.

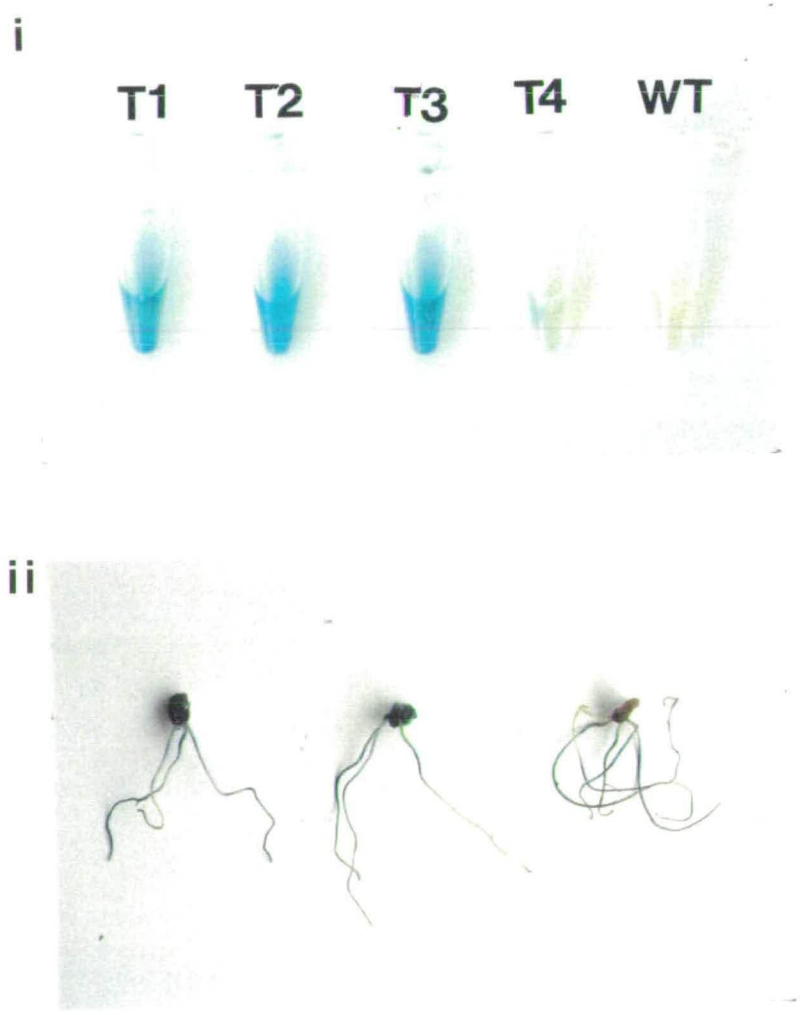


Table 6.1: The radial growth rate of *I. bolleyi* transformants and of the wild-type strain AB1 (mm 24 h^{-1}) on PDA without hygromycin B, and sporulation (x 10 spores ml^{-1}) of *I. bolleyi* transformants and of the wild-type strain AB1, in Czapek-Dox Liquid Medium without hygromycin B, after 72 h incubation at 25°C. (means ± SEM for three replicates).

Transformant	Radial grow rate (mm 24 h^{-1})	Sporulation (x 10^4 ml^{-1})
T1	3.0 ± 0.0	0.0 ± 0.0
T2	2.0 ± 0.3	0.0 ± 0.0
T3	5.4 ± 0.0	87.3 ± 46.2
T4	3.1 ± 0.2	45.5 ± 11.3
Wild-type	3.2 ± 0.0	81.3 ± 18.5

When assessed after the first cycle of culturing on PDA, all four transformants had considerably higher levels of activity than was recorded for the wild-type culture (Table 6.2). GUS activity for all transformants was similar, at around 4 nanomoles of methylumbelliferyl produced per minute, compared to the wild-type GUS level which was negligible at less than 0.01 nanomoles of methylumbelliferyl produced per minute.

When the transformants were re-tested after six cycles of single-spore transfer on PDA, their GUS activity remained unchanged, at around 4 nanomoles After the single spore cultures of each transformant had been through 6 cycles of conidiation, GUS activity as determined fluorimetrically, was around 4 nanomoles of methylumberlliferyl produced per minute for each transformant. The wild-type again gave a negligible value at less than 0.01 nanomole.

Table 6.2: GUS activity, determined by fluorimetric analysis (nanomoles of 4-methylumbelliferyl (MU) produced per minute) of wild-type *I. bolleyi* strain AB1 and *I. bolleyi* transformants (T1, T2, T3, and T4) in pure culture (means ± SEM for 3 replicates); after 6 cycles of conidiation on hygromycin-free medium and on wheat seed and roots (mean ± SEM for 10 replicates).

	GUS activity (nanomoles MU min-1)		
Transformant	**Pure culture**	**6 conidiation cycles**	**wheat seed and roots**
T1	4.40 ± 1.9	4.21 ± 2.0	1.15 ± 0.3
T2	4.49 ± 2.4	4.32 ± 1.7	1.01 ± 0.4
T3	4.22 ± 1.8	4.17 ± 1.3	1.37 ± 0.5
T4	4.88 ± 1.2	4.52 ± 2.1	0.98 ± 0.6
Wild-type	<0.01 ± 0.0	<0.01 ± 0.0	<0.01 ± 0.0

6.3.4 Fluorimetric analysis of wheat seeds and wheat seedling roots following inoculation with I. bolleyi *transformants.*

Wheat seedlings were inoculated with each transformant as described in Section 6.2.6. Following 21 days incubation, the seedlings were harvested and GUS activity of the transformants on the seeds and roots were determined fluorimetrically as described in Section 2.2.5. GUS activity was detected for all four transformants on wheat roots, but at a level of around 1 nanomole of methylumberlliferyl produced per minute in comparison to around 4 nanomoles of methylumberlliferyl produced per minute, for pure cultures. (Table 6.2). This difference is probably related to the relative amounts of fungal biomass in the two systems, although this was not assessed. The GUS activity recorded for the roots

inoculated with the *Idriella bolleyi* strain AB1 was less than 0.01 nanomole of methylumberlliferyl produced per minute

6.3.5 Histochemical localisation of GUS activity on roots inoculated with I. bolleyi *transformants.*

Wheat seeds and wheat seedling roots were inoculated with each transformant and following 14 days incubation, the seedlings were harvested and the seeds and roots were incubated in X-gluc solution (Section 6.2.6).

In all cases, the seeds and roots were coloured blue after incubation in X-gluc solution, indicating that ß-glucuronidase activity was expressed on or in the plant tissues colonised by the transformants (Figure 6.4(ii)). Most seeds were completely blue suggesting that they had been heavily colonised by the transformants, and on all plants the blue colouration extended 5 to 6 cm down the length of each root again indicating a high degree of fungal colonisation. In contrast, no blue colouration was observed on the seeds and roots of the plants inoculated with the wild-type *I. bolleyi* strain AB1 after incubation in X-gluc solution.

6.4 Discussion

The GUS gene coded by the *UidA* gene in *Escherichia coli* was initially developed for use in the nematode *Caenorhabditis elegans* (Jefferson *et al.,* 1987b). Since then GUS has been developed as a reporter gene for a number of higher plants including tobacco and potato (Jefferson *et al.,* 1986; Jefferson, 1987; Jefferson *et al.*, 1987a). The use of ß-

glucuronidase as a marker is favoured for a number of biological systems because of its extreme stability. It can be assayed under any physiological pH, its optimum being 5.2 to 8.0. ß-glucuronidase is fairly resistant to thermal inactivation, along with being tolerant to many detergents and widely varying ionic conditions (Jefferson, 1987).

In recent years, the development of GUS as a reporter system in fungi has become more common, particularly to enable monitoring of industrial and plant pathogenic fungi. Industrial uses include a tool for the analysis of protein secretion and sorting (Roberts *et al.*, 1989). Several plant pathogenic fungi have been transformed with GUS to allow effective assessment of fungal-fungal and fungal-plant interactions. Those fungi successfully transformed with the *E. coli* GUS gene include *Aspergillus nidulans, A. niger* and the tomato pathogen *Fulvia fulva* (Roberts *et al.*, 1989), *Fusarium oxysporum* (Eparvier & Alabouvette, 1994), *Cochliobolus heterostrophus* (Mönke & Schäfer, 1993), *Bipolaris sorokiniana* (Liljeroth *et al.*, 1993) and *Pseudocercosporella herpotrichoides* (Bunkers, 1991). Development of such reporter systems in filamentous fungi is still in its early stages, mainly because most of these are genetically uncharacterised (Roberts *et al.*, 1989).

Idriella bolleyi was successfully transformed with the pGUS5 plasmid, and four of the transformants recovered expressed both hygromycin resistance and GUS activity. The pGUS5 plasmid, also used in the transformation of *B. sorokiniana*, was developed by cloning the glyceraldehyde 3-phosphate dehydrogenase gene, GPD-1, from *C. heterostrophus* (Mönke & Schäfer, 1993). The expression of both the hygromycin B phosphotransferase resistance gene from *E. coli* and the ß-glucuronidase gene (GUS A), from *E. coli*, is under the control of this promoter (van Wert & Yoder, 1992). Many fungi have been transformed with plasmids under the control of the GDP-1 promoter, suggesting that it can be used by a remarkable variety of fungi including *Septoria nodorum* (Cooley *et al.*, 1988) and *Leptosphaeria maculans* (Farman & Oliver, 1988). The successful

recovery of transformants of *I. bolleyi* confirms the value of this promoter for yet another fungus.

Conditions seemed critical for successful transformation of *I. bolleyi*. Increasing the protoplast number increased the transformation frequency. Mönke & Schäfer (1993) found that transformation frequency was highly influenced by protoplast number in *C. heterostrophus*. Protoplast numbers of 10^5 and 10^7 gave transformation frequencies of 1 transformant per 1000 viable protoplasts, and 1 transformant per 12000 respectively. Protoplast numbers of 10^6 gave an optimal transformation frequency of 1 transformant per 800 viable protoplasts. Referring to the large numbers of protoplasts required in transformation, in one experiment Couteaudier *et al.* (1993) stated that transformation of *Fusarium oxysporum* with a GUS gene failed due to the low efficiency of transformation in filamentous fungi. Further analysis of optimal transformation conditions is required for *I. bolleyi*.

Hygromycin resistance did not prove to be totally satisfactory for the recovery of *I. bolleyi* transformants, due to the high level of natural resistance to hygromycin in the wild-type strains. The high concentration of protoplasts plated resulted in a degree of growth that although suppressed, was enough to prevent or mask the growth of resistant colonies. Langin *et al.* (1990) stated that in the absence of selection pressure for growth, untransformed strains can hide the transformed phenotype, preventing their direct selection. Because of the high protoplast number used in the transformation of *I. bolleyi,* use of the GUS^+ phenotype rather than selection for hygromycin resistance, would be unsuitable as even less selection pressure would inevitably result in greater difficulties in recovering transformants. In view of this, development of a plasmid with a more efficient selective marker would be desirable for *I. bolleyi*.

In initial experiments, fast-growing colonies resistant to hygromycin were observed on plates where either control of plasmid-treated protoplasts were allowed to regenerate. None of the original hygromycin resistant colonies recovered from the plasmid-treated protoplasts tested positive for GUS activity. Those colonies that were sub-cultured to new plates containing hygromycin B either grew uninhibited or showed staling. It is possible that these colonies were showing transient expression of the hph gene for hygromycin resistance, but because of the production of resistant colonies from untreated (control) protoplasts, it is likely that most fast growing colonies developed as a result of spontaneous mutation for hygromycin resistance in *I. bolleyi*. In the instances where growth became inhibited shortly after sub-culturing, this may suggest reversion of the mutation. Where growth remained uninhibited on the hygromycin medium, this may suggest that the spontaneous mutation was stable.

Mönke & Schäfer (1993) showed transient gene expression after transformation of *Cochliobolus heterostrophus* with the pGUS5 plasmid. Their results showed that the method of selection influenced the stability of gene expression. All colonies selected for hygromycin resistance tested positive for GUS activity, and expression was stable; this was confirmed by southern analysis showing integration of the vector plasmid into the fungal genome. No colonies selected for the GUS activity showed hygromycin resistance, and these colonies also lost their GUS activity after 45 h of sub-culturing onto fresh medium. Southern analysis showed no vector DNA present in these colonies. Oliver *et al.* (1987) found that selection for hygromycin B resistance in *Fulvia fulva*, after transformation with the pAN7-1 plasmid from *A. nidulans*, produced transformants that were stable in this phenotype and southern analysis confirmed plasmid integration into the fungal genome. No loss in hygromycin resistance was observed after growth and sporulation on a non-selective medium.

Results from *I. bolleyi* differed from those of Mönke & Schäfer (1993) and Oliver *et al.* (1987). In experiments where transformants were recovered, some colonies which showed hygromycin resistance did not show GUS activity and some colonies with hygromycin resistance lost this resistance after sub-culturing onto fresh PDA containing hygromycin B at 100μg ml^{-1}. It is possible that the hygromycin resistant phenotype was lost as a result of reversion of a natural spontaneous mutation back to the wild-type phenotype, as discussed previously. With an increased hygromycin B concentration at 200μg ml^{-1} in the Complete Regeneration Medium, no spontaneous resistant colonies were observed on the control plates. In view of this, colonies derived from plasmid-treated protoplasts and that showed only initial resistance to hygromycin B (200μg ml^{-1}) may have shown transient gene expression for hygromycin resistance but this phenotype was lost as a result of the plasmid failing to integrate into the fungal genome. Liljeroth *et al.* (1993) also observed transient expression of hygromycin resistant phenotype with *B. sorokiniana,* where 99% of colonies originally growing on hygromycin B medium failed to grow after transfer to fresh medium. From those colonies that continued to grow, only one did not show GUS activity.

The GUS activity of the four stable *I. bolleyi* transformants was broadly similar, consistent with the report by Eparvier & Alabouvette (1994) that the GUS activity of pathogenic and non-pathogenic strains of *Fusarium oxysporum* did not vary much. In contrast, Couteaudier *et al.* (1993) found variability between transformants of *Fusarium oxysporum,* and concluded that the variation was caused by different integration positions of the gene cassette. These workers suggested that copy number was not determining GUS activity, as a transformant with only one copy of the plasmid had the highest GUS activity. Bunkers (1991) found variation in GUS activity amongst transformants of *Pseudocercosporella herpotrichoides.* In this case all transformants showed multiple insertions of the GUS gene and the sites of integration were variable, as determined by

restriction analysis. Bunkers (1991) suggested that GUS activity was related to copy number in *P. herpotrichoides.*

The retention of GUS activity of *I. bolleyi* transformants after six cycles of single spore selection and growth on non-selective medium strongly suggests that the plasmid was integrated into the fungal genome. However, there was insufficient time to verify this by southern analysis. It was however, noted that the transformants deviated significantly from the wild-type strain in terms of radial extension rate, conidium production or conidium shape in culture. Two transfomants did not show any sporulation after 4 days incubation and another transformant produced similar levels of spores to the wild-type, but the spores showed abnormal morphology. These differences would be consistent with integration of the plasmid into different regions of the fungal genome, where integration might have disrupted normal gene expression. Further molecular analysis of these transformants might thus help to identify the genes that control sporulation in *I. bolleyi.*

GUS transformants could be extremely useful for ecological studies provided that the transformants are stable, as suggested by the work here, and provided that they behave like the wild-type strains in all other reports. Perhaps none of the four transformants generated in this study would be ideal for ecological studies, because all of them differed from the wild-type in growth or conidiation in laboratory culture. Nevertheless, all four transformants were found to grow on seeds and roots of wheat seedlings in perlite, and they also expressed GUS activity on the plates. It would be interesting to use these transformants in further studies on plants growing in soil, with and without competition from wild-type strains of *I. bolleyi.* Most *in vivo* work studying plant-fungal or fungal-fungal interactions has relied on simple re-isolation methods on agar (Singh, 1979), or microscopic examination of plant material for fungal structures (Kirk & Deacon, 1987b), or use of selective media for isolation of fungicide-resistant strains (Lascaris & Deacon, 1991). GUS

could provide a more accurate quantitative measurement. Eparvier & Alabouvette (1994) successfully used GUS transformed strains of *Fusarium oxysporum* in soil studies with flax root colonisation. By relating GUS activity to total fungal biomass on the root, they were able to show active competition between pathogenic and non-pathogenic strains of this fungus, relevant to the biocontrol of *Fusarium* wilt disease by non-pathogenic strains.

In order to do such ecological work, it may be necessary to relate GUS activity to fungal biomass. Ergosterol content in roots has frequently been used as a measure of fungal biomass (Gorden & Webster, 1984; Newell *et al.*, 1988). Liljeroth *et al.* (1993) found a positive correlation between ergosterol content and GUS activity in barley roots infected with a GUS transformed strain of *B. sorokiniana*. This method of fungal biomass measurement however, can have drawbacks. Culture age can influence ergosterol content in fungal mycelium (Torres *et al.*, 1992), and Liljeroth *et al.* (1993) stated that GUS expression may also differ with mycelial age. Eparvier & Alabouvette (1994) developed a more accurate technique of measuring fungal biomass in competitive root colonisation. Polyclonal antibodies were produced against *Fusarium oxysporum* and although they were not specific for a particular strain of *F. oxysporum*, they still enabled measurement of most of the total fungal biomass in the root tissue. The antigens could be easily detected by ELISA, and GUS activity per unit of fungal biomass could be calculated. Equivalent methods now need to be developed for *I. bolleyi.*

Couteaudier *et al.* (1993) found that the use of X-gluc enabled GUS-transformed hyphae of *F. oxysporum* to be seen on root tissues. Colonisation of wheat seedling roots by *I. bolleyi* was also visible when roots were incubated in X-gluc solution. This could be valuable for studying the pattern of colonisation of the roots by *I. bolleyi* when the fungus is applied to seeds or other localised regions of plants. Lascaris & Deacon (1991) found that *I. bolleyi* could be spread down the roots in percolating water, presumably by

movement of spores. The use of GUS-transformed strains provides a potential means of following this process more closely and identifying the precise sites on roots where *I. bolleyi* can become locally established.

CHAPTER 7

CONCLUDING DISCUSSION

Idriella bolleyi has received attention for commercial development simply because it fulfils the desired specifications for development of a successful biocontrol agent. *Idriella bolleyi* is commonly present on the roots and stem bases of cereals (Sprague, 1948; Salt, 1977) where it controls take-all (Kirk & Deacon, 1987a), eyespot (Reinecke & Fokkema, 1981) and possibly other root and stem base pathogens through competitive niche exclusion of naturally senescing roots and basal stem tissue (Kirk & Deacon, 1987b). *I. bolleyi* shows tropism to dead as opposed to living root hairs (Allan *et al.*, 1992) and its potential as a biocontrol agent is enhanced by its ability to sporulate abundantly in shaken liquid culture (Deacon, 1988; Jabudansa *et al.*, 1993). *Idriella bolleyi* retains its viability when the culture biomass is dried onto cereal seeds and it is able to colonise the root system from spores carried down the rhizosphere in percolating water (Lascaris & Deacon, 1991). The work presented in this thesis is a further contribution to developing *I. bolleyi* as a potential biocontrol agent.

From this work, *I. bolleyi* was found to be an extremely physiologically variable fungus. Such variability provides a great opportunity for exploitation in developing and improving biological control inoculum. Cook (1992) described the tendency of biological control researchers to evaluate a number of strains and then pick one or two of them for use as models in studies of mechanisms. Cook (1992) emphasised that the natural environment is a centre of diversity and that populations of micro-organisms are likely to contain genetically diverse organisms. In view of this, strains could be made more effective by genetics and selection of organisms that posses essential traits.

In vitro experiments with randomly selected *I. bolleyi* strains showed that *I. bolleyi* strains differed in their tolerance to water-stress with one strain, AB1, being comparable in tolerance to *Fusarium culmorum,* the foot-rot cereal pathogen associated with drought stress. In contrast, another strain, T560, was as intolerant as the take-all pathogen *Gaeumannomyces graminis* var. *tritici* which infects in wetter conditions. The third strain, J10, was found to be intermediate to the other strains in water-stress tolerance. This range of inter-strain variability to water-stress has not before been reported for a single fungus. This degree of strain variability may not be unique to *I. bolleyi* by any means, however, to date information of strain variability in other biocontrol agents is minimal as such studies have been largely neglected by most biocontrol researchers in development of their agents. Cook & Papendick (1972) recorded variation in three strains of *Fusarium culmorum* from two geographical areas of the USA. Their results suggested that the differences in water-stress recorded at both sites for all three strains, may be ecotypes adapted to conditions under which they occur. The water-stress tolerance of *I. bolleyi* strains did not reflect their origins as the most water-stress tolerant strain AB1 was isolated from flax under water-logged conditions (Black & Brown, 1986). It is believed that the strains used in this *in vitro* study may have changed physiologically due to prolonged laboratory culturing.

Strain variation may be targeted to control specific pathogens that are associated with different environmental conditions, or specifically with the root or stem base. Although strain variation for *I. bolleyi* has been established, it is not known whether strains are specifically adapted to the water potentials associated with either the stem base or roots of cereal plants. Field stem and root isolates of *I. bolleyi* are likely to accurately represent the degree of variability in *I. bolleyi*, however, isolates showing both high and low osmotolerance were found together on both the roots and basal stem tissue of mature cereal plants. This finding suggests that strains could be selected for their level of water-stress tolerance regardless of their origin, and strains showing different ecological attributes could

be mixed in a seed-applied inocula to allow the strains to colonise both the roots and stem base. *In vivo* experimentation showed that two strains in a mixed inoculum could colonise straw pieces to a similar degree and to a similar degree to the corresponding single strain inocula under a competitive soil environment. Strains in mixed strain inocula may have an equal chance of adequate colonisation under field conditions. Mixed strain inocula would serve two purposes, firstly it may allow better biocontrol over a greater range of environmental conditions, and secondly it would provide a greater amount of genetic diversity in a population where sexual genetic exchange may be absent.

The success of a biocontrol agent will depend on its ability to proliferate in the environment and its ability to compete with the resident microflora. Both these factors will be influenced by the environmental conditions affecting the activity of soil-borne micro-organisms. The importance of adaption for plant pathogens has been emphasised, enabling them to survive environmental change by generating fungicide resistance and overcoming host resistance (Klittch & Leslie, 1988). However, environmental adaption is likely to be just as significant for the survival of biocontrol agents. Many fungi are able to adapt to changing conditions by exchange of genetic material either sexually or in the case where sexual exchange is rare or absent, genetic diversity may rely on heterokaryosis and the parasexual cycle (Sidhu, 1983).

Idriella bolleyi has no known sexual cycle, so genetic exchange and the maintenance of genetic diversity must take place by asexual means. In this study, *I. bolleyi* nitrate non-utilising (*nit*) mutants with different mutational designation were able to complement one another in culture, the resulting prototrophic growth indicated possible heterokaryosis. Despite this, *I. bolleyi* was consistently seen to have uninucleate cells even upon the formation of anastomosis bridges between cells, suggesting that if heterokaryosis did occur the heterokaryotic phase may not have been sustainable. The lack of a

maintained heterokaryotic phase implies that this form of genetic exchange may be limited or rare in *I. bolleyi*. If this is the case, genetic diversity may be brought about by non-Mendelian mechanisms of genetic change.

The extreme strain variation of *I. bolleyi* could be explained by a number of novel mechanisms for genetic change including the presence of a transposable element. Cultural instability has been noted in *I. bolleyi* strain AB1, conversely strain T560 appears to be highly stable. Klittch *et al.* (1988) found that sectoring in *Fusarium moniliforme* was variable for strains and this was likely to represent the degree of active transposable elements within each strain. A transposable element was isolated from *Neurospora crassa,* this transposon was found to alter the phenotype of the fungus (Kinsey & Helber, 1989). In a comprehensive paper, Kistler & Miao (1992) have discussed a range of other novel mechanisms for genetic change. Those mechanisms which have come to light in the past five years include the presence of extensive chromosomal polymorphism within fungal populations. Polymorphisms are thought to occur at a high rate and are brought about by reciprocal translocation, deletion, aneuploidy etc. This variation in chromosomes was found to be even more apparent among field isolates of strictly asexual reproducing fungi (Kistler & Miao, 1992). Non-essential chromosomes, such as 'B' chromosomes have been found in some fungi it is has been suggested that these non-essential chromosomes may carry pathogenicity genes and so may have some influence on the variability of pathogenicity of some plant pathogens (Miao & VanEtten, 1992). Various kinds of cytoplasmic elements are now known to be transmitted by non-Mendelian mechanisms and transmission is not dependent on stable cellular fusion. These elements include cytoplasmic RNAs, dsDNA and invertons (plasmids) (Klister & Miao, 1992).

If heterokaryosis exists in *I. bolleyi* then this would allow recombination of the genomes of two strains. The *nit* mutants from different strains of *I. bolleyi* did not

complement one another when paired in culture and this inability to complement one another showed that the strains were vegetatively incompatible, the incompatibility being governed by loci involved in self and non-self recognition (Leslie, 1993). Protoplast fusion using *nit* mutants enabled vegetative incompatibility to be overcome between two *I. bolleyi* strains, strain AB1 and strain T560. Prototrophic growth occurred as a result of the cross and some of these prototrophs continued to show wild-type growth upon sub-culturing, although many also showed a high level of sectoring implying genetic instability. The fusion products were not tested to distinguish between possible heterokaryons, recombinants or any other form of post-fusion genetic event. Harman & Stasz (1991) carried out an inter-strain cross with the asexual biocontrol agent *Trichoderma harzanium* and highlighted evidence that showed a post-fusion genetic event that did not involve heterokaryosis or the classical parasexual cycle, and which led to the growth of prototrophs. Their results indicated that the transfer of genetic material from one parental nucleus to another occurred via a transformation system and they emphasised that alternative forms of genetic transfer could occur in asexual fungi where parasexuality is not known to exist. Fusion of two strains of *T. harzanium* by such a genetic event led to the recovery of a progeny that was superior at protecting seeds against a range of diseases and was more strongly rhizosphere competent than either parent. The ability to successfully fuse two strains of *I. bolleyi* regardless of whether heterokaryosis occurs, offers the possibility that strains could be selected for certain desirable attributes and fused in order to produce novel genotypes.

Protoplasts not only have use for the recombination of genomes, but the removal of the cell wall also has advantages for genetically manipulating organisms by introducing a selective gene (Tilburn *et al.,* 1983). Protoplasts were successfully used to introduce a β-glucuronidase (GUS) gene into *I. bolleyi*. Although the protoplasting conditions require

further optimisation, there was sufficient regeneration of the protoplasts to allow recovery of transformants.

The GUS reporter gene is a specific and sensitive way for studying many aspects of growth and survival within a number of systems including a soil environment. *In vivo* experimentation is particularly difficult without a selective marker. To date *in vivo* work with *I. bolleyi* has relied on the production of fungicide-resistant strains which require reisolation from soil or from plant tissue (Lascaris & Deacon, 1991) . The GUS system may prove to be advantageous to this method, as preliminary results with *I. bolleyi* transformants showed that GUS activity could be measured fluorimetrically in the root tissue of wheat seedlings, also roots could be incubated in X-gluc solution which resulted in a blue precipitation on the tissue at the sight of β-glucuronidase activity, so enabling visual assessment of tissue colonisation.

The *I. bolleyi* transformants recovered showed some disruption in their physiology compared to the wild-type. Spore production and growth rate were both affected and so the use of these particular transformants in ecological studies remains questionable. They do however, highlight the possible value of such transformants in population studies if transformants could be recovered that do not deviate significantly from the wild-type physiology. For example, *in vivo* work showed that *I. bolleyi* could increase its population after inoculation onto sterile and unsterile seed in a competitive soil environment. Spore numbers were assessed by counting fungicide-resistant single spore colonies following selective dilution plating. A GUS marked fungus could be used more easily and more accurately to determine the total fungal biomass on the seed using fluorimetric measurements, this would also take into account the level of tissue colonised as well as the spore numbers. As mentioned previously, Lascaris & Deacon (1991) showed that *I. bolleyi*

spores could disperse down the rhizosphere and colonise the root system. This application of a GUS strain would allow more precise monitoring of the dispersal of this fungus.

Much of the failure of biological control agents is attributed to their practical limitations: - the use of single strain inoculum over a range of environmental conditions, their use at non-specific sites and their uneconomical application. Deacon (1991), has emphasized the need to select for biological control agents at the specific desired niche where the control of a pathogen is required. The work presented here further highlights the need for careful strain selection when a suitable agent and control niche have been found. It seems paramount that a range of strains should be assessed for a number of desirable attributes when developing an agent, to expect a single strain to perform over a range of sites and seasonal conditions seems almost naive. The use of mixed strain inoculum to reduce some of the ecological constraints that could limit a single strain inoculum has been discussed, as has the importance of genetic variability within a mixed strain which may help the fungus to adapt to selective environmental pressures. This could be essential for maintaining a biocontrol agents longevity within the soil.

It is important that we look to the future when developing biological control agents. It is becoming more important to consider genetic manipulation of organisms to perhaps make them more specific to a niche or to improve their fitness in the environment. Much of this work has aimed to evaluate the potential of crossing strains with desirable attributes through the use of protoplast technology or introducing a particular beneficial gene by transformation. Genetic manipulation will require a greater understanding of the more novel forms of genetic exchange that have been coming to light more recently (Kistler & Miao, 1992). Such forms of genetic transfer may be particularly important for asexual biological control agents and it may be to our benefit to exploit these systems.

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APPENDIX

Appendix table 3.1: Number of straws (means of 5 replicates, 23 straw pieces per replicate) from which *Fusarium* sp. grew after 2 weeks burial in soil at different water potentials. Straws had been inoculated with different strains of *I. bolleyi* and were plated onto fungicide-free water agar.

***Idriella bolleyi* inoculum**	**Soil water potential (-MPa)**							
	-7.0	**-5.0**	**-1.5**	**-0.6**	**Row mean**	**SED**	**5% LSD**	**1% LSD**
T560-R2	18.4	14.8	12.6	15.2	**15.3**	**1.29**	**2.60**	**3.42**
T560-R2/AB1-IZ	19.0	6.2	5.2	2.8	**8.3**			
AB1-IZ	23.0	14.2	7.8	7.2	**13.1**			
Column mean	**20.1**	**11.73**	**8.5**	**8.4**				
SED	**1.49**							
5% LSD	**3.00**							

Appendix table 3.2: Number of straws (means of 5 replicates, 23 straw pieces per replicate) from which *Fusarium* sp. grew after 6 weeks burial in soil at different water potentials. Straws had been inoculated with different strains of *I. bolleyi* and were plated onto fungicide-free water agar.

Idriella bolleyi inoculum	Soil water potential (-MPa)					
	-7.0	-5.0	-1.5	-0.6	Row mean	SED
T560-R2	20.6	14.8	6.8	3.4	**11.4**	**1.16**
T560-R2/AB1-IZ	19.8	19.8	4.4	3.0	**11.8**	
AB1-IZ	16.4	20.0	8.0	8.4	**13.2**	
Column mean	**18.9**	**18.2**	**6.4**	**4.9**		
SED	**1.34**					
5% LSD	**2.71**					
1% LSD	**3.56**					

Appendix table 3.3: Number of straws (means of 5 replicates, 23 straw pieces per replicate) from which *Gliocladium* sp. grew after 2 weeks burial in soil at different water potentials. Straws had been inoculated with different strains of *I. bolleyi* and were plated onto fungicide-free water agar.

Idriella bolleyi inoculum	Soil water potential (-MPa)					
	-7.0	-5.0	-1.5	-0.6	Row mean	SED
T560-R2	4.2	2.6	1.2	0.2	**2.1**	**1.07**
T560-R2/AB1-IZ	7.0	4.4	3.2	0.4	**3.8**	
AB1-IZ	4.8	6.6	0.4	1.6	**3.4**	
Column mean	**5.3**	**4.5**	**1.6**	**0.7**		
SED	**1.23**					
5% LSD	**2.49**					
1% LSD	**3.28**					

Appendix table 3.4: Number of straws (means of 5 replicates, 23 straw pieces per replicate) from which *Gliocladium* sp. grew after 6 weeks burial in soil at different water potentials. Straws had been inoculated with different strains of *I. bolleyi* and were plated onto fungicide-free water agar.

***Idriella bolleyi* inoculum**	**Soil water potential (-MPa)**					
	-7.0	-5.0	-1.5	-0.6	Row mean	SED
T560-R2	18.0	16.2	5.4	3.8	**10.9**	**1.40**
T560-R2/AB1-IZ	16.2	20.2	12.8	3.6	**13.2**	
AB1-IZ	14.4	16.4	10.0	3.4	**11.1**	
Column mean	**16.2**	**17.6**	**9.4**	**3.6**		
SED	**1.63**					
5% LSD	**3.28**					
1% LSD	**4.34**					

Appendix table 3.5: Number of straws (means of 5 replicates, 23 straw pieces per replicate) from which *Trichoderma* sp. grew after 2 weeks burial in soil at different water potentials. Straws had been inoculated with different strains of *I. bolleyi* and were plated onto fungicide-free water agar.

***Idriella bolleyi* inoculum**	**Soil water potential (-MPa)**							
	-7.0	-5.0	-1.5	-0.6	Row mean	SED	5% LSD	1% LSD
T560-R2	0.6	2.8	9.6	5.0	**4.4**	**0.80**	**1.60**	**2.14**
T560-R2/AB1-IZ	1.2	1.6	2.4	7.8	**3.4**			
AB1-IZ	0.8	6.4	11.2	6.0	**6.1**			
Column mean	**0.87**	**3.6**	**7.6**	**6.3**				
SED	**0.92**							
5% LSD	**1.84**							
1% LSD	**2.47**							

Appendix table 3.6: Number of straws (means of 5 replicates, 23 straw pieces per replicate) from which *Trichoderma* sp. grew after 6 weeks burial in soil at different water potentials. Straws had been inoculated with different strains of *I. bolleyi* and were plated onto fungicide-free water agar.

Idriella bolleyi inoculum	Soil water potential (-MPa)							
	-7.0	-5.0	-1.5	-0.6	Row mean	SED	5% LSD	1% LSD
T560-R2	0 0	3.0	5.6	4.2	**5.7**	**1.38**	**2.76**	**3.66**
T560-R2/AB1-IZ	1.0	2.0	6.4	9.0	**4.6**			
AB1-IZ	0.2	3.4	16.2	17.0	**9.2**			
Column mean	**0.4**	**2.8**	**9.4**	**13.4**				
SED	**1.59**							
5% LSD	**3.18**							
1% LSD	**4.22**							

Appendix table 3.7: Number of straws (means of 5 replicates, 23 straw pieces per replicate) from which *Cephalosporium* sp. grew after 2 weeks burial in soil at different water potentials. Straws had been inoculated with different strains of *I. bolleyi* and were plated onto fungicide-free water agar.

***Idriella bolleyi* inoculum**	**Soil water potential (-MPa)**					
	-7.0	**-5.0**	**-1.5**	**-0.6**	**Row mean**	**SED**
T560-R2	0.0	4.6	11.4	17.8	**8.5**	**1.17**
T560-R2/AB1-IZ	0.4	8.4	12.0	13.2	**8.5**	
AB1-IZ	0.0	0.9	19.2	16.4	**9.1**	
Column mean	**0.01**	**4.6**	**14.2**	**15.8**		
SED	**1.36**					
5% LSD	**2.76**					
1% LSD	**3.62**					

Appendix table 3.8: Number of straws (means of 5 replicates, 23 straw pieces per replicate) from which *Cephalosporium* sp. grew after 6 weeks burial in soil at different water potentials. Straws had been inoculated with different strains of *I. bolleyi* and were plated onto fungicide-free water agar.

Idriella bolleyi inoculum	Soil water potential (-MPa)					
	-7.0	-5.0	-1.5	-0.6	Row mean	SED
T560-R2	0.0	7.4	8.2	18.2	**8.5**	**1.10**
T560-R2/AB1-IZ	0.0	3.8	11.6	19.2	**8.7**	
AB1-IZ	0.0	7.2	11.0	16.8	**8.7**	
Column mean	**0.0**	**6.1**	**10.3**	**18.1**		
SED	**1.28**					
5% LSD	**2.58**					
1% LSD	**3.40**					

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Strain Variation in Tolerance of Water Stress by *Idriella* (*Microdochium*) *bolleyi*, a Biocontrol Agent of Cereal Root and Stem Base Pathogens

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(Received for publication 21 December 1993; revised manuscript accepted 16 April 1994)

Three randomly chosen isolates of Idriella bolleyi *differed markedly in tolerance of water stress down to* − *5 MPa* in vitro. *The differences were seen with respect to osmotic potential of media adjusted with KCl or matric potential of media adjusted with polyethylene glycol 8000. They were consistent when assessed by linear extension of colonies, spore germination, biomass production in liquid culture and sporulation in liquid culture. In comparative tests, one strain of* I. bolleyi *showed similar stress tolerance to that of* Fusarium culmorum, *noted as a pathogen of cereals in dry conditions, whereas another strain of* I. bolleyi *showed low tolerance equivalent to that of the take-all fungus* Gaeumannomyces graminis *and a* Phialophora *sp. from maize. The extreme variation in water stress tolerance of* I. bolleyi *might be used to select strains for biocontrol of different cereal root- and foot-rot pathogens or strains might be combined in seed-applied inocula for consistency of biocontrol in different site or seasonal conditions.*

Keywords: *competition*, Fusarium culmorum, Gaeumannomyces graminis, *osmotic potential, matric potential, soil, spore germination, take-all, water potential*

INTRODUCTION

Idriella bolleyi (Sprague) von Arx [= *Microdochium bolleyi* de Hoog & Hermanides-Nijhof] is a cosmopolitan fungus that is common on roots and stem bases of cereals and grasses (Sprague, 1950). It is implicated in biocontrol of several cereal pathogens, including the take-all fungus *Gaeumannomyces graminis* on wheat (Kirk & Deacon, 1987a) and in golf-course turf (Baldwin *et al.*, 1991), the eyespot fungus *Pseudocercosporella herpotrichoides* (Reinecke *et al.*, 1979; Reinecke & Fokkema, 1981) and *Fusarium* spp. (Reinecke *et al.*, 1979). It has no direct antagonistic effect on these fungi *in vitro*. Instead, it is thought to act by competition for host-derived nutrients that pathogens would use to support the early stages of infection (Deacon, 1991; Kirk, 1984). In particular, *I. bolleyi* can exploit the naturally senescing cereal root tissues during early growth of the crop (Kirk & Deacon, 1987b) and probably also the naturally senescing coleoptile and first-formed leaf sheaths (Lewis & Deacon, 1982). Consistent with this,

spores of *I. bolleyi* were found to germinate differentially towards dead as opposed to living cereal root hairs, ensuring rapid colonization of the senescent tissues (Allan *et al.*, 1992).

I. bolleyi has several features of potential value as a commercial biocontrol agent. It sporulates abundantly in shaken liquid culture (Deacon, 1988; Jadubansa *et al.*, 1993). It retains viability when culture biomass is dried on to cereal seeds (J. W. Deacon, unpublished data) or on to sand grains (used, but not stated, in the study by Baldwin *et al.*, 1991). It sporulated abundantly on seeds in vermiculite and the spores were carried down the roots in percolating water (Lascaris & Deacon, 1991). Its rapid spread on roots in soil is also compatible with spore movement in the rhizosphere (Kirk & Deacon, 1987b).

The work described here is a further contribution to the development of *I. bolleyi* as a potential seed-applied biocontrol agent for cereals. We recognized that *I. bolleyi* might be of most value as a general-purpose biocontrol agent, reducing the activities of several different pathogens in the particular seasonal or site conditions that favour these. Of the many environmental variables, availability of water has a major effect on activities of fungi (Ayres & Boddy, 1986), including pathogens and their potential biocontrol agents (Cook & Baker, 1983). For example, *Fusarium* foot-rot of cereals typically is associated with drought stress, and *F. culmorum* and related fusaria are highly tolerant of water stress *in vitro* (Cook & Christen, 1976). Conversely, the take-all fungus infects in wetter conditions and is intolerant of water stress *in vitro*. We therefore studied the inter-strain variability of *I. bolleyi* to water stress *in vitro*, so that strains might be targeted for control of particular pathogens (Deacon, 1991) or strain mixtures might be used to improve the consistency of biocontrol over sites and seasons (Deacon, 1994).

MATERIALS AND METHODS

Fungi

Three strains of *I. bolleyi* were chosen at random from our culture collection as follows: strain T560 from barley roots, Southern England (Dr G. A. Salt, 1974); strain AB1 from flax, Northern Ireland (see Black & Brown, 1986); strain J10 from wheat, Edinburgh (Dr J. J. Kirk, 1977). Also used were *F. culmorum* (strain CD9, stock culture collection), *G. graminis* var *tritici* Walker (strain LD1, from wheat near Edinburgh, 1992) and *Phialophora* sp. (strain IMI 187786, from maize). All strains were mass mycelial transfers from stock cultures, except for *I. bolleyi* strain AB1 which was a single-spored culture.

Media

Standard liquid medium comprised (g l^{-1} distilled water): Na_2HPO_4 (0.75), $MgSO_4.7H_2O$ (0.12), NH_4NO_3 (0.40), KH_2PO_4 (0.75), NaCl (0.10), Difco yeast extract (0.10), glucose (1.80) and Difco malt extract (1.00). Potato dextrose broth (PDB) was prepared by boiling 200 g peeled, diced potato for 20 min then straining through muslin and making up to 1 l with distilled water and 20 g glucose. PDA (Oxoid, Basingstoke, UK) was used at 39 g l^{-1} distilled water.

Before they were autoclaved (121°C, 15 min) the media were adjusted to different water potentials from − 0.1 MPa (controls) to − 5 MPa, using either KCl or polyethylene glycol (PEG) 8000 (Sigma Chemical Co., Poole, Dorset, UK; this product was formerly catalogued as PEG 6000). The concentrations were as follows (g l^{-1}): for KCl, − 0.1 MPa (none), − 1.1 MPa (10.06), − 1.9 MPa (17.90), − 3.0 MPa (28.44), − 3.9 MPa (36.50), − 5.0 MPa (47.56); for PEG 8000: − 0.1 MPa (none), − 1.1 MPa (150), − 1.9 MPa (212), − 3.0 MPa (265), − 3.9 MPa (297.5), − 5.0 MPa (345). The water potentials of the adjusted media were checked with an osmometer for osmotic potential (KCl-adjusted media) or a thermocouple psychrometer for matric potential (PEG-adjusted media).

Colony Extension Rates

Agar discs (5 mm) were taken from the margins of 7 day-old colonies of all fungi on PDA and placed centrally on PDA plates supplemented with KCl or into PEG-supplemented PDB (15 ml) in sterile Petri dishes. PEG had to be used in the liquid medium because it prevented the gelling

of agar. All dishes were incubated at 25°C and colony diameters were recorded daily for 7 days. The dishes containing PEG-supplemented liquid medium were handled carefully to avoid disturbance of the mycelia, and growth was measured by placing them on to a marked grid. In all cases, radial growth rates were calculated as mean ± SEM for three replicates, but for ease of comparison they are presented as percentage of growth in the control (− 0.1 MPa). Statistical differences between strains of *I. bolleyi* were calculated in two ways. On PDA, where control growth rates of strains were almost identical, the measured growth rates of strains at each water potential were subjected to analysis of variance. For the liquid medium, where control growth rates of the strains differed, each replicate at each PEG concentration was expressed as percentage of control growth rate and the percentages were subjected to analysis of variance after arcsine transformation; however, data for − 1.1 MPa could not be analyzed because they exceeded 100% for *I. bolleyi* strain J10.

Biomass Production

Conical flasks (250 ml) containing KCl-adjusted standard liquid medium (100 ml) were inoculated with single PDA discs (5 mm) of *I. bolleyi* then incubated for 7 days at 25°C on an orbital shaker at 125 rpm. The flask contents were then filtered under vacuum so that the biomass was retained on pre weighed 9.0-cm Whatman filter paper circles. These were rinsed then dried to constant weight at 80°C to obtain biomass yields. There were three replicate flasks per treatment. The same procedure was tried for PEG-adjusted medium, but abandoned because PEG was retained on the filter papers used to collect the biomass, precluding accurate measurement.

Spore Germination

Conidial suspensions of *I. bolleyi* were obtained by flooding 8-day PDA cultures with 10 ml sterile distilled water, then dislodging the spores by rubbing the agar surface with a glass rod. The suspensions were transferred to sterile tubes and washed three times by centrifugation (3000 × *g*, 4 min) with distilled water. The final suspensions were adjusted to *c.* 15×10^5 spores ml^{-1} distilled water. Aliquots (1 ml) were mixed thoroughly with 9 ml standard liquid medium adjusted to different water potentials with KCl or PEG 8000. Drops of the suspensions were transferred to sterile glass coverslips which were then inverted on to glass rings on microscope slides and sealed with Vaseline to prevent drying. The hanging drops were incubated at 25°C for 14 h, which had previously been found to be the optimum time for assessment of germination. Germination was assessed microscopically at X700 magnification, using three replicate drops per treatment and counting 200 spores per drop. Spores were considered to have germinated when the germ tube length equalled or exceeded the length of the spore. Standard errors were always very low, so for ease of comparison the data are presented as percentage germination.

Sporulation in Liquid Culture

Conical flasks (250 ml) contained 50 ml standard liquid medium adjusted to different water potentials with KCl or PEG 8000. They were inoculated with spore suspensions of *I. bolleyi*, prepared as above, so that the initial spore concentration in the flasks was *c.* 5×10^4 ml^{-1}. The flasks were incubated at 25°C on an orbital shaker at 120 rpm. Aliquots (0.5 ml) of the media were removed aseptically every 24 h for 7 days and spore numbers were assessed with a haemocytometer. There were three replicate flasks per treatment.

RESULTS

Colony Extension on KCl-adjusted Agar

The three strains of *I. bolleyi* grew at similar rates (3.0–3.6 mm $24\ h^{-1}$) on control agar plates, but showed significant differences in growth on KCl-adjusted agar (Figure 1). Strain AB1 grew at undiminished rate down to at least − 5 MPa, and its growth was enhanced at intermediate water potentials compared with the control. Strain T560 showed a progressive fall in colony extension rate with increase of water stress: it grew very slowly at − 3 MPa and lower. Strain

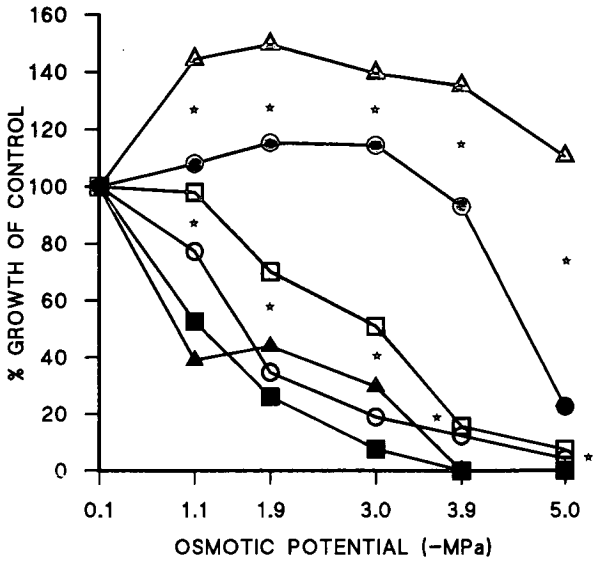

FIGURE 1. Radial extension on agar osmotically adjusted with KCl, as a percentage of control (− 0.1 MPa). Extension rates of controls (mm 24 h^{-1}, means ± SEM for three replicates) were: *F. culmorum* (●) 4.3 ± 0.2; *G. graminis* var. *tritici* (■) 3.0 ± 0.6; *I. bolleyi* AB1 (△) 3.6 ± 0.6; *I. bolleyi* T560 (○) 3.6 ± 0.6; *I. bolleyi* J10 (□) 3.4 ± 0.2; *Phialophora* sp. (▲) 3.4 ± 0.1. Asterisks denote significant differences in growth between all *I. bolleyi* strains at each water potential, based on analysis of variance of actual growth measurements.

J10 was intermediate in response; its growth rate was significantly less than that of strain AB1 at all KCl levels from − 1.1 to − 5.0 MPa, and significantly higher than that of strain T560 at all KCl levels. For comparison, *F. culmorum* was tolerant of KCl down to at least − 3.9 MPa, but its growth was reduced considerably at − 5 MPa (Figure 1). Both *G. graminis* and *Phialophora* sp. showed poor tolerance of KCl: they made little growth at − 3 MPa and none at − 3.9 MPa.

Colony Extension in PEG-adjusted Media

F. culmorum was the most tolerant of PEG-induced stress (Figure 2) and *G. graminis* was poorly tolerant; *Phialophora* sp. was not used in this test. Compared with the pathogens, all three strains of *I. bolleyi* showed intermediate responses to PEG-induced stress, but strain AB1 was significantly more tolerant than both other strains at − 1.9 to − 3.0 MPa (data for − 1.1 MPa could not be tested), and strain J10 was significantly more tolerant than strain T560 at − 1.9 MPa.

Biomass Production in Shaken Liquid Culture

The three strains of *I. bolleyi* gave similar yields in control flasks and in media adjusted with KCl down to − 1.9 MPa (Figure 3). However, strain T560 showed a marked fall in yield at potentials of − 3 MPa and lower, whereas strains AB1 and J10 showed undiminished yields at − 3.9 MPa and still grew well at − 5 MPa.

Spore Germination

The three strains of *I. bolleyi* were clearly and statistically separable in germination response to different water potentials (Figure 4); the standard errors of the germination counts were always very low, so that all clearly separable points in Figure 4 are significantly different. Strain AB1

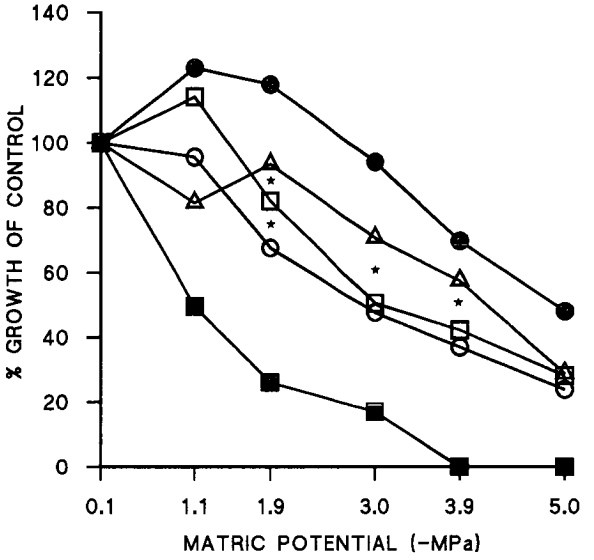

FIGURE 2. Radial extension in medium matrically adjusted with PEG 8000, as percentage of control (− 0.1 MPa). Extension rates of controls (mm 24 h^{-1}, means ± SEM for three replicates) were: *F. culmorum* (●) 1.2 ± 0.3; *G. graminis* var. *tritici* (■) 1.4 ± 0.6; *I. bolleyi* T560 (○) 1.1 ± 0.5; *I. bolleyi* J10 (□) 1.0 ± 0.1; *I. bolleyi* AB1 (△) 1.3 ± 0.1. Significant differences between *I. bolleyi* strains at each matric potential shown by asterisk, based on analysis of variance of arcsine-transformed data (not applicable to − 1.1 MPa).

germinated maximally down to − 5 MPa with PEG 8000 and at nearly 70% with KCl at − 5 MPa. Strain K560 showed much reduced germination at even − 1.9 MPa with either PEG or KCl, and its germination was inhibited at − 5 MPa. Strain J10 was intermediate in behaviour. All three strains were more tolerant of PEG than of KCl at equivalent water potentials, but this difference did not obscure the strain differences in tolerance of water stress.

Spore Production in Shaken Liquid Culture

In the controls (− 0.1 MPa), all three strains of *I. bolleyi* began to sporulate at 2–3 days, during exponential growth, and gave maximum spore yields ranging from *c.* 6 to 18 × 10^6 ml^{-1} culture broth. With KCl, all strains produced at least some spores down to − 5 MPa (Figures 5–7). Other features of the results were difficult to interpret because the time-course of sporulation in KCl-adjusted media often differed from that in controls and some of the early-formed spores germinated to produce mycelia so that the spore numbers sometimes declined from peak values. However, at some of the assessment times, all isolates produced more spores at − 1.9 and − 3 MPa than they did in the controls at equivalent times. It was also seen (Figure 6) that strain T560 had the clearest demarcation of behaviour at the different water potentials: its sporulation relative to the controls was significantly enhanced at − 1.1, − 1.9 and − 3 MPa, but not at − 3.9 and − 5 MPa.

The findings were different for PEG-adjusted media (Figures 8–10) than for KCl. Sporulation was often suppressed relative to the controls, and especially at the higher concentrations of PEG. Strains T560 and J10 produced few or no spores at − 3, − 3.9 and − 5 MPa, whereas strain AB1 produced at least some spores at all levels of water stress. It was noted that all strains differentiated to form clusters of chlamydospore-like cells in the presence of PEG at water potentials of − 1.1 MPa and lower, so the cultures progressively melanized with time. This was not seen in the controls or in KCl-adjusted media.

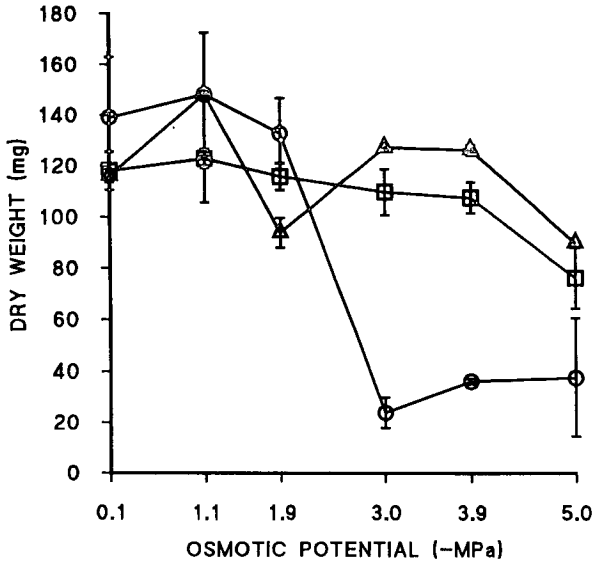

FIGURE 3. Dry weights of *I. bolleyi* AB1 (△), T560 (○) & J10 (□) in liquid medium osmotically adjusted with KCl (mean ± SEM for three replicates).

DISCUSSION

We assessed the effects of water stress on the three stages of activity of *I. bolleyi* that are most relevant to biocontrol—spore germination, hyphal growth and sporulation. This fungus has a

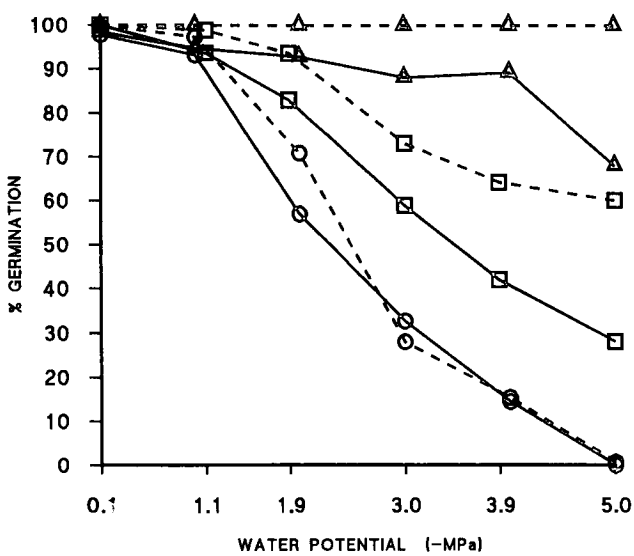

FIGURE 4. Percentage germination of *I. bolleyi* AB1 (△), T560 (○) & J10 (□) at different osmotic potentials (KCl solid lines) or matric potentials (PEG 8000, broken lines). Data based on counts of 200 conidia in each of three replicates. Standard errors (not shown) calculated for the original data were smaller than the symbol sizes here.

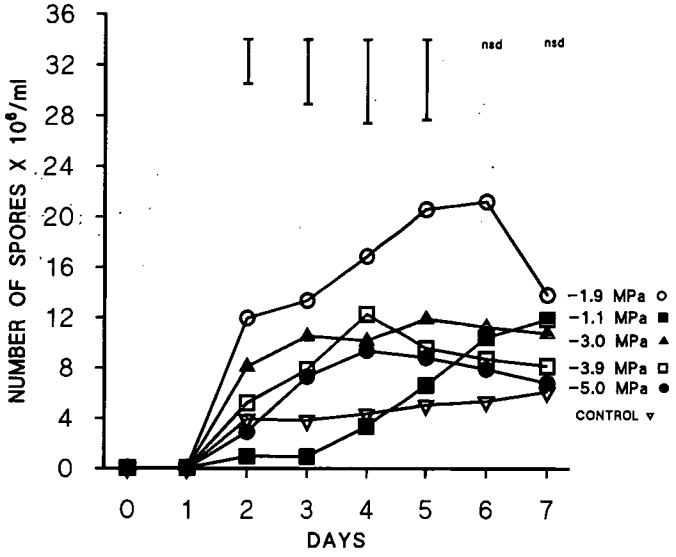

FIGURE 5. Spore production by *I. bolleyi* (AB1) in liquid medium adjusted with KCl; means of three replicates. Bars above the graphs show 5% L.S.D. between treatments at each time; nsd = no significant difference.

simple life cycle: spore germination leads to a short phase of vegetative growth then further spores are budded from the hyphae or the fungus converts to clusters of chlamydospore-like cells (Cooper & Gadd, 1984). Sporulation occurs during exponential growth *in vitro* (Jadubansa *et al.*, 1993), the germlings can produce further spores within 24 h (Allan *et al.*, 1992) and the spores can even germinate directly to produce further spores by microcycle conidiation (Lascaris &

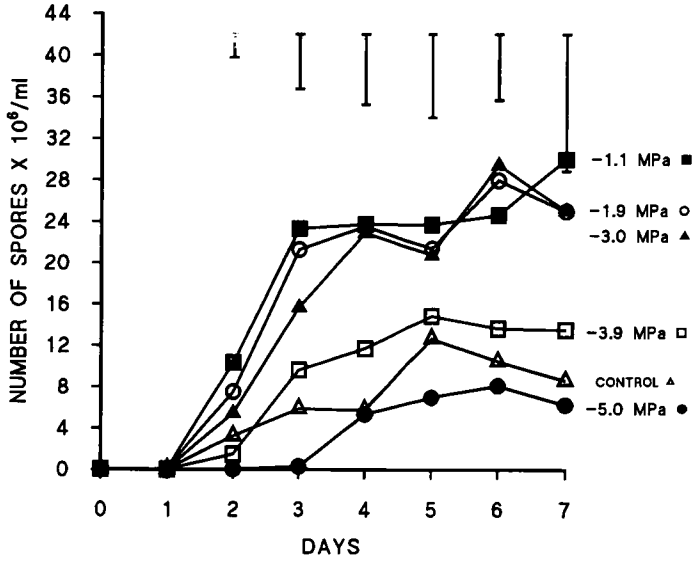

FIGURE 6. Spore production by *I. bolleyi* (T560) in liquid medium osmotically adjusted with KCl. Means for three replicates; bars show 5% L.S.D. between treatments at each time.

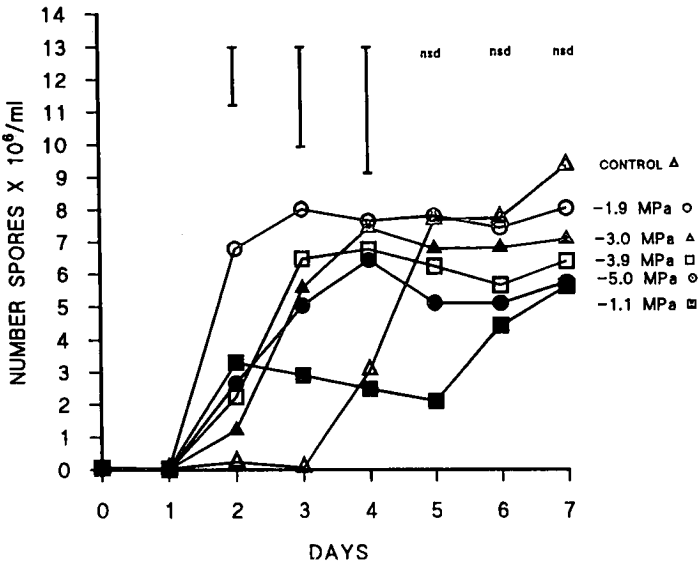

FIGURE 7. Spore production by *I. bolleyi* (J10) in liquid medium adjusted with KCl; means for three replicates. Bars above the graphs show 5% L.S.D. between treatments at each time; nsd = no significant difference.

Deacon, 1994). All these features could contribute to efficient exploitation of resources and thus competitive exclusion of pathogens.

It is often difficult to interpret the effects of water potential on fungi *in vitro* or to relate these effects to growth in natural systems. We used two approaches to try to overcome these problems. Firstly, we compared the behaviour of *I. bolleyi* strains with that of two cereal pathogens that have been intensively studied for water-stress tolerance. In this respect, our results for *F.*

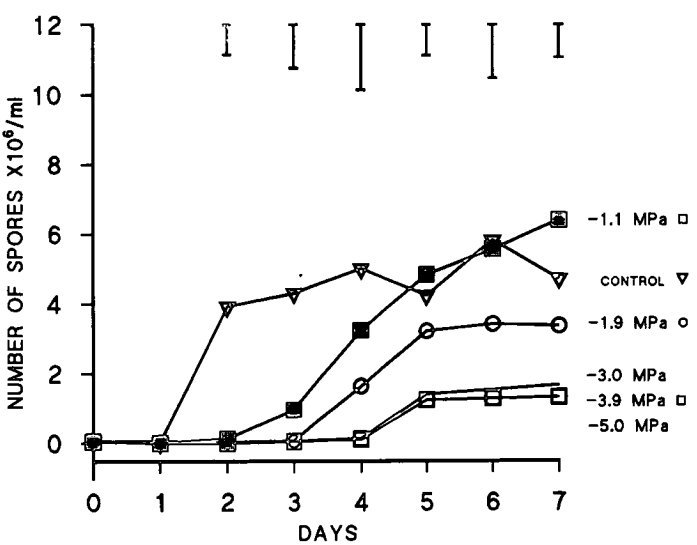

FIGURE 8. Spore production by *I. bolleyi* (AB1) in liquid medium adjusted with PEG 8000; means of three replicates. Bars above the graphs show 5% L.S.D. between treatments at each time.

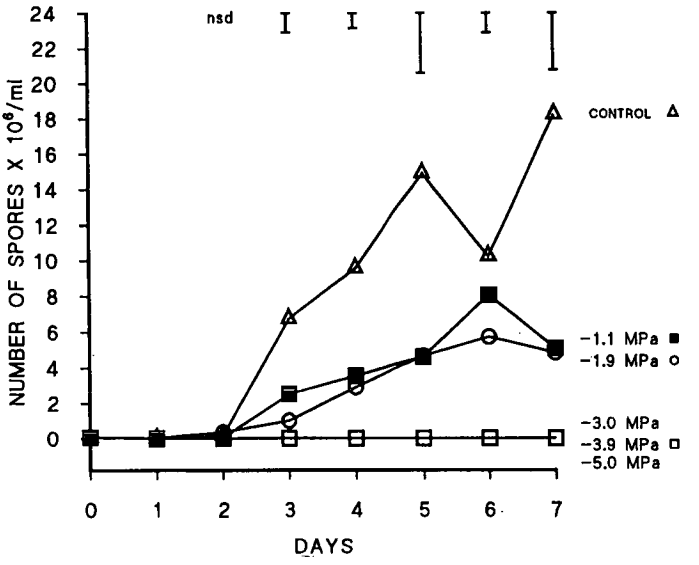

FIGURE 9. Spore production by *I. bolleyi* (T56) in liquid medium adjusted with PEG 8000; means of three replicates. Bars above graphs show 5% L.S.D. between treatments at each time; nsd = no significant difference.

culmorum and *G. graminis* agree with many previous reports (e.g. Cook *et al.*, 1972); the former is tolerant of water stress and the latter intolerant. Relative to the controls, we also observed an increase in growth of *F. culmorum*, but not of *G. graminis*, at low levels of water stress, as others have noted (Cook *et al.*, 1972; Griffin, 1972). Secondly, we used both KCl and PEG 8000 to adjust the water potential. The salt acts by generating osmotic potential (= solute potential) although at high concentrations it might also have a directly inhibitory effect (Papendick &

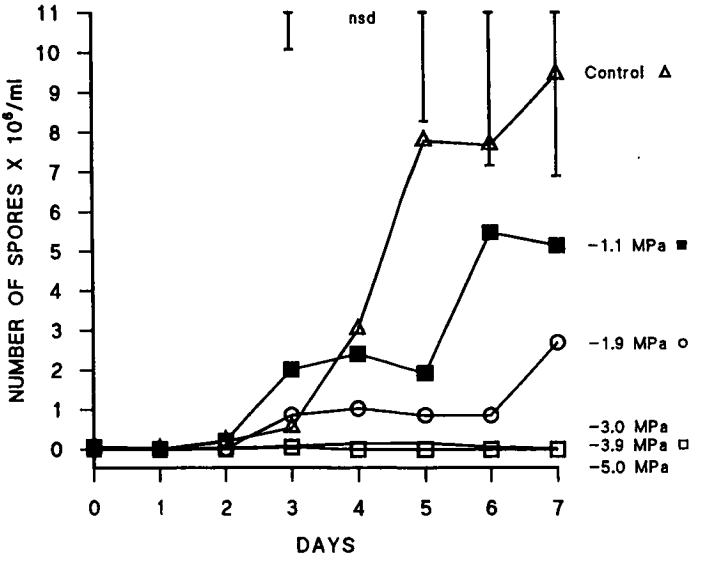

FIGURE 10. Spore production of *I. bolleyi* (J10) in liquid medium matrically adjusted with PEG 8000. Means of three replicates; bars show 5% L.S.D. between treatments at each time (nsd = no significant difference).

Mulla, 1986). In contrast, PEG 8000 binds water and thus generates matric potential; at water potentials below − 1 MPa less than 10% is contributed by osmotic potential, the rest being matric potential (Steuter *et al.*, 1981). Osmotic potential would be relevant to growth within plant tissues; wheat tissues under dryland conditions in the Pacific north-west of the USA are commonly − 2.5 to − 3.5 MPa between the tillering and heading stages (Papendick & Cook, 1974). Matric potential could be more relevant to growth in soil or on the root surface (Cook & Papendick, 1972), and presumably also to growth in senescing plant tissues where the host has lost control over the water relations of its cells.

The most notable feature of our results is that strains of *I. bolleyi* differed markedly from one another in tolerance of stress imposed by both osmotic and matric potential. The range of variation in this species was much greater than ever before reported for a single fungus. Indeed, one strain approached the water stress-tolerance of *F. culmorum* while another was almost as intolerant as *G. graminis*, and yet the three *I. bolleyi* strains had been tested at random. Their origins could not have been used to predict their stress tolerance because the most stress-tolerant strain (AB1) originated from diseased flax, where prolonged waterlogging of the soil is thought to have predisposed the crop to attack by weak parasites (Black & Brown, 1986).

Stetter and Leroul (1979) reported that strains of *I. bolleyi* vary in their effects on barley seedlings in a laboratory assay. Strains from continuous barley cropping were mildly deleterious whereas strains from first barley crops had no deleterious effect or were mildly growth-promoting. The deleterious effect of strains from continuous cropping was lost after laboratory culturing. That report and the work presented here suggest that *I. bolleyi* is a physiologically variable fungus from which strains might be selected for specific, stable, desirable attributes in biocontrol. One of these attributes might be a degree of water-stress tolerance that matches that of specific pathogens. Ramakrishna *et al.* (1993) showed that interactions of fungal species on stored cereal grains were strongly influenced by water potential of the grain, and the outcome of interactions could be predicted from the stress tolerance of the species *in vitro*. Cook and Baker (1983) discussed how similar interactions could influence competition between non-pathogens and pathogens in the early stages of infection of plants. It has been argued that one of the major reasons for inconsistent performance of biocontrol inocula in field sites is that single clonal strains of biocontrol agents are expected to compete for resources with indigenous organisms in conditions that vary between sites and seasons (Deacon, 1994). The variation in water-stress tolerance among strains of *I. bolleyi* raises the possibility that strains might be combined in seed inocula to overcome this problem or to colonize both roots and basal stem tissues which exist in somewhat different environments. With these points in view, we are investigating the effects of water stress on competitive interactions of *I. bolleyi* strains and also testing the stress-tolerance of strains isolated from cereal roots as opposed to basal stem tissues.

ACKNOWLEDGEMENTS

We are grateful to Drs G. A. Salt and A. E. Brown for supply of strains; also to Dr N. Magan for much helpful advice in relation to water potential. L. I. Douglas was supported by a Postgraduate Studentship from the Ministry of Agriculture, Fisheries and Food, which we gratefully acknowledge.

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