The role of colonisation of soil and wheat roots by *Trichoderma koningii* in biological control of *Gaeumannomyces graminis var. tritici*.

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CONTENTS

ABSTRACT

The aim of this project was to track *Trichoderma koningii* in soil, the rhizoplane and wheat roots. The extent of rhizoplane and root colonisation by *T. koningii* was assessed to determine whether these factors play a significant role in the control of take-all disease caused by *Gaeumannomyces graminis* var. *tritici* (Ggt). *T. koningii* is a known biocontrol agent for take-all disease of wheat caused by Ggt. Increasing our understanding of the mechanisms by which *T. koningii* reduces take-all disease will lead to greater consistency of disease control.

Using a marker gene to track *T. koningii*, the location and extent of root colonisation could be quantified. The *gus*A gene was selected as a marker. This gene acts by transcribing an enzyme that cleaves β-glucuronides. In the presence of different substrates blue colouration or fluorescence can be used to locate a fungus carrying the gene within plant tissue and also to quantify the active biomass of the fungus. The *gus*A and *hph* (hygromycin B phosphotransferase) genes were co-transformed into protoplasts of *T. koningii*. The resultant transformants were unstable after five generations. An alternative transformation procedure was used in which a 2 kb segment of DNA from *T. koningii* was integrated into plasmids carrying the *gus*A and *hph* genes. These transformants were stable on media after ten generations. The *gus*A gene was detected in the transformants by PCR analysis and Southern hybridisation and histochemical tests showed the gene was being expresssed. The transformants could not be distinguished morphologically from the untransformed isolate and they inhibited the growth of Ggt on agar.

Wheat root and rhizosphere colonisation by *T. koningii* were not impaired

by the tracking gene. In sterile soil up to 30 % of the wheat roots were found to be colonised by *T. koningii* when plated after surface disinfestation. While the fungus moved both up and down wheat roots, colonisation was greatest in the 2.5 cm closest to the crown. Native isolates of *T. koningii* were not found beyond the outer cortex of wheat roots. However it was shown that both the indigenous isolates of *T. koningii* and the introduced isolates colonised up to 80 % of the rhizosphere. These figures are much higher than has previously been reported.

Isolates of *T. koningii* expressing the *gus*A gene were added to soil in which wheat seedlings were grown. Histochemical assays, PCR analysis and Southern hybridisation of the roots failed to find any indication of the presence or expresson of the *gus*A gene. However when *T. koningii* was reisolated from wheat roots, the rhizosphere and soil surface, some of the recovered isolates were GUS positive when assayed histochemically. The *gus*A gene was detected when Southern hybridisation was performed on the DNA from the recovered isolates that had been amplified by PCR.

Nutrition may play a role in the higher rate of GUS expression in *T. koningii* grown on wheat roots compared with soil. GUS expression was higher in isolates grown on nutrient rich media than on a nutrient poor medium. Also a higher proportion of isolates expressing the *gus*A gene were recovered from roots than from the soil surface. The metabolic load incurred when expressing the *gus*A gene may be too great so that the gene is not expressed under nutritional stress.

Although the *gus*A gene could not be used to track *T. koningii* in wheat roots the interaction between *T. koningii* and *Gaeumannomyces graminis* var. *tritici* in roots and soil was examined using a selective medium. *T. koningii*

reduces take-all disease by inhibiting hyphal regeneration of Ggt in the bulk soil. The two fungi do not have to be co-located in soil for effective biocontrol, probably due to the rapid growth rate of *T. koningii*. However, addition of *T. koningii* was ineffective after Ggt had colonised soil. Root and rhizosphere colonisation could not be related to disease suppression. The extent of rhizosphere and root colonisation was similar in wheat plants with severe take-all disease and healthy plants. Therefore inhibition of Ggt in soil appears to be critical for successful biocontrol by *T. koningii*.

This thesis contains no material which has been accepted for the award of any other degree or diploma in any university. To the best of my knowledge the thesis contains no material previously published or written by another person, except where due reference is made in the text of the thesis. I consent to this thesis being made available for photocopying and loan once it has been accepted for the award of a Ph.D.

Sonya Dyer.

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CHAPTER 1. INTRODUCTION AND LITERATURE REVIEW

1.1 Introduction

Biological control using naturally occurring antagonistic microorganisms has potential to inhibit soil and root pathogens of many economically important crops. However, this approach is not widely used due to the inconsistency of control in different seasons and locations (Wong & Southwell 1980, Rovira *et al.* 1992). To enable biocontrol to be a commercially viable option, the factors limiting the success of the antagonistic microbes need to be identified and their ecology well understood. When a fungus is added to soil or plants as a biocontrol agent it is difficult to distinguish it from the native fungal flora and in particular from indigenous members of the same or closely related species. Monitoring the presence, location, abundance and activity of the antagonist is crucial to understanding its action in a production system. In order to monitor performance of an introduced strain it is necessary to be able to reisolate it hence, a means of tracking the antagonist is required. A number of tracking methods have been tried including isolation on selective media, marker genes, isozymes and DNA-based probes. Generally tracking techniques have been more successful with bacteria than fungi (Kluepfel 1993, Ryder 1993) however, they are being developed for fungi. To date tracking of fungi has not been widely tested outside a sterile environment.

Trichoderma Pers. ex Fr. is regarded as one of the most widely distributed of all genera of soil fungi (Domsch *et al.* 1980) and members of the genus are well known as potential biocontrol agents. Species of *Trichoderma* (including *T. koningii*) protect seedlings against species of *Pythium* which cause

damping off (Kraft & Papavizas 1983, Taylor *et al.* 1991), reduce viability of the sclerotia of *Sclerotinia sclerotiorum* (Jones & Watson 1969) and *S. rolfsii* (Wells *et al.* 1972, Henis *et al.* 1984), prevent blossom end-rot of apples caused by *Botrytis cinerea* (Tronsmo 1991) and are antagonistic in soil to *Rhizoctonia solani* (Chet & Baker 1980, 1981, Kohl & Schlosser 1991). Strains of *Trichoderma* species are currently available commercially to control foliar and root pathogens of turf (Lo *et al.* 1995), silver leaf disease of peach and pip fruit trees caused by *Chondrostereum* species, *Fusarium* and *Rhizoctonia* on cuttings, ornamentals and turf. Root rot caused by *Armillaria* in kiwifruit vines has been controlled by species of *Trichoderma* and members of this genus have also been used to protect posts and poles against wood decay. In addition, *Trichoderma koningii* has been recognised as being potentially important in controlling take-all disease of wheat caused by *Gaeumannomyces graminis* (Sacc.) von Arx & Oliver var. *tritici* Walker (Ggt). To make effective use of these biocontrol products an improved understanding of the ecology of *Trichoderma* is required.

1.2 Biology of *Trichoderma*

1.2.1 Taxonomy

Trichoderma is morphologically similar to the closely related genera *Verticillium* and *Acremonium*. On agar medium all have rapidly spreading colonies of loose texture (Pitt & Hocking 1985). Because a number of species of *Trichoderma* act as biocontrol agents it is important to be able to tell them apart from each other. Identification of species within the genus *Trichoderma*

is widely recognised to be difficult (Papavizas 1985). The genus is divided into five sections based on the morphology of conidiophores, phialides and conidia when cultures are grown on 2 % malt extract agar. *T. koningii* Oudem belongs to the section *Trichoderma*, in which, the teleomorphs (if known) are from the genus *Hypocrea*. The section includes four aggregate species. Members of the aggregate in which *T. koningii* fits have conidiophores bearing regularly verticillate branches. Phialides on these branches produce smooth-walled ellipsoidal conidia which are green to brownish in colour (Bissett 1991). Amplification of the 28S rDNA using the polymerase chain reaction (PCR) has enabled the phylogenetic relationship amongst isolates of *T. virens*, *Gliocladium* species and related teleomorphs to be studied (Rehner & Samuels 1994). It was concluded that *Gliocladium* is polyphyletic and that *G. penicilloides*, *G. roseum* and *T. virens* are genetically distinct. This work shows that there is potential for using molecular techniques with sufficient sensitivity to distinguish species, however, to date this has not been attempted.

1.2.2 Ecology

T. koningii is ubiquitous in soil. It inhabits the leaf litter layer and the A and B horizons in natural forests, ranging from conifers to acacias, grasslands and soils cultivated with grapevines, citrus and broad-acre crops. *T. koningii* has also been isolated from peat bogs, swamps, salt marshes and coastal cliffs. The species also occurs frequently in plant material exposed to soil such as roots, stumps or decaying leaves and stems (Domsch *et al.* 1980). *T. koningii* is widely viewed as a saprophyte rather than a root coloniser (Domsch *et al.* 1980, Papavizas 1985, Ahmad & Baker 1988). However, it has been isolated

from roots of a wide variety of crop plants as well as species of *Hakea* and *Pinus* (Domsch *et al.* 1980, Falloon 1982, Allsopp *et al.* 1987). The significance of root and rhizosphere colonisation by *T. koningii* has not been studied although this is important, in achieving biological control.

T. koningii has been shown to have both negative and positive effects on plant growth. Enhanced plant growth in the presence of *T. koningii* could be via antagonism towards pathogens which is the basis on which potential biocontrol microbes are selected. Infestation of soil with *T. koningii* has resulted in increased shoot growth, root dry weight or fruit production in wheat (Simon 1989b), radish seedlings (Windham *et al.* 1985) and tomatoes (Latunde-Dada 1993). It has been postulated that this is due to the production of a growth regulating factor (Windham *et al.* 1986). However, this mechanism clearly does not operate for all plant species because growth promotion of apple seedlings has not been observed (Roiger & Jeffers 1989) and growth of maize and spruce seedlings may be retarded by the presence of *T. koningii* in soil (McFadden & Sutton 1975, Falloon 1985), possibly due to production of koninginins (polyketides) which have been observed to inhibit growth of etiolated wheat coleoptiles (Parker *et al.* 1995).

A further complication is the interaction between *T. koningii* and soil-borne microorganisms. When inoculated onto wheat, successful biological control depends upon *T. koningii* effectively competing with the microflora naturally present in soil. The competitive advantage may vary according to the microhabitat within soil being examined. There are few reports on repression of *T. koningii* by other fungi and bacteria. Germination of conidia of *T. koningii* is inhibited *in vitro* (Schuepp & Green 1968) and in soil (Schuepp & Frei 1969,

Mitchell & Dix 1975). Growth of *T. koningii in vitro* has been reported to be retarded by hyphal contact with *Tuber melanosporum* (Domsch *et al.* 1980). *T. koningii* competes with other *Trichoderma* species on spruce needles. Temperature and the identity of the challenging species determines which becomes dominant (Widden 1984). In a study on maple and pine litter *T. koningii* was an effective competitor over a wide range of temperatures but performed best at 20-25 °C. Litter type did not have much effect on the competitive ability of *T. koningii* (Widden & Hsu 1987).

In media with a low iron content (< 10 µg/g) *Pseudomonas* spp. may inhibit growth of *T. koningii*. In addition to competition for iron, *T. koningii* is sensitive to the fluorescent pigment produced by *Pseudomonas* spp (Hadar, Harman & Taylor 1984). Hyphal growth by *T. koningii* on water agar was inhibited 5 to 10 % by *Agrobacterium tumefaciens*, *Xanthomonas campestris* pv. *campestris*, *Erwinia carotovora* pv. *carotovora* and *Pseudomonas syringae* pv. *glycinea* (Leben 1984). Some of these bacteria were observed to spread from their original position along hyphae of *T. koningii*. Bacteria isolated from take-all conducive soil inhibited radial growth of *T. koningii* on PDA by 28 % and impaired its ability to reduce saprophytic growth of Ggt (Simon & Sivasithamparam 1988d). We need to understand the complexities of the interactions between *T. koningii*, plants and other microorganisms to utilise this fungus successfully as a biological control agent.

1.2.3 Inhibition of Disease by *T. koningii*.

As mentioned earlier, *T. koningii* is reported to be an effective biological control agent against a variety of soil-borne pathogens, including fungi that

produce sclerotia or cause seedling death or take-all disease of wheat. *T. koningii* reduces seedling damping-off caused by *Pythium* species (Hadar *et al.* 1984, Lifshitz *et al.* 1986, Nelson *et al.* 1988, Roiger & Jeffers 1991) and *Rhizoctonia solani* (Abdel-Rahim & Abu-Surriah 1989, Tzavella *et al.* 1991, Liu 1991) and seedling crown and root rot caused by *Phytophthora cactorum* (Roiger & Jeffers 1991). Seedling survival has also improved as a result of the addition of *T. koningii* to soil precolonised with *Sclerotium rolfsii*, *Fusarium* species (Monaco *et al.* 1991) and *Verticillium dahliae* (Georgieva 1992). *T. koningii* parasitises sclerotia and inhibits apothecial development (Trutman & Keane 1990, Luth *et al.* 1992, Latunde-Dada *et al.* 1993). *T. koningii* reduces take-all disease of wheat in pots in a controlled environment and in field trials using cylinders (30 cm diam) to contain each treatment (Simon 1989, M. Ryder, personal communication 1994). While *T. koningii* inhibits infection of pruned apricot trees by *Cytospora cincta* (Rozsnay *et al.* 1992) little work has been done on inhibition of bacterial growth by this fungus. Tri-D25 (Nu Erth Horticultural and Rural Supplies, South Australia) containing *T. koningii* is available commercially as a product that is claimed to increase the beneficial microbial population around the roots of cuttings, bulbs, seeds and seedlings. This implies that the product will decrease disease, but precise benefits to plants are not stated. Observations of disease control such as these do not, especially when derived from studies on plants in soil, give direct information on mechanisms. Studies on mechanisms have usually been carried out on agar, with all the attendant limitations of artificial conditions. Nevertheless this is the only type of information currently available.

The interaction of *T. koningii* with fungi has primarily been studied *in*

vitro. Members of the genus *Trichoderma* have been found to act extensively as mycoparasites (Dos Santos & Dhingra 1982, Lynch 1990). Coiling of hyphae of *T. koningii* around fungal hyphae resulting in cessation of growth of the latter has been reported (Table 1.1). *T. koningii* degrades the cellulose walls of Oomycetes by producing endo-1,4-β-glucanases, a cellobiohydrolase and β glucosidases (Yeoh *et al.* 1984, Halliwell *et al.* 1985, Wood & M^{c}Crea 1986, Ishihara *et al.* 1989). Amylase, β-fructofuranosidase, α-glucosidase, ribonuclease, phenol oxidase and proteolytic enzymes have also been identified as enzymes secreted by *T. koningii* (Domsch, Gams & Anderson, 1980). As glucans and chitins are in most fungal cell walls production of enzymes by *T. koningii* that break down these substances may be important in biocontrol mechanisms. Proteases could also contribute to the antagonistic activity (Lynch 1990).

T. koningii produces antibiotics that inhibit growth of some fungi (Table 1.2). These antibiotics include dermadin and trichoviridin which contain an isocyanide group (Tamura *et al.* 1975), isonitrins (Okuda *et al.* 1982), koninginin B (Cutler *et al.* 1991), koningic acid (Endo *et al.* 1985, Sakai *et al.* 1990), ergokoninin A and ergokoninin B (Augustiniak *et al.* 1991). Production of 6-n pentyl-2H-pyran-2-one and 4.8-dihydroxy-2-(1-hydroxyheptly)-3,4,5,6,7,8-hexahydro-2H-1-benzopyran-5-one by *T. koningii* inhibits growth of Ggt in culture media (Simon & Sivasithamparam 1988, Dunlop *et al.* 1989). However the action of antibiotics depends on the stage of development of the fungus challenged. For example 6 pentyl-alpha-pyrone reduced hyphal growth of *Phytophthora cinnamomi*, but induced oospore formation in the same fungus (Pratt *et al.* 1972, Benoni *et al.* 1990). Antibiotics produced by conidia of

T. koningii inhibited growth of bacteria found in a wheat-pasture soil (Simon & Sivasithamparam 1988).

Production of volatile metabolites by *T. koningii* impairs hyphal growth of some fungal species (Table 1.3). However, the volatile compounds homothallin I and II produced by *T. koningii* have been reported to stimulate oospore production by *Phytophthora cinnamomi* (Anderson *et al.* 1978, O'Brien 1991). Competition may play a role in the microbial biocontrol by *Trichoderma* species, as an independent phenomenon, or alongside antibiosis, production of volatile metabolites or hyperparasitism (Chet 1987).

There is some evidence that *T. harzianum* and *T. viride* stimulate hypersensitive responses in citrus seedlings and tobacco leaves (Quass *et al.* 1993, Sharon *et al.* 1993). This interaction between *T. koningii* and plants has not been further investigated. There is clearly a gap between our understanding of mechanisms that operate *in vitro* and those that act in soil or plants. The aim of the work carried out in this project is to determine how *T. koningii* controls take-all disease in soil and in particular to devise methods for tracking *T. koningii* in wheat roots and the rhizosphere.

Table 1.1 Fungal species the growth of which is reduced by hyphal interaction (coiling around hyphae (C) and/or mycoparasitism (M)) by *T. koningii* when tested *in vitro*.

Fungi	Hyphal Interaction	Medium	Reference
Pyronema domesticum	C	2 % MEA[a]	Denis & Webster 1971c
Sclerotinia sclerotiorum	C, M	WA[b]	Trutmann & Keane 1990
Pythium ultimum	C	2 % MEA	Denis & Webster 1971c
Mucor hiemalis	C	2 % MEA	Denis & Webster 1971c
Mucor mucedo	C	2 % MA[c]	Tronsmo & Dennis 1978
Ganoderma lucidum	M	2 % MEA	Shukla & Uniyal 1989
Lentinus edodes	C	2 % MEA	Denis & Webster 1971c
Phellinus noxius	C	WA	Tong-Kwee & BoonKeng 1990
Rigidoporus lignosus	C	WA	Tong-Kwee & BoonKeng 1990
Heterobasidion annosum	C	2 % MEA	Denis & Webster 1971c
Rhizoctonia solani	C	2 % MEA	Denis & Webster 1971c
	C, M	PDA[d]	Tzavella *et al.* 1991
Fusarium oxysporum	C	2 % MEA	Denis & Webster 1971c
Sclerotium rolfsii	C	WA	Tong-Kwee & BoonKeng 1990
	C	PDA	Deb & Dutta 1988
Botrytis cinerea	C	2 % MA	Tronsmo & Dennis 1978

[a] malt extract agar, [b] water agar, [c] malt agar, [d] potato dextrose agar

Table 1.2 Fungal species the growth of which is reduced by production of antibiotics by *T. koningii* when tested *in vitro*.

Fungi	Medium	Reference
Gaeumannomyces graminis var. *tritici*	1/5 PDA[a] MA[b]	Simon *et. al.* 1988 Innocenti 1991
Phytophthora cinnamomi	1/5 PDA	Simon *et al.* 1988
Pythium middletonii	1/5 PDA	Simon *et al.* 1988
Mucor mucedo	2 % MA	Tronsmo & Dennis 1978
Ganoderma boninense	MA	Tong-Kwee & BoonKeng 1990
Ganoderma philippi	MA	Tong-Kwee & BoonKeng 1990
Rigidoporus lignosus	MA	Tong-Kwee & BoonKeng 1990
Phellinus noxius	MA	Tong-Kwee & BoonKeng 1990
Heterobasidion annosum	2 % MEA[c]	Denis & Webster 1971a
Cytospora cincta	PDA	Rozsnay *et al.* 1992
Sclerotium rolfsii	MA	Tong-Kwee & BoonKeng 1990
Fusarium oxysporum	1/5 PDA	Simon *et. al.* 1988
Alternaria solani	NS[d]	Kumar & Singh 1983
Botrytis cinerea	2 % MA	Tronsmo & Dennis 1978
Rhizoctonia solani	1/5 PDA	Simon *et al.* 1988
	PDA	Khara & Hadwan 1990
		Worasatit *et al.* 1994

[a] potato dextrose agar, [b] malt agar, [c] malt extract agar, [d] not stated

Table 1.3 Fungal species the growth of which is reduced by production of volatile metabolites by *T. koningii* when tested *in vitro*.

Fungi	Medium	Reference
Pyronema domesticum	2 % MEA[a]	Denis & Webster 1971b
Pythium ultimatum	2 % MEA	Denis & Webster 1971b
Mucor hiemalis	2 % MEA	Denis & Webster 1971b
Mucor mucedo	2 % MA[b]	Tronsmo & Dennis 1978
Heterobasidion annosum	2 % MEA	Denis & Webster 1971b
Phellinus noxius	MA	Tong-Kwee & BoonKeng 1990
Fusarium oxysporum	2 % MEA	Denis & Webster 1971b
Rhizoctonia solani	2 % MEA	Denis & Webster 1971b
Botrytis cinerea	2 % MA	Tronsmo & Dennis 1978
Sclerotium rolfsii	2 % MEA	Deb 1990
	MA	Tong-Kwee & BoonKeng 1990

[a] malt extract agar, [b] malt agar

1.3 Biocontrol of Take-all Disease by *Trichoderma*

1.3.1 Take-all Disease of Wheat

Temperate wheat crops are likely to suffer yield loss from take-all disease (Garrett 1981) caused by Ggt. Ggt survives in soil and dead organic matter exclusively as hyphae (Cunningham 1981). The fungus extensively colonises the rhizosphere of wheat plants and other monocotyledons found in wheat cropping rotations, but it can also survive saprophytically (Glenn *et al.* 1988). Grain development is impaired as hyaline hyphae from the cortex grow through the endodermis to the stele, where translocation in the phloem is severely inhibited. Severely infected plants are stunted and ripen prematurely and as a result grain yield may be halved (Wiese 1987). Take-all disease causes between $100-200 million worth of damage annually to wheat crops in southern Australia (D. Roget, personal communication 1996).

Current methods available to control take-all disease in wheat include weed control and crop rotation. Elimination of grasses which are alternate hosts and rotation with non-cereal crops are presently the most widely used measures for the control of take-all in southern Australia (Kollmorgen 1982). Crop rotation and removal of weeds such as barley (*Hordeum* spp.), brome (*Bromus* spp.), silver (*Vulpia* spp.) and rye grass (*Lolium rigidum*) lowers the inoculum potential of Ggt in two ways. The food base for pathogenic growth of the take-all fungus is removed and strong competition from soil microbes supported by nongramineacous crops reduces saprophytic survival of Ggt. Application of a triazole-based fungicide coated on double superphosphate fertiliser which is added to furrows at the time of planting has been successful in reducing take-all

disease (Ballinger & Kollmorgen 1988, Cotterill *et al.* 1992) but this practice is not cost effective in poor seasons.

1.3.2 Biocontrol of Take-all Disease

Biological control of take-all has been studied because of the lack of other commercially feasible methods to control this disease. Soil microbes have been recognised as causing decline of take-all in paddocks after several years of wheat monoculture (Gerlagh 1968, Shipton 1975, Cook & Rovira 1976, Rovira & Wildermuth 1981, Cook & Weller 1987). Numerous laboratory and glasshouse experiments using a range of different microorganisms have been conducted with the aim of finding a promising biocontrol agent for Ggt. Bacteria, fungi, actinomycetes (Zogg & Jaggi 1974, Zogg 1976, Sivasithamparam & Parker 1978), weakly pathogenic viruses (Lapierre *et al.* 1970, Lemaire *et al.* 1970, Rawlinson *et al.* 1973), mycophagous amoebae (Homma *et al.* 1979, Chakraborty & Warcup 1983), and earthworms (Stephens *et al.* 1994) have all been shown to reduce take-all disease of wheat in soil. *T. koningii* has been shown to inhibit Ggt in soil, hence it has potential to act as a biocontrol agent of take-all disease in the field.

In comparison to pot tests, relatively few microbes have been tested using standard cropping procedures in the field. *Pseudomonas* species have been the most widely tested in the field, and have shown yield increases of approximately 20 % (Weller & Cook 1983, Lamers *et al.* 1988 Wuthrich & Defago 1991, Zaspel 1992). Yield has been used as a measure of take-all control under conditions where crop growth has been severely limited by Ggt. *Bacillus pumilis* and *Bacillus subtilis* applied as seed coatings have also

increased the yield of wheat exposed to Ggt. *Bacillus pumilis* gave the best yield response (39-114 %) (Capper & Campbell 1986, Zaspel 1992, Nayudu *et al.* 1994). Yield increases attributed to fungal biocontrol agents are generally higher than those found with bacteria.

A number of different approaches have been used to investigate the effects of fungi and bacteria on Ggt. Most studies involving inoculation with bacteria rely on naturally occurring populations of Ggt in soil. However, trials with fungi have used artificial inocula of both Ggt and the potential biocontrol agent. In consequence the two types of inocula cannot be directly compared. The most commonly tested fungi have been those that are closely related to the pathogen. Of these, the most successful biocontrol agents include *Gaeumannomyces graminis* var. *graminis* (Ggg) (Wong & Southwell 1980, Wong 1994) and a Ggg/*Phialophora* mixture (Speakman 1984). A sterile red fungus (Dewan & Sivasithamparam 1989) and *T. koningii* (Duffy & Weller 1992), inoculated on ryegrass, have also shown yield increases in wheat of at least 70 % in field trials. While impressive yield increases are reported in response to fungal inocula, the amounts of inoculum used to achieve these results are too high to be commercially viable.

Emphasis has been placed on identifying the mechanisms by which biocontrol agents inhibit the growth of Ggt, in order to reduce the considerable variability seen in field trials. Antibiotics produced by both fungi and bacteria can control Ggt. The antibiotics produced differ between isolates (for a more detailed overview see Weller 1988 and Maurhofer *et al.* 1994). *Pseudomonas fluorescens* is the most widely recognised antibiotic-producing bacterium. After identifying an inhibitory mechanism *in vitro* has proved very difficult to show that

the mechanism is effective in soil. Only the antibiotics phenazine-1-carboxylic acid and 2,4-diacetylphloroglucinol have been detected in the wheat rhizosphere (Thomashow *et al.* 1990, Haas *et al.* 1991, Keel *et al.* 1992). Siderophore production by bacteria, resulting in a depletion of iron availability, has been shown to reduce the growth of Ggt effectively (Wong & Baker 1984, Weller *et al.* 1988). Competition for infection courts on plant roots between Ggt and potential biocontrol fungi can be mediated by antibiosis. Competition is a significant means by which antagonistic fungi can reduce ectotrophic growth of Ggt and has been demonstrated to contribute to the effectiveness of Ggg (Wong 1975), *Phialophora radicicola* (Deacon 1974, Speakman & Lewis 1978) and a sterile red fungus (Dewan & Sivasithamparam 1988, 1989). *Pseudomonas fluorescens* inoculated on seed colonises roots from the seed to just behind the growing apex and so has the advantage of early colonisation of the root surface (Weller 1984). Colonisation of wheat roots by an antagonistic fungus prior to infection by Ggt may induce host resistance mechanisms. This is seen as depositions of lignin and suberin in the endodermis and xylem vessels and an increase in the activity of polyphenol oxidase resulting in accumulation of caffeic acid which hinders penetration of the stele by hyaline runner hyphae of Ggt (Cowan 1978). Bacteria have not been shown to induce host resistance.

Five species of *Trichoderma* have shown antagonism to Ggt in acidic soil (pH 4.8-5.9) (Slagg & Fellows 1947, Skipsna 1963, Ponomareva 1965, Maas & Kotze 1987, Ghisalberti *et al.* 1990). Simon & Sivasithamparam (1988b) identified a soil from Western Australia that suppressed take-all of wheat in which *Trichoderma* species made up 71-82 % of the total fungal population

isolated by dilution plating on Martin's agar medium. *T. koningii* was the species most frequently encountered and was found to reduce saprophytic growth of Ggt in soil sandwiches and take-all disease in pot tests.

Biocontrol using *T. koningii* has great potential both on its own and as part of an integrated approach to disease control. In order to get consistent control, optimal conditions need to be provided to the antagonist and this requires a clear understanding of the behaviour of *T. koningii* in the rhizosphere and the mechanisms of control. A means of tracking *T koningii* so that it can be differentiated from the background population, its location identified and its abundance assessed would be a very useful tool in understanding its antagonistic activity. It is important to be able to distinguish the ways in which *T. koningii* operates so that the timing and means of application can be optimised. *T. koningii* might inhibit saprophytic growth of Ggt, in which case it should be applied to soil at the end of the growing season prior to the next seasons wheat crop. This would ensure the inoculum potential is reduced before it has the chance to cause serious economic loss. Alternatively the antagonist may effectively colonise the wheat rhizosphere or roots such that Ggt is excluded. In this case, the antagonist would be best added at planting and would not be required throughout the soil but may be applied in the furrow or as a seed dressing. A marker would greatly aid our detection of the phases of the disease cycle in which *T. koningii* is active.

1.4 Tracking *T. koningii* - What are the Options?

Detecting the presence and or abundance of a particular fungus in sterile conditions is considerably easier than in a natural system. Assessment of

fungal biomass is not species specific unless a single fungus is assessed in a sterile environment. The quantity of fungi in a sterile environment can be assessed by chitin, ergosterol, ATP, respiration (CO_2) and fluorescein diacetate esterase activity (Lynch 1990). A chitin assay can be used to detect most common fungi because, unlike bacteria and plant cells, members of the Chytridiomycota, Zygomycota and Ascomycota have a high chitin content in the cell walls of hyphae and spores. However, chitin content does not increase proportionally with fungal growth and insect contamination of samples can cause misleading results (Pitt & Hocking 1985, Schnurer & Borjesson 1996). Ergosterol, a major steroid produced by fungi, can be used to detect fungi which are metabolically active. The ergosterol assay is reported to have a high sensitivity and can be performed quickly (Pitt & Hocking 1985). While an ATP or respiration (CO_2) assay can be used to measure fungal activity, they cannot be used to assess fungal presence in plants or field soil. The disadvantage of a fluorescein diacetate esterase assay is that fluorescein diactetate may be differentially taken up by hyphae, compared with reproductive structures (Ribeiro 1978). ATP, chitin, ergosterol, fluorescein diacetate and respiration have been used to monitor activity in a potting mix in which *T. harzianum* dominated (1 % w/w) (Lumsden *et al.* 1990). None of these methods are specific for a particular species and each has inherent limitations. Hence, in non-sterile potting mix the estimates of fungal activity/biomass differed widely between each approach.

To understand the means by which *T. koningii* inhibits take-all disease in the field it needs to be differentiated from other members of the microflora so it can be tracked and quantified in non-sterile soil. The greatest challenge of

tracking a fungus in soil is distinguishing it from indigenous members of the same species. Detecting the presence and abundance of fungi in non-sterile soil or plants can be difficult. Approaches currently used include microscopy, isolation onto selective media, isozymes, immunological methods, making genetically different strains and the use of specific DNA probes and genetic markers.

The presence, spatial arrangement and biomass of fungal mycelium in a host plant or soil can be detected microscopically following the application of stains or fluorescent brighteners (Tsao 1970, Gisi & Schwinn 1976, Soderstrom 1977, Ribeiro 1978). Novel techniques including immunological methods and marker genes can be coupled with microscopy permitting precise identification of vegetative hyphae. The hyphae of *Rhizoctonia solani* have septation and branching patterns which are highly characteristic and allow them to be distinguished when growing intermingled with hyphae of *T. hamatum* (Elad *et al.* 1983). Hyphal length can be used as an indicator of biomass. However, most fungi cannot be distinguished microscopically and this would certainly be true for Ggt and *T. koningii* which both have hyaline hyphae of similar diameter and lack other distinguishing features. Microscopic examination was therefore not attempted in my project.

Isolation on selective media is the simplest approach to detect *T. koningii* in the rhizosphere and root. At least five media have been used to recover the genus *Trichoderma* (Davet 1979, Elad, Chet & Henis 1981, Papavizas & Lumsden 1982, Elad & Chet 1983, Harman, Taylor & Stasz 1989). However, not all species of *Trichoderma* grow on these media and isolates that do grow require subculturing onto malt extract agar and microscopic examination before

they can be identified to species level.

Use of a selective medium is an inexpensive technique to process large numbers of samples. Depending on the fungus studied, the detection limit is about 5 cfu/g soil (Pitt & Hocking 1985). This approach only identifies living cells, is not strain specific and hence would not differentiate an introduced isolate of *T. koningii* from other species of *Trichoderma* resident in roots or soil. When detecting systemic infection by plating plant pieces the assumption is made that surface disinfestation has killed any spores or hyphae on the exterior of the pieces. No indication of the mass of hyphae present in a soil sample or a piece of plant can be obtained when using selective media. The viability of hyphal cells after dilution plating is probably highly variable between species and the dilution technique used. *T. koningii* sporulates prolifically on organic matter exposed to light. Conidia survive plating well and can be counted easily. This means that high colony forming unit counts can be obtained when conidia are abundant in soil, not necessarily reflecting the vegetative activity of the fungus.

Fungicides and antibiotics can be used as components in selective media. An isolate which is resistant to an antibiotic or fungicide or has abnormally coloured spores can then be tracked and identified in an environment in which other members of the same species are present. Resistance to cycloheximide has been used to assess soil and rhizosphere colonisation by *T. harzianum* (Chao *et al.* 1986, Sivan & Chet, 1989). However, the nutritional status of a medium may dictate whether antibiotic resistance is expressed (Ryder 1993).

Resistance to fungicides by isolates found naturally (Abd-El Moity *et al.*

1982) or generated by UV radiation (Locke *et al.* 1985, Baker & Scher 1987) has enabled *Trichoderma* species to be used as part of integrated disease control programmes. Chemical mutation has also resulted in benomyl resistance in isolates of *T. harzianum, T. viride, T. koningii,* and *T. polysporum* (Chang *et al.* 1986, Ahmad & Baker 1987a, 1988a). Fungicide-resistant isolates may be tracked in soil and plants. However, this approach may require plating on a broadly selective medium followed by testing for fungicide resistance. Chemical and UV mutation of isolates of *Trichoderma* has induced fungicide resistance and improved rhizosphere competence and antagonistic activity in agar and/or soil in comparison to the wild-type isolates (Papavizas *et al.* 1982, Papavizas & Lewis 1983, Ahmad & Baker 1987a, 1988a). Caution must be taken because chemical and UV exposure can affect other fungal characteristics.

Spontaneous mutants are easily found or induced and are inexpensive to follow in soil or plants. However, tracking a spontaneous mutant requires use of a selective medium. When using chemical resistance to track a fungal isolate there needs to be a low natural background of resistance and/or a low rate of reversion. Alternatively the desired isolate can be double marked (Ryder 1993). Extensive testing may be required to ensure that the mutation to resistance has not impaired growth, physiological activity or competitiveness within the artificial and natural habitats of microorganisms. Mantyla *et al.* (1992) found that extensive alterations in genome organisation had occurred in *T. longibrachiatum* that had been exposed to UV light to increase production of cellulolytic enzymes. Microorganisms modified by exposure to chemical or UV mutagens are not defined as being genetically modified under current Australian legislation

hence, they are free to be used in the field. Insertion of a single gene at a known locus may be much safer than random mutation induced chemically or by UV light. However, broadscale use of strains generated using these techniques is not permitted.

Isolates of *T. viride* and *T. harzianum* with abnormally coloured spores, and therefore recognisable, have been induced by irradiation (X-rays or UV light), exposure to N-methyl-N'-nitro-nitrosoguanidine (Greenshpan & Galun 1971, Papavizas 1981) and protoplast fusion (Harman, Taylor & Stasz 1989). Isolates created by protoplast fusion were effective antagonists against *Pythium*, *Rhizoctonia solani*, *Fusarium graminearum*, and *Sclerotium rolfsii* in soil (Harman, Taylor & Stasz 1989). Fargasova *et al.* (1985) found that some isolates of *T. viride* with coloured spores had their conidiation structures altered morphologically, produced fewer conidia and had a slower growth rate than the parent. However, in other fungi such as *Aspergillus flavus*, isolates with coloured spores have been used to track soil populations and progression through plants (J.I. Pitt personal communication).

Fatty acid methyl ester (FAME) analysis is a method that has the potential to track fungi. FAME can be used to identify an unknown isolate. A fatty acid profile that distinguishes a fungus from other species and or members of the same species is required, but culturing conditions must be standardised and current data bases do not have extensive data on fungal profiles. FAME profiles have been used to assess interspecific variation among isolates of *Pythium* (C. Pankhurst personal communication) and Glomalean mycorrhizal fungi (Martinez *et al.* 1991, Bentivenga & Morton 1994, Graham *et al.* 1995) and intraspecific differences in *Rhizoctonia solani* (Stevens Johnk & Jones 1992,

Stevens Johnk & Jones 1993) and Glomalean fungi (Bentivenga & Morton 1994, Graham *et al.* 1995).

While fungal biomass of a particular species/isolate in soil or plant matter cannot be directly determined using FAME analysis the relative amount of a fatty acid marker can be estimated as a proportion of the total fatty acid content. The total fatty acid content of a sample is highly correlated with biomass (C. Pankhurst personal communication, Tunlid & White 1992, Olsson *et al.* 1995, Graham *et al.* 1995). It is possible to differentiate metabolically viable from non-viable biomass using FAME (White & MacNaughton 1997).

To track *T. koningii*, fatty acid markers distinct from other fungi need to be identified. This necessitates testing conidia and hyphae (Graham *et al.* 1995) but might have the advantage that the two forms could be distinguished in soil and roots. The lower limit for detection would also need to be assessed. For Glomalean fungi the lower limit of detection is between 130 and 500 spores (Graham *et al.* 1995). FAME requires specialised equipment, is expensive and comparison of profiles requires detailed analysis, hence it was not considered for this project.

It is possible, but difficult, to compare isozyme banding patterns between species and among strains of *T. harzianum, T. polysporum, T. hamatum, T. koningii* and *T. viride* (Zamir & Chet 1985, Stasz *et al.* 1988). Isozymes (16) have been used to conduct a phylogenetic analysis on five species of *Trichoderma*, however *T. polysporum, T. koningii* and *T. viride* were not readily distinguished from each other (Stasz *et al.* 1989). Isozymes have been used to detect interstrain fusion between isolates of *T. harzianum* (Harman *et al.* 1989, Stasz & Harman 1990). Isozyme analysis begins with a pure fungal

culture and hence requires preliminary isolation on a selective medium and subculturing to produce colonies not contaminated with spores or hyphae from other isolates. Isozyme banding patterns could indicate the presence of a known isolate of *T. koningii*, but could not quantify how extensively it had colonised the rhizosphere or root. Isozyme analysis is substrate-dependent and is not suited to routine surveying of a large number of samples.

Antibody-based techniques can be used to identify species of *Trichoderma*. Polyclonal antisera to *T. harzianum* have been raised, but cross reactivity with other fungi is a problem (Carter & Lynch 1991). While mono and polyclonal antibodies have been developed to quantify *Trichoderma* species *in vitro* (Jackson & Talburt 1988, Mischak *et al.* 1989, Zurbriggen *et al.* 1990) little research has been done in soil. Monoclonal antibody-based immunological assays have been used to detect conidia of *T. harzianum* and *T. viride* (Thornton & Dewey 1996) and mycelium of *T. harzianum* (Thornton *et al.* 1994) in a peat bran medium. However, the antibodies to mycelium also cross reacted with other *Trichoderma* species and species from the related genus *Hypocrea*. Quantification of a specific isolate is possible by tagging antibodies with a fluorochrome then using a flow cytometer (Pickup & Saunders 1990). This technique can be very expensive. Autofluorescence of background material such as soil, especially soil high in organic matter, may disguise the presence of fluorescent mycelia and is hence a restriction of this technique. For the purposes of this study antibodies are not useful because of the high likelihood of cross reactivity between strains and the detection of dead cells.

DNA probes can be used to identify a sequence that is naturally occurring or introduced via genetic manipulation in DNA extracted from soil,

plant tissue or colonies grown in pure culture. Differences in restriction fragment length polymorphism (RFLP) patterns of ribosomal, mitochondrial or chromosomal DNA can be used to distinguish between closely related species of fungi which colonise soil and plants (Goodwin *et al.* 1990, Nazar *et al.* 1991, Ophel-Keller *et al.* 1995). As well, randomly amplified polymorphic DNA (RAPD) and ribosomal analyses can be used. A number of these methods have been applied to *Trichoderma*. RFLP analysis has been used to distinguish members of the genus *Trichoderma* from other filamentous fungi (Meyer *et al.* 1991, Mule 1993). To identify species within the genus *Trichoderma* RFLP analysis, RAPD analysis, internally transcribed spacers (ITS) and hypervariable loci have been used. According to the relative position of the genes for cellulase production *cbh*1 and *cbh*2 in Southern blots, Morawtz *et al.* (1992) were able to differentiate three species of *Trichoderma*. *T. harzianum* has been divided into three groups based on RFLP, randomly amplified polymorphic DNA analysis and sequencing of the ITS 1 region of rDNA (Muthumeenakshi *et al.* 1994). This differentiation was necessary to identify aggressive strains of the species that colonise mushroom compost and interfere with mycelial growth of mushrooms (*Agaricus bisporus*). When the genus *Trichoderma* was analysed for the presence of hypervariable loci it was reclassified into 5 groups (Meyer *et al.* 1992). Group II contains the morphologically distinct species *T. polysporum, T. longibrachiatum, T. koningii* and *T. pseudokoningii*. This type of analysis is not able to distinguish *T. koningii* from other members of the genus.

DNA methodologies can be highly sensitive and specific. Using slot blot hybridisation many samples can be processed quickly and routine analysis with

a probe is possible (Whisson *et al.* 1995, Herdina *et al.* 1996). Quantitation using probes can be performed using the most probable number procedure followed by most probable number-DNA hybridisation or PCR (Frederickson *et al.* 1988, Picard *et al.* 1992) Fluorescent tagging of oligonucleotide probes allows *in situ* visualisation (Ryder 1993), which could be very useful tracking and perhaps quantifying fungal growth in the rhizosphere and root. However, as species in the genus *Trichoderma* are recognised to be aggregates (Rifai 1969), strain-specific probes can be difficult to find.

In order to track a fungus with potential for biocontrol in roots and soil a particular isolate needs to be differentiated from others in the same species. Isolates of *T. viride, T. harzianum* and *T. virens* have been found to contain mitochondrial DNA plasmids (Meyer 1991). Nearly all strains had a unique pattern which could be used to track an isolate in soil where the mtDNA patterns of natural isolates are known. There are no available studies indicating mtDNA plasmids occur in *T. koningii*. Polymerase chain reaction with random primers enabled identification of ten out of twenty three *T. harzianum* isolates, however distinctive patterns were not found for *T. viride* and *T. hamatum* (Zimand *et al.* 1994). A biocontrol isolate of *T. harzianum* was distinguished from other isolates of this species using a set of several primers (Zimand *et al.* 1994). This approach requires culturing of the organism, as direct RAPD-PCR from plant and soil is not possible because host material is also amplified (Guillaumin *et al.* 1996). Also, the amount of DNA influences RAPD patterns (Erlich 1989).

A marker gene would be a viable option to track *T. koningii* in roots and soil. An introduced gene sequence would enable an isolate to be distinguished from members of the same species. The presence of an isolate

carrying a marker gene could be quickly and easily assessed by plating soil or plant pieces on an agar medium. As the DNA sequence of a marker gene is known, probes can also be used to monitor the marked isolate. Transformation of protoplasts in polyethylene glycol and $CaCl_2$, high voltage electroporation (Goldman *et al.* 1990, 1993) and particle gun bombardment (Lorito *et al.* 1992) have been used to insert genes into *Trichoderma* species. Transformation of protoplasts usually generates the most stable transformants. Marker genes that have been inserted into members of the *Trichoderma* genus include genes which code for antibiotic resistance *hph* (hygromycin B phosphotransferase) (Herrera-Estrella *et al.* 1990), fungicide resistance eg *bml* (benomyl resistance) (Ulhoa *et al.* 1992), genes which allow growth on a nutritionally incomplete medium eg *arg*B (without arginine) (Penttila *et al.* 1987) and *pyr* (without uracil) (Gruber *et al.* 1990a, b, Smith *et al.* 1991) and a gene which allows visualisation *in situ* (*gus*A, β-glucuronidase) (Thrane *et al.* 1995). Some of the genes which have been used for transformation markers in *Trichoderma* spp. can only be used if mutant strains are available. The only member of the *Trichoderma* genus carrying a marker gene which has been tested in soil is *T. harzianum* marked with the *amd*S (acetamidase) or *gus*A gene (Pe`er *et al.* 1991, Thrane *et al.* 1995). Whereas a number of genes for antibiotic resistance have been transformed into fungi, few visual marker genes are available. The *lux* (luciferase) gene can identify the location and quantify the presence of bacteria (Grant *et al.* 1991, de Weger *et al.* 1991, Rattray *et al.* 1992, 1993) and a fluorescent green protein has also been transformed into bacteria (Chalfie *et al.* 1994, Matthysse *et al.* 1996). While it is theoretically possible to link the *lux* gene to a fungal promoter, to date there have been few reports of this in the

literature.

Each method of microbial detection outlined above has been successfully used to identify the presence of a fungus. There is no single best technique, rather the questions being asked dictate the option most suited to the investigation. Insertion of a genetic marker into a fungus is an approach that allows an isolate to be studied in the rhizosphere and the plant root and shoot systems. A gene such as *gus*A should allow these studies to be undertaken.

1.5 The *gus*A Gene

The *gus*A gene codes for β-glucuronidase (GUS) which catalyses the hydrolysis of glucuronides (Jefferson 1989). β-glucuronidase is an enzyme in the hexuronide-hexuronate pathway in *Escherichia coli* and is also found in *Salmonella* and *Shigella* species (Davies *et al.* 1994). The *gus*A gene was initially developed as a reporter gene system for use in nematodes (Jefferson *et al.* 1986, 1987) and has also been used to detect gene expression in plants (e.g. Jefferson *et al.* 1987, Jefferson 1989, Lojkowska *et al.* 1993). Insertion of the *gus*A gene into strains of *Saccharomyces cerevisiae* which were then inoculated into grape juice has enabled studies of the competition between introduced and natural yeasts to be conducted (Petering *et al.* 1991a, b). The *gus*A gene has been transformed into a large range of filamentous fungi including *T. reesei* and *T. harzianum*. In most cases the *gus*A construct pNOM102 has been used, but other plasmids are also available (Bhairi & Staples 1992, Woloshuk & Payne 1994). As *gus*A is not a selectable marker a second gene is used to for initial detection of transformants. Genes used in

this role have included *arg*B, *pyr*G, *hph*, *nia*D (nitrate reductase), *bml*, and *cbh*2 (cellobiohydrolase II). The co-transformation frequency of *gus*A with these genes has ranged from 25 to 92 % (de Ruiter-Jacobs *et al.* 1989, Murray *et al.* 1992, Smit & Tudzynski 1992, Ashby & Johnstone 1993).

Root and rhizosphere colonisation by an isolate of *T. koningii* transformed with *gus*A and inoculated into soil could be detected by plating on a *Trichoderma*-selective medium with and without surface disinfestation, followed by testing for cleavage of GUS substrates. Without using a marked isolate it would be difficult to show that the fungus isolated is the same as that used to inoculate the soil.

The substrate X-Gluc (5-bromo-4-chloro-3-indolylβ-D-glucuronide) forms an indigo precipitate when cleaved by β-glucuronidase. With X-Gluc, *gus*A has been used to demonstrate ectotrophic and internal growth of transformed strains of *Fusarium oxysporum* on wheat and flax roots (Couteaudier *et al.* 1993, Turlier *et al.* 1994). Rhizosphere, epidermal and cortical colonisation by *T. harzianum* transformed with *gus*A was studied using X-Gluc concurrently with my research project (Green & Funck Jensen 1995). Understanding the spatial orientation of *T. koningii* on wheat roots may be very important in optimising its competitiveness with Ggt. It should also be possible to identify the root tissues in which a fungal isolate marked with *gus*A is present. Expression of the *gus*A gene did not affect the pathogenicity of *Bipolaris sorokiniana* in barley roots or *Fusarium oxysporum* in flax and muskmelon (*Cucumis melo*) roots (Couteaudier *et al.* 1993, Liljeroth *et al.* 1993, Eparvier & Alabouvette 1994). However these roots were grown under artificial conditions in filter papers, nutrient solution or sterile soil. The real potential can only be judged after testing in field soil.

An alternative substrate used to detect GUS activity is MUG (4-methylumbelliferyl β-D-glucuronide). Because the *gus*A gene is constitutively expressed, fluorescence emitted in the presence of MUG has been able to be used to quantify fungal biomass in flax roots, cultivated in a nutrient solution, (Couteaudier *et al.* 1993), barley roots on filter paper (Liljeroth *et al.* 1993), steamed peat (Green & Funck Jensen 1995), tomato cotyledons (Oliver *et al.* 1993), excised apple leaves (Yourman *et al.* 1992) and maize kernels (Brown *et al.* 1995). The potential exists to assess the fungal biomass as well as the presence of an isolate containing the *gus*A gene in soil, the rhizosphere and within the wheat root system.

While *gus*A can be used to quantify the biomass of fungi, there are limitations of the GUS system. It is assumed that all living cells of a fungus express the gene equally. There is conflicting evidence as to the importance of the number of copies of *gus*A integrated in relation to GUS expression (Bunkers 1991, Couteaudier *et al.* 1993). The site of integration has also been observed to influence expression of the gene (Couteaudier *et al.* 1993). Another consideration is that the GUS system has rarely been used in non-sterile field soil. While most plants and fungi do not contain the *gus*A gene (Jefferson 1987, Liljeroth *et al.* 1993) some soil inhabiting bacteria do. GUS expression by indigenous bacteria can be controlled with an antibiotic such as chloramphenicol (K. Wilson, personal communication 1994). X-Gluc is an expensive substrate which limits the assessment of large numbers of samples. These disadvantages need to be taken into account in developing a useful tracking methodology.

Few workers have undertaken quantitative studies on the survival,

establishment or proliferation of *Trichoderma* species in the plant root or rhizosphere (Papavizas 1985). If *T. koningii* is to be used as a biocontrol agent monitoring its passage through soil and determining its presence or absence in the rhizosphere and root would aid our understanding of its ecology and enable us to optimise conditions for its antagonistic action to Ggt. Survival, abundance and metabolic activity are important when looking at persistence in soil and colonisation of plant roots over time. For a biocontrol agent to be effective understanding when and where it is active is crucial. A marker gene should also enable studies on the presence and extent of root/rhizosphere colonisation. At the time the work described in this thesis was commenced use of the *gus*A marker gene appeared to be the option best suited to this task.

1.6 Aims of this research project

The overall aim of this research was to develop a method that can be used to track inoculant strains of *T. koningii* in soil, the rhizosphere and roots. The specific aims of this project were:

1. Determine the extent of root and rhizosphere colonisation by *T. koningii*.
2. Determine if the presence of *T. koningii* in the rhizosphere/root inhibits take-all disease caused by Ggt.
3. Transform the *T. koningii* isolate AST-1, which inhibits take-all disease, with the *gus*A gene.
4. Assess the stability of transformants on media and in soil.

CHAPTER 2. THE INTERACTION OF *T. KONINGII* WITH *G. GRAMINIS* VAR. *TRITICI* AND WHEAT ROOTS

2.1 INTRODUCTION

There are numerous examples of *Trichoderma* species preventing disease when coated on seed (Chet & Baker 1981, Kommedahl *et al.* 1981, Wu 1982, Hadar *et al.* 1983). Addition of *T. koningii* to soil can reduce take-all disease of wheat in pot tests and field trials (Dewan & Sivasithamparam 1988, Simon 1989b, Rovira *et al.* 1992, Duffy *et al.* 1996). To control root invading pathogens, an antagonistic fungus must inhibit the pathogen in bulk soil, induce plant defence mechanisms or inhibit the pathogen in the rhizosphere and/or root. Mechanisms by which *T. koningii* has been shown to control take-all disease include reduced saprotrophic growth of Ggt, as shown in soil sandwiches (Simon & Sivasithamparam 1988e). Furthermore the reduction of take-all disease was increased when both fungi were added to soil two weeks before seeding (Simon 1989b). However, induced resistance has not been reported for any species of *Trichoderma.*

There is evidence that fungi that reduce take-all disease also colonise the rhizosphere effectively (Deacon 1974, Speakman *et al.* 1978, Speakman & Krueger 1984, Narita & Suzui 1991, Shivanna *et al.* 1996). Whether the antagonist colonises the rhizosphere after reducing the Ggt population in bulk soil or acts in the rhizosphere to reduce colonisation by Ggt and hence take-all disease, is difficult to determine. In contrast to this, there is a widespread view that natural populations of *Trichoderma* spp. do not proliferate in the rhizosphere (Papavizas 1981, Chao *et al.* 1986, Ahmad & Baker 1987a).

Isolates of *Trichoderma* with potential for biological control were mutated for antibiotic production. Rhizosphere-competent mutants were selected to protect vegetable seedlings from preemergence damping off (Ahmad & Baker 1981, 1987a, Papavizas *et al.* 1982).

More detailed work has shown that the mode of application greatly influences disease control by *Trichoderma* species in soil, perhaps helping to provide an explanation for the contradictory results. Use of fungi in their hyphal state has been much more successful than spores in promoting rhizosphere colonisation and disease control (Lewis & Papavizas 1984, Elad *et al.* 1980), because conidia are more sensitive than growing hyphae to fungistasis (Lockwood 1977). Hyphae added on a carrier have a food base, are actively growing and have many points to grow from. Those of *Trichoderma* compete with the resident soil microflora as they grow through soil towards roots and once there they can effectively colonise the rhizosphere (Beagle-Ristiano 1985, Sivan *et al.* 1987, Pe`er *et al.* 1991).

The simplest approach to tracking a fungus in a plant, the rhizosphere or bulk soil is by plating on a selective medium. This is an inexpensive technique which can be used to process large numbers of samples. Selective media generally act in one of two ways. The medium may inhibit growth of all but the targeted fungus, or change colour enabling the identification of a particular genus or species. An ideal selective medium is simple to make, inexpensive and chemically defined. To enumerate soil populations of *Trichoderma* species at least 30 colony forming units must be able to grow discretely on a single plate. The colonies of *Trichoderma* must be easily distinguished. As well, a medium on which *T. koningii* is floccose is desirable

so the colonies can be subcultured with ease.

The aim of the work described in this chapter was to determine the extent of root colonisation by *T. koningii* when a) uninhibited by other soil microbes (a non-competitive environment) and b) in natural field soil. In order to achieve this a selective medium for *Trichoderma*, in particular *T. koningii* was developed. Experiment one was set up to determine if *T. koningii* colonised wheat roots in a non-competitive environment, and if so, to assess whether *T. koningii* moved along the roots from the point of infestation. Questions asked in a second experiment conducted in field soil were:

1. Does *T. koningii* act in the bulk soil or in the rhizoplane and root to control take-all disease?
2. Can *T. koningii* reduce take-all disease if Ggt has already colonised the soil?
3. To reduce take-all disease does *T. koningii* have to be in the same location in soil as Ggt?
4. Does the sequence in which the root comes into contact with the antagonist and pathogen influence control of take-all disease?

2.2 METHODS

2.2.1 Fungi

The isolates of *Trichoderma koningii* Oudem AST-1 (IMI 299426), 7a (IMI 308475) and 7c used in this investigation, were collected by A. Simon from soil at Newdegate in Western Australia (Simon & Sivasithamparam 1987d, 1988f). They had been in the collection of M. Ryder (CSIRO Division of Soils, Adelaide, South Australia) for at least five years. At the beginning of this study the

isolates were cultured in sterile soil and, after reisolating, grown on Malt Extract Agar (MEA, Pitt & Hocking 1985). Single spores were isolated on Water Agar (WA, Difco) and transferred to Czapek Yeast Extract Agar (CYA, Pitt & Hocking 1985). The isolate AST-1 was used for all experiments unless otherwise stated.

The isolate of *Gaeumannomyces graminis* (Sacc.) von Arx & Oliver var. *tritici* Walker, (Ggt 8), used in this investigation was recovered from infected wheat roots collected near Avon, South Australia in 1979 by H. McDonald (CSIRO Division of Soils, Adelaide, South Australia).

2.2.2 A Selective Medium for *Trichoderma*

Preliminary tests showed that the three isolates of *T. koningii* tested (AST-1, 7a, 7c) grew poorly on the media currently in use for selective isolation of species of *Trichoderma*. The selective medium of Elad & Chet (1983) was modified and called STSM. Captan, PCNB, Fenaminosulf and KCl were deleted and the concentration of chloramphenicol reduced. STSM consisted of $MgSO_4$ (0.2 g), KH_2PO_4 (0.9 g), NH_4NO_3 (1 g), glucose (3 g), agar (20 g), chloramphenicol (0.1 g), rose bengal (0.15 g), dicloran (1 ml of 2% solution) in 1 L DI H_2O. All ingredients were added before autoclaving. A variety of soil-borne and plant pathogenic fungi from the collection of K. Ophel-Keller (SARDI, Plant Research Centre, Adelaide, South Australia) and three isolates of *T. koningii* were assessed for growth on STSM to determine its potential selectivity towards *T. koningii* (Table 2.1). Spores and hyphae were picked up on a needle and inoculated at three points on each of three plates. After incubation at 25 °C for 3 d in darkness, the presence or absence of colonies was scored.

Table 2.1 Fungal species tested on STSM to assess its potential for isolating *Trichoderma* species.

Fungi	Host	Location	Collected by
Alternaria sp. 21005	soil	SA*	C. Franco
Aspergillus niger	unknown	unknown	J. Kelly
Bipolaris sp.	*Pisum sativum*	Adelaide, SA	R. Cook
Cladosporium sp.	*Pisum sativum*	Adelaide, SA	R. Cook
Curvularia sp. 2028	*Hordeum vulgare*	SA	R. Moen
Fusarium culmorum 5618	*Triticum aestivum*	SA	R. Moen
Fusarium graminearum 2183	*Triticum aestivum*	SA	R. Moen
Fusarium avenaceum 1171	unknown	NSW	L. Burgess
Hymenoscyphus ericae	unknown	unknown	M. Tester
Leptosphaeria namari DAR 51197	couch grass	Tamworth, NSW	P.T.W. Wong
Penicillium sp. 21008	soil	Bordertown, SA	C. Franco
Penicillium sp. 21021	soil	Bordertown, SA	C. Franco
Pisolithus tinctorius	unknown	unknown	unknown
Phoma epicoclens	unknown	unknown	unknown
Phoma medicaginis	*Pisum sativum*	Adelaide, SA	R. Cook
Rhizopus sp.	bread	Sydney, NSW	P.T.W. Wong
Sclerotinia sp.	unknown	unknown	University of Adelaide
Sordaria sp.	unknown	unknown	unknown
Trichoderma koningii AST-1, 7a, 7c	soil	Newdegate, WA	A. Simon

* SA - South Austalia, NSW - New South Wales, WA - Western Australia

STSM was compared with Dichloran Rose Bengal Chloramphenicol Agar (DRBC, a general purpose medium for fungal isolation) and several media which have primarily been used to select for *Trichoderma*. Preliminary work indicated that Captan was inhibitory to *T. koningii* and media with and without this Captan were tested (+/-C) (see Appendix 1). Three isolates of *T. koningii* (7a, 7c, AST-1) were used. In the first experiment conidia were dispersed in 0.2 % agar and 0.05 % Tween 80 (Pitt & Hocking 1985) and inoculated at three points on three plates of each medium. After incubation at 25 °C for 3 d in darkness the maximum diameter of each colony was measured (9 colonies in total). In the second test a suspension of conidia (10^6/ml) was made in 0.1 % peptone (Oxoid), dilution-plated (10^{-5} - 10^{-8} dilutions) onto each medium and incubated at 25 °C in darkness. Three replicate plates were used per dilution. Soil from the Kapunda field trial site (section 2.2.4) was also diluted in 0.1 % peptone (10 g soil in 90 ml peptone, 10^{-2}, 10^{-3} dilutions) and plated onto each medium. Two replicate plates were used for each dilution on each medium. These plates were incubated in the dark for 3 d, then exposed to light by placing them under a light bank or on a windowsill.

Media which gave the highest counts of *Trichoderma* from Kapunda soil were selected for further work. Soil samples (14), representing the major soil types in South Australia in which take-all disease occurs, were dilution-plated on each of these media to determine which gave the highest number of readily identifiable colonies of *Trichoderma* in the shortest period of time.

2.2.3 Preparation of Fungal Inoculum for Pot Tests

Gamma-irradiated ryegrass seeds (*Lolium rigidum*) (75 g) were soaked in deionised water in a 600 ml bottle overnight. Excess water was drained off and the bottles of ryegrass seeds autoclaved (120 °C 20 min) on 3 consecutive days. Inoculum consisted of plates of *T. koningii* grown on MEA (7 d) or Ggt grown on Potato Dextrose Agar (PDA, Difco) (14 d). Each culture was roughly cut up and shaken through a bottle of sterile ryegrass seeds. Ryegrass seed cultures were incubated at 25 °C and shaken every 3 to 4 d to ensure even colonisation. Cultures of *T. koningii* and Ggt produced in this way were dried and frozen after 14 and 21 d respectively. Control inoculum was prepared by inoculating the fungal cultures on ryegrass seeds and autoclaving (120 °C, 20 min) prior to their addition to soil.

2.2.4 Soil

Surface soil (0-10 cm) was collected from the CSIRO trial site at Kapunda, which is 80 km north of Adelaide, South Australia. The soil which is a red-brown earth, classified as Calcic Natrixeralf (Soil Survey Staff 1990) has a texture of loam - fine sandy, pH 5.1 (H_2O); pH 4.7 ($CaCl_2$), Ec = 30mS cm^{-1}, total C = 1.2 %). Soil was stored in plastic-lined steel tins and sieved (5 mm) as required. Water was added to 13 % w/w in both experiments. Each layer was watered individually.

2.2.5 Seed

Wheat seeds (*Triticum aestivum* cv Spear) were surface disinfested by immersion in 95 % ethanol for 1 min followed by 0.5 % NaOCl for 3 min, then rinsed twice in sterile distilled water (2 min/rinse). The seeds were germinated on sterile wet paper towels at 25 °C in darkness for 20-24 h.

2.2.6 Harvest

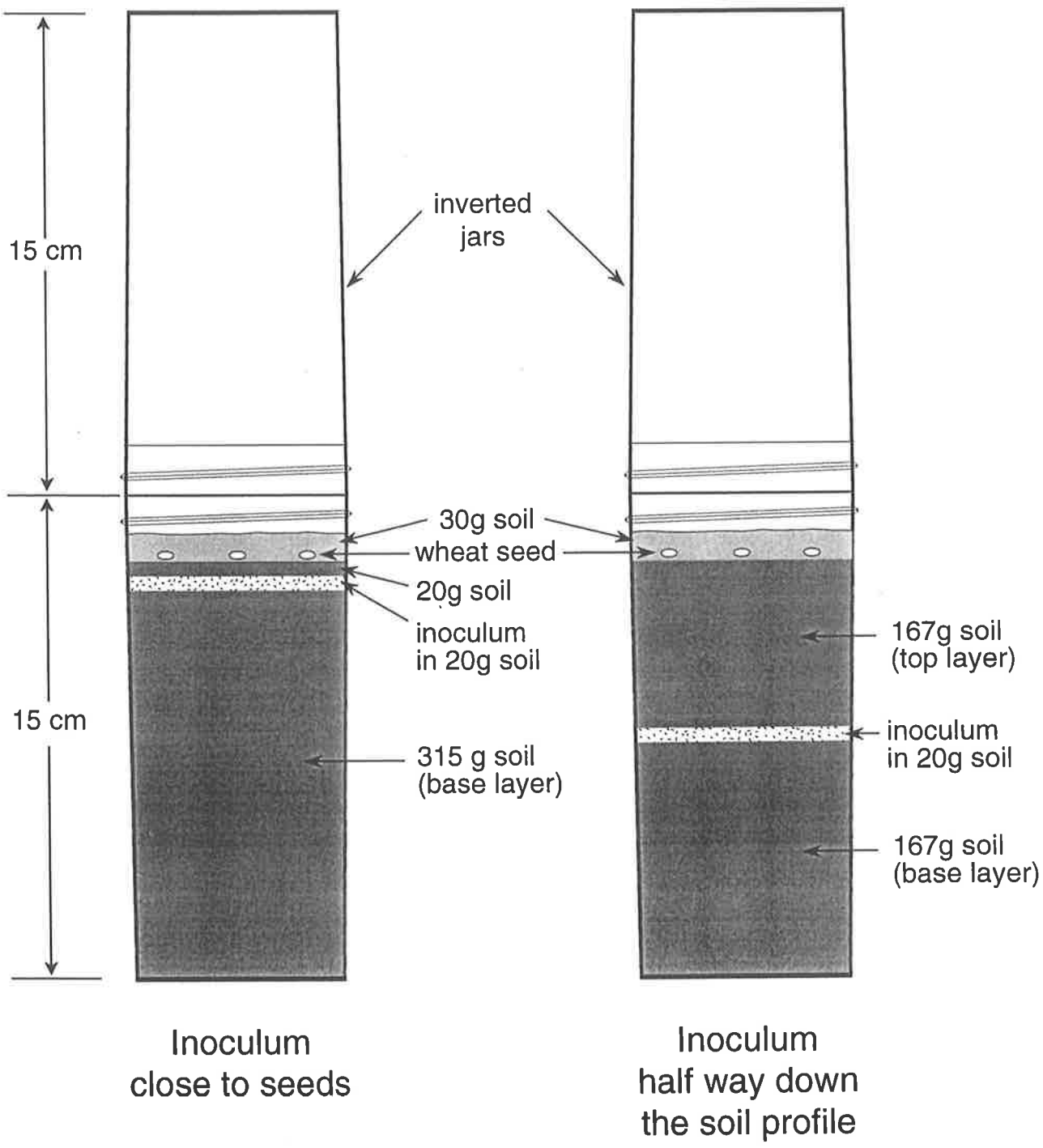
Plants were washed from soil 7, 14 or 21 d after planting. Plant samples (4 plants per pot) were split into two treatments. Roots from two plants were cut into 2.5 cm pieces and plated on STSM. To assess for internal colonisation, roots of the other two plants were surface disinfested (0.5 % NaOCl, 0.0005 ml Tween 80/100 ml for 2 min) then cut into 2.5 cm pieces and plated on STSM. These plates were incubated at 25 °C in darkness for 4 d, then exposed to light (either on a windowsill or under a light bank) to stimulate sporulation and thus enable identification of colonies of *Trichoderma*.

2.2.7 Experiment 1. Colonisation of Wheat Roots by *T. koningii* When Grown in Sterile Soil

The aim of this experiment was to determine if *T. koningii* moved within wheat roots from the point at which it was added to soil. Soil was sterilised in polycarbonate jars (500 ml) or McCartney bottles by autoclaving (121 °C 20 min) on 3 consecutive days. Ryegrass seed inoculum of *T. koningii* (0.2 % of the total dry weight of soil in the jars) was mixed through 20 g of sterile soil in McCartney bottles and then placed as a band either close to the seed or approximately half way down the soil profile in the jars (Figure 2.1). The top

band of inoculum sat on the base layer of soil (315 g). Soil (20 g) covered this layer of inoculum. The bottom band of inoculum was sandwiched between two batches of 167 g soil. The pregerminated seeds (4/jar) were covered with 30 g soil. Soil adhering to sides of the jar above this level was removed. The jar containing soil was covered with a second jar and the junction sealed with clear tape to ensure sterile growth conditions. Jars were placed in an temperature controlled tank maintained at 15 ^{o}C. The soil surface was level with the wooden supports ensuring the soil and roots were not exposed to light. The lower 7 cm of these jars had also been painted black. Five replicate jars per treatment were used. A three way analysis of variance was performed on the proportion of root colonisation by *T. koningii*. Treatments to which *T. koningii* were not added were left out of the analysis.

Figure 2.1 The arrangement of layers of inoculum in sterile soil in which wheat was grown. The jar containing soil was covered with a second jar to maintain sterile growth conditions.



2.2.8 Experiment 2. The Interaction of Ggt and *T. koningii* in Field Soil.

In this experiment the interaction between *T. koningii* and Ggt was examined in wheat roots grown in non-sterilised field soil. The factorial experiment consisted of 4 fungal treatments x 4 positions of inoculum x 4 preincubation treatments x 3 harvest times x 4 replicates (Table 2.2).

The fungal treatments were i) live inoculum of *T. koningii* and live inoculum of Ggt; ii) live inoculum of *T. koningii* and dead inoculum of Ggt; iii) dead inoculum of *T. koningii* and live inoculum of Ggt; iv) dead inoculum of *T. koningii* and dead inoculum of Ggt. Dead inoculum was used to maintain comparable nutrient levels in the soil in all treatments.

Inoculum placement was varied in order to determine if the sequence in which the root came into contact with the fungi influenced control of take all disease. It also helped to determine whether *T. koningii* was inhibiting growth of Ggt in bulk soil or in the root/rhizoplane. As illustrated in Figure 2.2 the fungi were added either in separate layers or as a single layer (mixed inoculum). The layers of inoculum were placed close to the seed and/or half way through the soil profile in the pot. The position treatments were:

- *T. koningii* in the top layer, Ggt in the base layer
- Ggt in the top layer, *T. koningii* in the base layer
- *T. koningii* and Ggt in the top layer, no inoculum in the base layer
- no inoculum in the top layer, *T. koningii* and Ggt in the base layer

A 2:1 ratio of *T. koningii*:Ggt inoculum was used in all treatments. The total amount of ryegrass seed inoculum was always 0.3 % of the dry weight of soil in the pot. In treatments where inocula were in separate layers, 0.15 % (w/w) of ryegrass seed inoculum was added per band. However, in treatments

where both fungi were in the same layer, ryegrass seed inoculum was not added to the second layer. The negative control treatments for fungi added in different layers were the addition of 0.15 % precolonised, autoclaved ryegrass seed to the appropriate layer. When inocula of both fungi were added in the same layer the negative control treatment was 0.3 % precolonised, autoclaved ryegrass seed added to the corresponding layer only.

There were two times of inoculum addition: two weeks prior to seeding (preincubation) or at seeding. Preincubation of soil was examined to determine if *T. koningii* could

a) reduce growth of Ggt once the pathogen had an established mycelial network

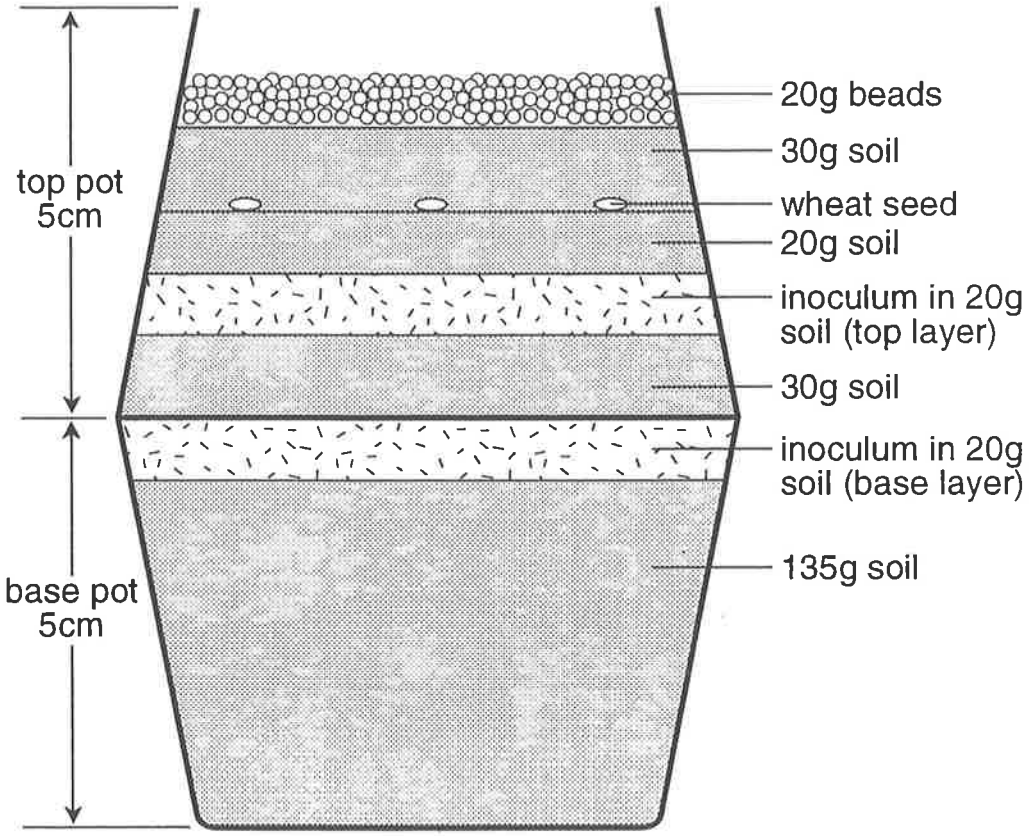
b) inhibit hyphal regeneration of Ggt

c) reduce take-all disease by interacting with the root before Ggt

The arrangement of fungi and soil is illustrated in Figure 2.2. Two 160 ml pots were used. The base was cut from one pot so the two pots could be easily joined after the preincubation period. The top and bottom layers were preincubated separately. To enable preincubation of the top layer, a lid was placed on the widest end of the pot. Inoculum in 20 g soil overlaid 30 g soil. Polyalkathene beads (20 g) were added to prevent desiccation. For the base layer, inoculum was mixed through 20 g soil which overlaid 135 g soil. To prevent soil from drying out polyalkathene beads (20 g) were contained within a cardboard rim. At planting beads were tipped off both layers, the lid removed and the two pots taped together. Soil (20 g) was placed on the top layer. This band of soil was not added until planting to enable seed germination unhindered by inoculum. Soil (30 g) covered the seed and was in turn overlaid with

polyalkathene beads (20 g).

Figure 2.2 The arrangement of layers of inoculum in a pot of Kapunda field soil.



Four replicate pots were used for each treatment. Pots were randomly placed in three blocks, maintained at 15 °C with a 12 hour period of light. Pots were watered to weight daily both in the preincubation period and after seeding.

Pots were harvested after 7, 14 and 21 days. After being washed free of soil, roots were examined for vascular discolouration typical of take-all disease. The number of roots with vascular lesions were scored as a proportion of the total number of roots. Roots were then assessed for rhizoplane and root colonisation by *Trichoderma* as outlined in section 2.2.6.

Three way analysis of variance (ANOVA) was conducted on the proportion of roots infected by *Trichoderma*. Following analysis, the residuals were examined and found to be normally distributed (Tables 2.8, 2.9). To determine if the proportion of roots with a vascular lesion differed between treatments statistical analysis was conducted using the generalised linear model. It was assumed the number of lesions had poisson errors (Table 2.6). A two way ANOVA was conducted on root and rhizoplane infection by *Trichoderma* separately (Table 2.7). Of the surface sterilised treatments those which did not have *T. koningii* added to the soil were left out of the analysis as their numbers were small.

Table 2.2 A summary of the treatments set up in unsterilised Kapunda soil to examine the interaction of *T. koningii* (T.k.), Ggt and wheat roots. Each treatment was harvested after 7, 14 and 21 days.

Added to soil:			Added to soil:		
T.k.	Ggt	Preincubated	T.k.	Ggt	Preincubated
Fungi in separate layers					
T.k. top Ggt base			**Ggt top T.k. base**		
+	+	both	+	+	both
+	+	T.k. only	+	+	T.k. only
+	+	Ggt only	+	+	Ggt only
+	+	none	+	+	none
+	-	T.k. only	+	-	T.k. only
+	-	none	+	-	none
-	+	Ggt only	-	+	Ggt only
-	+	none	-	+	none
-	-	yes	-	-	yes
-	-	none	-	-	none
Fungi in one layer (mixed inoculum)					
T.k. = Ggt at top level			**T.k. = Ggt on base level**		
+	+	yes	+	+	yes
+	+	none	+	+	none
+	-	yes	+	-	yes
+	-	none	+	-	none
-	+	yes	-	+	yes
-	+	none	-	+	none
-	-	yes	-	-	yes
-	-	none	-	-	none

2.3 RESULTS

2.3.1 A Selective Medium for *Trichoderma*

T. koningii grew as distinct, floccose colonies on STSM, which sporulated when exposed to light. Of the 17 other fungal species tested on STSM (Table 2.1), most failed to germinate or grow, however *Fusarium* colonies grew well. When soil was dilution-plated on the medium *Penicillium* colonies were prolific. Attempts to reduce the growth of these two genera on this medium were unsuccessful.

STSM was compared to other media reported to be selective for species of *Trichoderma*. Initially growth of *T. koningii* was assessed on each medium. Floccose colonies grew to greater than 10 mm diameter on MTSM-C, STSM and DRBC after 3 d incubation (Table 2.3). When conidia of *T. koningii* were point inoculated, large colonies (41-60 mm diameter) grew on Davet's medium. When conidia of *T. koningii* were plated on the selective media between zero and 10^5 colonies per ml were recovered. The highest numbers of colonies grew on MTSM-C, TSMC-C-F, C&H, A&L-C and STSM. Colonies on STSM were particularly easy to count as the rose bengal stain pronounced at a central point under each colony. After 18 d isolates of *T. koningii* were found on TME-SA and TSMC while colonies on these media were not visible after 5 d incubation. On Davet's medium colonies grew rapidly, so that a much higher dilution was required to count the number of conidia present in the suspension. The highest counts of *Trichoderma* colony-forming-units from soil were found on STSM and MTSM-C. Three other media also supported counts of *Trichoderma* in the

same order of magnitude after 5 d incubation. No colonies of *Trichoderma* were evident from soil samples plated on the A&L medium, DRBC, TSMC or TME-SA after 5 d incubation. *Trichoderma* propagules from soil were recovered on MTSM, whereas conidia of *T. koningii* in suspension did not germinate and grow on this medium.

Table 2.3. A comparison of the growth by *T. koningii* and other *Trichoderma* spp. on selective media (section 2.2.2).

Medium[a]	Conidia inoculated at 3 points and incubated for 3 d		Conidia dilution-plated from a suspension in peptone and incubated for		Soil sample dilution-plated and incubated for	
	colony diameter (mm)	floccose colonies	5 d	18 d	5 d	18 d
			cfu/ml		cfu/g	
MTSM	0-12	-	0	0	500	750
-C	18-27	+	3.5×10^5	NT	900	900
Davet's	41-60	+	*	NT	250	250
TME-SA	0	-	0	700	0	0
DRBC	11-16	+	5.0×10^3	NT	0	300
TSMC	0-5	-	0	2.5×10^4	0	200
-C-F	4-10	+/-	2.6×10^5	NT	350	350
-C+F	0-7	-	4.0×10^4	NT	150	150
C&H	5-10	+/-	3.5×10^5	NT	100	100
A&L	0-4	-	0	0	0	0
-C	4-7	-	2.4×10^5	NT	250	250
STSM	11-25	+	3.2×10^5	NT	900	900

[a] See Appendix 1 for details of media
* not plated at a sufficiently high concentration to allow colonies to be counted
-C without Captan
F Fenaminosulf
NT Not tested, as results were positive at 5 d.

When soil samples were plated on the selective media, STSM performed well in comparison to MTSM-C (Table 2.4) both with respect to time taken to sporulate and estimated population of *Trichoderma*. In three soils (Port Broughton, Minnipa, Walpeup) the estimated population size of *Trichoderma* was higher on STSM and MTSM-C than MTSM by an order of magnitude. STSM gave lower population counts than MTSM-C for 2 soils (Avon GC1, Palmer trial), but a higher count for soil from Booborowie. To be clearly identified as *Trichoderma* colonies need to be sporulating. The time taken to sporulate was shorter on MTSM+/-C (7 d) than on STSM which required 9 d.

Table 2.4. A comparison of the *Trichoderma* populations in 14 soil samples dilution planted and spread on three selective media (section 2.2.2).

	cfu of *Trichoderma* / g soil		
	Medium		
Soil sample from:	MTSM	MTSM-C	STSM
Avon 4	5	0	12
Avon 53	6	0	20
Avon GC1	2	5.0×10^2	0
Palmer 4	1	0	0
Palmer 53	0	0	3
Palmer trial	5.4×10^2	3.5×10^2	50
Roseworthy	1.5×10^2	1.5×10^2	3.5×10^2
Bute	5.5×10^2	4.0×10^3	2.0×10^3
Port Broughton	4.0×10^2	6.5×10^3	2.5×10^3
Birchip (Vic)	7	1	7
Booborowie	11	5	1.5×10^2
Minnipa	5.5×10^2	1.5×10^3	1.2×10^3
Kapunda	3.5×10^2	4.0×10^2	8.0×10^2
Walpeup	4.5×10^2	3.0×10^3	2.5×10^3

MTSM - Smith, Wilcox & Harman, 1990.
-C - without Captan
STSM - a modification of the medium of Elad & Chet, 1983.

2.3.2 Experiment 1. Colonisation of Wheat Roots Grown in Sterile Soil by *T. koningii*.

When *T. koningii* was added to sterile soil it could be reisolated from surface disinfested roots (Table 2.5). In sterile soil *T. koningii* colonised up to 88 % of wheat roots (Table 2.5). When *T. koningii* was added in the top layer the mean level of root colonisation increased between 7 and 14 d from planting then remained consistently high. When *T. koningii* was added at greater depth a significant increase in mean root infection was seen at each sampling time. After 7 d some roots had not reached the inoculum source placed half way through the pot profile, hence the level of root colonisation was much lower (3-6 %) than when *T. koningii* was added close to the seed. At later harvests the position at which *T. koningii* was added to soil did not significantly influence root colonisation. While *T. koningii* grew both up and down the root from the point of inoculation, the root segments closest to the seed were the most heavily infected. Analysis of data from the 14 and 21 d harvests showed the decrease in root infection towards the growing tip was linear ($P>0.05$). At 7 d addition of *T. koningii* to sterilised soil increased root growth. It was observed that wheat roots in treatments to which *T. koningii* had been added were up to 7.5 cm in length in comparison to 5 cm in soil which did not contain *T. koningii* (data not shown). This effect was lost after 14 days.

Table 2.5 Wheat root infection by *Trichoderma* after surface disinfestation and plating on a selective medium. Wheat plants were grown in sterile soil (section 2.2.7).

Time from planting (d)	Root infection (%) Distance from seed (cm) 0-2.5	2.5-5.0	5.0-7.5	7.5-10.0	mean
***T. koningii* added close to the seed**					
***T. koningii* added to soil**					
7	56^{cde}	18^{ab}	0^{a}	-	27^{b}
14	88^{f}	69^{def}	78^{ef}	53^{cd}	72^{d}
21	72^{def}	81^{f}	70^{def}	75^{def}	75^{d}
mean	72^{c}	56^{b}	49^{ab}	47^{ab}	
no *T. koningii* added					
7	0	0	-	-	0
14	0	2	0	-	4
21	2	2	0	0	1
mean	4	1	0	0	
***T. koningii* added half way down the pot profile**					
***T. koningii* added to soil**					
7	6^{a}	3^{a}	0	-	4^{a}
14	87^{f}	66^{def}	39^{bc}	20^{ab}	53^{c}
21	85^{f}	79^{ef}	68^{def}	66^{def}	74^{d}
mean	59^{bc}	50^{ab}	36^{a}	43^{a}	
no *T. koningii* added					
7	0	0	-	-	0
14	0	2	0	0	1
21	0	0	0	0	0
mean	0	1	0	0	

* Numbers followed by the same letters are not significantly different at the P>0.05 level. Data from pots which had *T. koningii* added close to the seed and pots which had *T. koningii* added half way down the pot underwent a single analysis. The means were analysed separately so a different lettering system has been used for the mean column and the mean row figures.

2.3.3 Experiment 2. The interaction of Ggt and *T. koningii* in field soil.

In the absence of *T. koningii*, addition of Ggt to soil resulted in severe disease (Table 2.6). The number of roots with vascular lesions was significantly lower when Ggt was preincubated in comparison to when it was added at seeding.

When *T. koningii* was added to soil in the absence of Ggt no take-all lesions were seen (Table 2.6). 10-29 % of root segments in these treatments were internally infected with *Trichoderma* (Table 2.7) and *Trichoderma* was reisolated from between 54 and 100 % of the root pieces indicating high levels of both cortical and rhizoplane infection (Table 2.8). Similar rates of rhizoplane colonisation were observed in soil to which the *Trichoderma* inoculum had not been added ($P>0.05$), indicating rhizoplane colonisation by the native population of *Trichoderma*. However, *Trichoderma* was infrequently isolated after surface disinfestation of roots grown in natural soil without added inoculum of *T. koningii* (Table 2.9). The time of sampling and distance from the seed did not significantly change the level of rhizoplane colonisation.

When *T. koningii* was added to soil, the extent of internal colonisation was similar at all three sampling times (Table 2.9). Root segments closest to the seed were more highly colonised than the lower half of the roots, irrespective of where *T. koningii* was introduced. *T. koningii* grew down the wheat roots, but the frequency of growth by the antagonist up the roots, towards the seed was much higher than downwards movement (Table 2.9). Only about 5 % of the lower half of the roots were internally colonised.

Take-all lesions were not seen when inoculum of *T. koningii* was added to soil at the same time as Ggt and the seed (Table 2.6), irrespective of the

position of the potential antagonist in the soil in relation to Ggt. Similarly, when *T. koningii* and Ggt were preincubated in the same layer in the pot there was no evidence of typical lesions of take-all disease. However, when the two fungi were preincubated separately before seed was added there was less disease (22 %) when the root came into contact with *Trichoderma* before Ggt than when Ggt was encountered first (48 %). When *Trichoderma* had precolonised the soil before Ggt was introduced, vascular discolouration was significantly reduced in comparison to when both fungi were added to soil at the same time. This reduction was greatest if the root came into contact with *Trichoderma* before Ggt. If soil was precolonised with Ggt the addition of *T. koningii* at planting had no effect.

Table 2.6 Vascular discolouration typical of take-all disease in wheat roots grown in the presence (+) and absence (-) of *T. koningii* (T.k.) and Ggt in field soil (section 2.2.8).

Added to soil:			roots with a vascular lesion (%)			
Trichoderma koningii	Ggt	Preincubated**	T.k. top*** Ggt base	Ggt top T.k. base	T.k. + Ggt top	T.k. + Ggt base
+	+	both	22^{b*}	48^{de}	1^{a}	0^{a}
+	+	*T. koningii* only	3^{a}	25^{bc}		
+	+	Ggt only	37^{cd}	40^{d}		
+	+	-	9^{a}	3^{a}	0^{a}	0^{a}
+	-	+	0^{a}	4^{a}	0^{a}	4^{a}
+	-	-	2^{d}	0^{a}	0^{a}	0^{a}
-	+	+	39^{d}	42^{d}	58^{efg}	46^{de}
-	+	-	56^{ef}	62^{fg}	71^{g}	64^{fg}
-	-	+	2^{a}	0^{a}	2^{a}	0^{a}
-	-	-	4^{a}	0^{a}	0^{a}	

* Numbers followed by the same letters are not significantly different at the P>0.05 level.
** - without preincubation of fungi in soil, + with preincubation of fungi in soil
*** for explanation of top and base see Figure 2.2

Table 2.7 Colonisation of wheat roots and rhizoplane by *Trichoderma* spp. Wheat plants were grown in Kapunda soil in the presence and absence of *T. koningii* and Ggt for 21 d, the roots were cut into 2.5 cm segments then plated on STSM with and without surface disinfestation (section 2.2.8).

Added to soil:			roots from which *Trichoderma* was isolated (%)			
Trichoderma koningii	Ggt	Preincubated**	T.k. top*** Ggt base	Ggt top T.k. base	T.k. + Ggt top	T.k. + Ggt base
With surface disinfestation						
+	+	both	12^{abc*}	16^{abcd}	22^{abcde}	16^{abcd}
+	+	*T. koningii* only	8^{a}	30^{df}		
+	+	Ggt only	19^{abcde}	18^{abcde}		
+	+	-	26^{bcde}	29^{de}	17^{abcd}	35^{e}
+	-	+	21^{abcde}	26^{bcde}	10^{ab}	16^{abcd}
+	-	-	12^{abc}	29^{de}	19^{abcde}	25^{abcde}
-	+	+	6	4	1	3
-	+	-	0	0	0	0
-	-	+	0	4	0	3
-	-	-	9	5	1	
Without surface disinfestation						
+	+	both	94^{ij}	91^{ghij}	$86^{defghij}$	$80^{bcdefghi}$
+	+	*T. koningii* only	98^{j}	74^{abcde}		
+	+	Ggt only	63^{a}	$76^{abcdefg}$		
+	+	-	$76^{abcdefg}$	63^{a}	66^{ab}	$80^{bcdefghi}$
+	-	+	89^{efghij}	$77^{abcdefgh}$	$82^{cdefghi}$	75^{abcdef}
+	-	-	66^{ab}	63^{a}	69^{abc}	$80^{bcdefghi}$
-	+	+	92^{hij}	$80^{bcdefghi}$	75^{abcdef}	$85^{defghij}$
-	+	-	68^{abc}	73^{abcd}	$83^{cdefghij}$	$81^{bcdefgh}$
-	-	+	$76^{abcdefg}$	90^{fghij}	69^{abc}	$87^{defghij}$
-	-	-	91^{ghi}	93^{ij}	93^{ij}	

* Numbers followed by the same letters are not significantly different at the P>0.05 level.
** - without preincubation of fungi in soil, + with preincubation of fungi in soil
*** For explanation of top and base see Figure 2.2

Table 2.8 Wheat root colonisation by *Trichoderma* after plating directly, without surface disinfestation, on a selective medium. Wheat plants were grown in field soil (section 2.2.8).

	Root infection (%)				
Time from	Distance from seed (cm)				
planting (d)	0-2.5	2.5-5.0	5.0-7.5	7.5-10.0	mean
***T. koningii* added close to the seed**					
***T. koningii* added to soil**					
7	86	92	100	66	86
14	76	74	81	76	76
21	58	71	70	67	66
mean	73	79	84	69	
no *T. koningii* added					
7	95	95	73	72	84
14	92	84	95	86	89
21	87	97	92	88	91
mean	92	92	87	82	
***T. koningii* added half way down the pot profile**					
***T. koningii* added to soil**					
7	94	93	100	100	97
14	67	75	65	76	71
21	55	54	71	73	63
mean	72	74	79	83	
no *T. koningii* added					
7	83	89	79	78	82
14	90	86	100	100	94
21	96	100	96	82	94
mean	90	92	92	87	

There were no significant differences between treatments when *T. koningii* was added close to the seed, nor were there any significant differences at the P>0.05 level between treatments when *T. koningii* was added half way down the pot profile.

Table 2.9 Wheat root colonisation by *Trichoderma* after surface disinfestation and plating on a selective medium. Wheat plants were grown in field soil (section 2.2.8).

	Root infection (%)				
Time from	Distance from seed (cm)				
planting (d)	0-2.5	2.5-5.0	5.0-7.5	7.5-10.0	mean
	***T. koningii* added close to the seed**				
***T. koningii* added to soil**					
7	40^{de*}	32^{de}	8^{abc}	0^{a}	20^{a}
14	27^{bcde}	29^{cde}	0^{a}	1^{a}	16^{a}
21	21^{abcde}	14^{abcd}	6^{ab}	7^{abc}	12^{a}
mean	29^{b}	25^{b}	5^{a}	5^{a}	
no *T. koningii* added					
7	0	0	0	0	
14	0	0	0	0	
21	14	11	4	5	
	***T. koningii* added half way down the pot profile**				
***T. koningii* added to soil**					
7	7^{ab*}	6^{a}	0^{a}	0^{a}	3^{a}
14	29^{bc}	11^{abc}	14^{abc}	0^{a}	14^{a}
21	52^{d}	22^{abc}	11^{abc}	31^{c}	29^{b}
mean	29^{b}	13^{a}	8^{a}	10^{a}	
no *T. koningii* added					
7	0	0	0	0	
14	0	0	0	0	
21	15	0	3	0	

* Numbers followed by the same letters are not significantly different at the P>0.05 level. The means were analysed separately so a different lettering system has been used for the mean column and the mean row figures. Data from pots which had *T. koningii* added close to the seed were analysed separately from data from pots which had *T. koningii* added half way down the pot profile.

2.4 DISCUSSION

As a prerequisite for studies on colonisation of wheat roots by *T. koningii*, an effective selective medium for *Trichoderma* was developed. The existing medium (MTSM) used Captan which is now recognised as a carcinogen and is not readily available in Australia. Unlike other species of *Trichoderma* the isolates of *T. koningii* tested are sensitive to Captan and PCNB so that in any event the media were not suitable for isolation of this species. When Captan was omitted from selective media, growth of colonies of *T. koningii* was seen from individual conidia and a higher number of *Trichoderma* colony-forming-units were isolated from soil. Several media were tested for isolation of *T. koningii* from plants, soil or a suspension and the best for the purposes of this study were found to be STSM and MTSM-C. STSM contains eight ingredients all of which are added before autoclaving in comparison to the 11 ingredients of MTSM-C which requires the antibiotics to be added after autoclaving. STSM restricts the growth of rapidly growing fungi such as *Rhizopus* and supports growth of at least 30 colonies of *Trichoderma* per Petri dish which are clearly identifiable and can be easily subcultured. Initial incubation needs to be in the dark to allow colony growth, the plates are then exposed to light for sporulation and identification. STSM was used in the studies of the interaction of Ggt and *T. koningii*.

Addition of *T. koningii* generally reduced take-all disease of wheat roots and the most important mode of action was inhibition of hyphal regeneration, rather than impairment of established mycelia of Ggt. When *T. koningii* and Ggt were added in the same layer of soil, or separate layers without preincubation, stelar discolouration was effectively controlled, supporting the hypothesis that

T. koningii acts by restricting hyphal growth of Ggt from ryegrass seed inoculum. However, when Ggt was preincubated in soil before *T. koningii* was added, severe take-all lesions were seen, indicating that *T. koningii* did not inhibit pre-established mycelium. This supports the results of a previous study which found a significant reduction in take-all disease by *T. koningii* when the pathogen and antagonist were mixed through soil (Simon 1989). Antibiosis has been reported to play a role in disease reduction by *T. koningii*. *T. koningii* inhibited spore germination but not mycelial development of *Glomus mosseae* (McAllister *et al.* 1994). These workers proposed that a soluble or volatile substance produced by *T. koningii* may be the cause of inhibition. Similarly, the reduction of Rhizoctonia root rot of wheat has been attributed to pyrone production by isolates of *T. koningii* (Worasatit *et al.* 1994).

Where preincubation was carried out and Ggt came into contact with the wheat root system before *T. koningii*, there was little or no disease control. Fellows & Ficke (1934) also found Ggt to cause greatest injuries when inoculated near the seed. Best results using *Trichoderma* as a biocontrol agent of soil-borne pathogens have been obtained if the antagonist comes into contact with the root before the pathogen (Lifshitz *et al.* 1986, de Jong *et al.* 1993). When *Trichoderma* is first to colonise the root it has the potential to deplete the nutrient supply of pathogens (Cook & Baker 1983) and is not likely to become displaced from the root/rhizoplane once established.

Inhibition of hyphal regeneration does not explain the results when *T. koningii* and Ggt were preincubated in separate layers (Table 2.6). No decrease in take-all disease was observed, because each half of the pot was preincubated separately so *T. koningii* could not have prevented the

regeneration of Ggt. When *T. koningii* and Ggt were preincubated separately there were fewer vascular lesions if the roots came into contact with *T. koningii* before Ggt (T.k. top, Ggt base). The antagonist may outcompete the pathogen for root exudates as a nutrient source when it is first to colonise the rhizoplane/root. Increased rhizoplane colonisation by *Trichoderma* was not seen (Table 2.7), but a major limitation is that the inoculant strain of *T. koningii*, could not be distinguished from the indigenous population of *Trichoderma*. There are several other explanations for the increase in disease control when *T. koningii* was added in the top layer. There may be more growth by *T. koningii* in soil with a higher oxygen content and exposure to light near the surface of the pot than at the level of the lower band of inoculum. Thus when *T. koningii* is added to the upper layer of soil it can rapidly colonise the soil and reduce the activity of Ggt. Roots that come into contact with *T. koningii* before Ggt may grow more rapidly (Table 2.5). Increased growth due to the presence of *Trichoderma* species has been observed in other studies (Baker 1988, Simon 1989b, Ousley *et al.* 1994). Alternatively, prior exposure of roots to the antagonist may induce resistance and thus reduce Ggt infection. This has not been observed to date with *T. koningii*, although it has been described as a protective mechanism operating against other plant pathogens (Liu *et al.* 1995 a, b, c, Strobel & Kuc 1995, Wei *et al.* 1996). When *T. koningii* was added in the lower layer it may have been unable to compete for nutrients, stimulate plant growth and/or induce host resistance before the roots came into contact with Ggt. Initial contact with Ggt might have caused root damage that counteracted these effects.

Reduction in take-all lesions appeared to be unrelated to the extent of

root colonisation by *T. koningii*. Addition of *T. koningii* to soil did not increase the total rhizoplane colonisation by *Trichoderma*. It is possible that *T. koningii* became established in the rhizoplane, but because the introduced antagonist could not be differentiated from the native population of *Trichoderma* this can not be verified. *Trichoderma* could only be isolated from surface disinfested roots when it had been added to the soil. Internal root colonisation by *Trichoderma* was not related to control of take-all. While it may be advantageous for *Trichoderma* to enter and colonise roots, this is not reflected in the control of take-all disease. Increasing the number of replicates might improve the ability to see significance in the extent of rhizoplane colonisation observed in each treatment. Other studies have noted differences in root colonisation when *T. koningii* colonised roots after another fungus. Populations of *T. koningii* on maize and lettuce roots were considerably reduced when *G. mosseae* was inoculated before *T. koningii* (McAllister *et al.* 1994).

In sterile soil 36 to 72 % of roots were colonised by *T. koningii* from the growing tip to the seed. Thus the rate of hyphal growth by *T. koningii* matched that of root growth. However, in non-sterile soil root colonisation by *T. koningii* was significantly higher close to the seed (29 %) than at the growing tips (5-10 %). The uneven root colonisation observed in field soil may be due to intense competition with other rhizoplane microrganisms for nutrients at the elongation zone of the root. This has been observed in other systems (Rovira 1965). Competition between *T. hamatum* and *Pseudomonas* species for iron, prevented protection of pea roots from *Pythium* spp (Hubbard *et al.* 1983). Antagonism to *T. koningii* by rhizoplane bacteria has also been shown to occur. Microbes in field soil that colonise the rhizoplane near the root tip may produce

metabolites that are toxic to *T. koningii* (Papavizas 1985).

The isolate of *T. koningii* used as inoculum acted quite differently from the indigenous population of *Trichoderma*. Indigenous species of *Trichoderma* extensively colonised the rhizoplane along the full length of the roots. Extensive hyphal growth and sporulation by *Trichoderma* species has been observed on the surface of wheat roots (Duffy *et al.* 1996) and rhizoplane colonisation from the point at which the seed was planted to depths of at least 10 cm from the soil surface has been reported in tomatoes and potatoes (Sivan *et al.* 1987, Bahme *et al.* 1988). While both the inoculant and the native isolates of *Trichoderma* extensively colonised the rhizoplane, the introduced isolate reduced take-all disease and colonised roots internally, unlike the native population of *Trichoderma*. *T. koningii* has been isolated from wheat roots previously (Simmonds & Leddingham 1937, Innocenti 1991), but only 0-2 % internal colonisation by *T. koningii* was observed in wheat roots at the seedling stage and the level of colonisation did not increase with the age of the plant (Dewan & Sivasithamparam 1988). However these roots were washed in 0.6 % NaOCl for 10 mins in contrast to 0.5 % NaOCl for 2 min used in my experiments.

Quantification of rhizoplane colonisation is difficult. In these experiments the proportion of washed root segments from which a colony of *Trichoderma* was isolated was used to measure rhizoplane colonisation. Each piece of root may have been colonised by one hypha or it may have been densely enclosed by a network of mycelium of *T. koningii*. Others studies have quantified populations as colony forming units per gram of root or rhizoplane soil. However whether each colony comes from a conidium, a clump of conidia, a chlamydospore or a small or a large hyphal strand is unknown. A direct or *in*

situ assessment using a quantifiable marker would greatly assist our understanding of the extent of rhizoplane colonisation.

Inhibition of initial hyphal growth appears to be a primary mechanism by which *T. koningii* controls Ggt. Therefore to control take-all disease most effectively *T. koningii* needs to interact with Ggt in the saprophytic soil phase. Coating wheat seed with *T. koningii* is not recommended as conidia can inhibit seed germination (data not shown). The antagonist needs to be distributed throughout each row at sowing rather than being added at just one position in order to maximise contact with Ggt in soil. The fungus appeared to act on Ggt largely in the bulk soil and to a lesser degree in the rhizoplane, but difficulty in distinguishing *T. koningii* from resident *Trichoderma* species made evaluation difficult. Colonisation of the rhizoplane and root appear to be unrelated to inhibition of take-all disease in this experiment. A marker that could distinguish an introduced isolate from the native population would be useful because assessment of root and rhizoplane colonisation is difficult using selective media. The marker could also be used to quantify the biomass of the inoculant in plant material so that segments of root sparsely colonised by the fungus could be differentiated from those that are extensively colonised.

CHAPTER 3. TRANSFORMATION OF *TRICHODERMA KONINGII*

3.1 INTRODUCTION

The aim of this investigation was to track and quantify *Trichoderma koningii* in the rhizosphere and roots of wheat plants, using the *gus*A gene transformed into the fungus to provide a marker, suitable for observation in the rhizosphere and within the root. There is no record of genes being transformed into *T. koningii*, although other *Trichoderma* species have been transformed using genes for antibiotic and fungicide resistance and auxotrophic markers (Pentilla *et al.* 1987, Pe`er *et al.* 1991, Smith *et al.* 1991, Gruber *et al.* 1990 a, b). The transformation of *T. koningii* with *gus*A would enable it to be tracked on wheat roots in the presence and absence of the take-all fungus and allow it to be distinguished from resident *Trichoderma* species, thus overcoming some of the problems described in chapter 2. One of the advantages of *gus*A is that it is a visual marker. It can be used to identify in which part of a root or the rhizosphere a fungus is growing, and by fluorescence, can be used to quantify the biomass of the marked fungus. When linked to an appropriate promoter (*gpd*, glycerol-3-phosphate dehydrogenase) the gene is constitutively expressed and background activity does not occur in most fungi and plants.

When using *gus*A for fungal transformation direct selection of transformants expressing the gene is not possible (Couteaudier *et al.* 1993) because of the prohibitive cost of GUS substrates, so a second selectable marker gene is also required. The gene most frequently co-transformed with

*gus*A is *hph* (hygromycin phosphotransferase) which allows selection on media containing hygromycin. Other genes which have been co-transformed into fungi are *pyr*G (orotidine-5'-phosphate decarboxylase), *bml* (conferring benomyl resistance) and *nia*D (nitrate reductase). These genes permit selection using growth on media containing uracil, benomyl and $NaNO_3$ respectively. In this investigation the use of *nia*D was inappropriate because wild-type *T. koningii* can grow on media containing $NaNO_3$ as the only nitrogen source (Danielson & Davey 1973). When *bml* and *gus*A were co-transformed, the transformants were not stable in agar culture (Bunkers 1991). The *hph* gene has been transformed into *Trichoderma harzianum*, *T. viride* and *T. hamatum* (Goldman *et al.* 1990, Herrera-Estrella *et al.* 1990, Lorito *et al.* 1992, Ulhoa *et al.* 1992). High co-transformation frequencies of *hph* and *gus*A have been found for *T. harzianum*, *Pseudocercosporella herpotrichoides*, *Bipolaris sorokiniana*, *Leptosphaeria maculans*, and *Fusarium oxysporum*, and the co-transformants have retained their pathogenicity (Bunkers 1991, Liljeroth *et al.* 1993, Oliver *et al.* 1993, Eparvier and Alabouvette 1994, Thrane *et al.* 1995). Hence, the *hph* gene conferring hygromycin resistance appears to be the best option for a secondary marker to co-transform into an isolate of *T. koningii* with the *gus*A gene and was chosen for this study.

3.2 METHODS

3.2.1 Fungi

The isolates of *Trichoderma koningii* Rifai AST-1 (IMI 299426), 7a (IMI 308475) and 7c came from the collection of M. Ryder (CSIRO Division of Soils, Adelaide, South Australia). The isolates 7a and 7c were only used in

hygromycin sensitivity tests (3.2.2). All other work used AST-1.

3.2.2 Growth on Media Containing Hygromycin

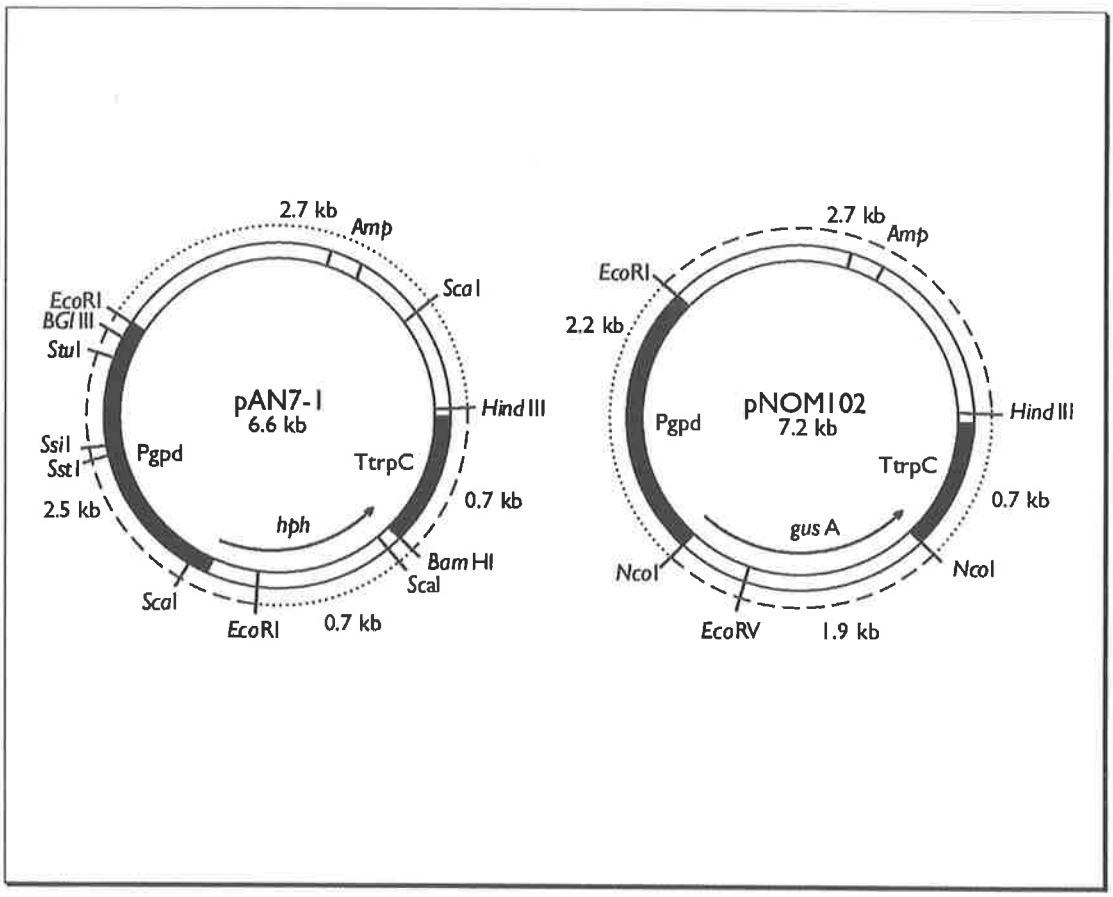
Growth on media containing hygromycin was assessed using three isolates of *T. koningii*. Four concentrations of hygromycin B (Boehringer Mannheim, Australia) (15, 50, 100, 200 µg/ml) were added to potato dextrose agar (PDA, Difco) containing 1.2 M sorbitol that had been cooled to approximately 50 °C. Sorbitol was included as this was the medium which would be used for regeneration of putative transformants. A needlepoint of conidia was combined with 0.2 % agar and inoculated at three places on each plate (Pitt & Hocking 1985). Plates were incubated at 25 °C in darkness. Maximum colony diameter was measured daily for 14 d.

3.2.3 Plasmids

The plasmids pAN7-1, carrying the gene for hygromycin resistance (*hph*) (Punt *et al.* 1987), and pNOM102, carrying the *gus*A gene (Roberts *et al.* 1989), were used for the co-transformation of *T. koningii* (Figure 3.1). Plasmids were provided by R. Oliver (University of East Anglia, Norwich, United Kingdom). Both genes were originally isolated from *Escherichia coli* and are flanked upstream by the glyceraldehyde 3-phosphate dehydrogenase (*gpd*) promoter and downstream by the *trp*C transcription termination signal. The promoter and terminator signals originate from *Aspergillus nidulellus* Samson & Gams (syn. *A. nidulans*) and were therefore likely to be appropriate for transformation into

fungi such as *T. koningii*. For the complete sequence of the *gus*A gene see Jefferson *et al.* 1986. Transformation of plasmid DNA into *E. coli* JM109 was achieved using the $CaCl_2$ procedure (Sambrook *et al.* 1989). Plasmids were maintained in *E. coli* strain JM109 on Luria Bertani (LB) broth (Sambrook *et al.* 1989) containing ampicillin (40 µg/ml). Plasmid DNA was extracted by alkaline lysis as outlined by Sambrook *et al.* (1989).

Figure 3.1 Maps of the plasmids pAN7-1 containing the *hph* gene and pNOM102 containing the *gus*A gene compiled from Punt *et al.* 1987, Roberts *et al.* 1989, Murray *et al* 1992.



3.2.4 Protoplast Preparation

Protoplasts of *T. koningii* were generated using the method of Tilburn *et al.* (1983), with modifications by Herrera-Estrella *et al.* (1990). Conidia were inoculated into 50 ml potato dextrose broth (PDB, Pitt & Hocking 1985 Appendix 2) and incubated at 28-32 °C for 20 h at 300 rpm on an orbital shaker. The broth was removed by vacuum filtration through a Whatman number 1 filter paper and the germinated spores were resuspended in 15 ml $MgSO_4$ (1.2 M in 10 mM sodium phosphate buffer pH 5.8) (Sambrook *et al.* 1989). Lysing enzymes (chiefly cellulase, proteinase and chitinase) (75 mg) from *T. harzianum* (Sigma Catalogue no. L-2265) were added and the solution incubated for 1 h at 27-28 °C, 150 rpm. Protoplasts were separated from mycelial debris by overlaying the protoplast solution with an equal volume of 600 mM sorbitol in 100 mM Tris-HCl, pH 7 and centrifuging at 7500 rpm for 15 min. The band that formed between these two layers contained the protoplasts and was collected with a sterile Pasteur pipette, washed twice in 1.2 M ST (Appendix 2) at 7500 rpm for 15 min and resuspended in 200 µl 1.2 M STC. This suspension contained 10^5-10^7 protoplasts per ml.

3.2.5 Transformation of *T. koningii* AST-1

The transformation procedure of Herrera-Estrella *et al.* (1990) was used. Plasmid DNA (5 µg) of pAN7-1 and pNOM102 were added to the protoplast suspension in 20 µl TE8 (10 mM Tris-HCl, 1 mM EDTA, pH 8). A control transformation with TE8 was performed. An aliquot (50 µl) of polyethylene glycol 6000 (PEG) was added and the mixture incubated in a water-ice bath for 20 min. A further 2 ml of the PEG solution was added and the solution

incubated at 25 °C for 10 min. After the addition of 4 ml of 1.2 M STC (Appendix 2), the solution was combined with 6 ml of PDB containing 1.2 M sorbitol.

Protoplasts were incubated at 25 °C for 2 h. Aliquots (0.2 ml) of the protoplast suspension were mixed with 15 ml tempered potato dextrose agar (PDA, Difco) or PDA containing 1.2 M sorbitol and set in Petri dishes. A top layer of 7 ml of tempered PDA containing 1.2 M sorbitol and 200 µg/ml hygromycin B was poured over half the PDA and sorbitol plates. Fourteen replicate plates were used per treatment. After 3 d incubation at 25 °C in darkness, a total of 80 discrete colonies from single protoplasts were subcultured onto PDA with hygromycin (100 µg/ml) (PDA+H). Only clearly distinct colonies were counted. Potential transformants that grew underneath the layer containing hygromycin were not counted. Co-transformation was assessed by transferring a needlepoint of hyphae from these putative transformants to PDA+H (100µg/ml) containing 5-bromo-4-chloro-3-indolyl β-D-glucuronide (X-Gluc) (0.4 g/L). Growth on PDA+H showed transformation had occurred with pAN7-1 and the presence of a blue precipitate when incubated with X-Gluc indicated transformation with pNOM102.

3.2.6 Transformation of *T. koningii* with Modified Plasmids.

Stable transformants expressing the *gus*A gene were not obtained in the first transformation series in which pNOM102 and pAN7-1 were co-transformed into *T. koningii* (see results) so a second method was tried. Addition of a 2.4 kb segment of DNA from *T. harzianum* to a plasmid carrying the *hph* gene resulted in more stable transformants of the fungus than those made with the *hph*

plasmid alone (Herrera Estrella *et al.* 1990). This approach was followed in a second transformation series.

In the second attempt at transformation a 2 kb fragment of DNA from *T. koningii* AST-1 was inserted into both pNOM102 and pAN7-1 as follows. DNA was digested with *Hind*III and purified by extraction with phenol-chloroform. The plasmids were linearised by cutting at the *Hind*III site then dephosphorylated essentially as described by Sambrook *et al.* (1989), except that the first aliquot of calf intestinal alkaline phosphatase (CIP) was added and incubated at 37 °C for 30 min, followed by 15 min at 55 °C. The second aliquot of CIP was added and the solution incubated at 37 °C for 15 min followed by 45 min at 55 °C. Ligation of the dephosphorylated plasmids and digested DNA from *T. koningii* was conducted as outlined in Sambrook *et al.* (1989). A ratio of 9:1 *Trichoderma*:plasmid DNA was used. The ligation mix was transformed into competent *E. coli* cells (DH5α), then plated on LB agar with 50 µg/ml ampicillin.

Transformation was confirmed by extractions of plasmid DNA using the mini-scale alkaline lysis procedure of Sambrook *et al.* (1989) with modifications (P. Harvey, personal communication 1995) as follows: Cells were resuspended in 100 µl of plasmid solution I and 2 mg/ml lysozyme and placed on ice for 10 minutes. Freshly made plasmid solution II was added and incubated on ice for 10 mins. After the addition of 3 M sodium acetate pH 4.8 (150 µl), the solution was placed at -20 °C for 10 min and centrifuged at 12 000 rpm for 10 min. The supernatant was extracted twice with phenol:chloroform:isoamyl alcohol (25:24:1), and the DNA precipitated with ethanol and resuspended in 30 µl TE8 buffer. Following digestion with *Hind*III two clones of each of pNOM102 and

pAN7-1 containing *T. koningii* fragments about 2 kb in size were transformed into protoplasts of *T. koningii* as previously described (section 3.2.5). The colonies on PDA with a hygromycin (H) overlay grew into one another; 80 isolates were therefore subcultured onto PDA+H, followed by malt extract agar (MEA, Pitt & Hocking 1985 Appendix 2) to conidiate. Single spores were then inoculated onto PDA. These colonies were tested for their ability to grow on PDA+H and cleave the GUS substrates X-Gluc and MUG (4-methylumbelliferyl β-D-glucuronide).

3.2.7 Detection and Expression of *gus*A in the Transformants

3.2.7.1 Growth on agar and histochemical tests

The protocol by which transformant stability was assessed is outlined in Figures 3.1 and 3.2. For the first series the co-transformants were subcultured five times on non-selective (PDA) and selective (PDA+H) media. The transformants generated with unmodified plasmids, which were still alive after 5 transfers on PDA+H, had their stability further assessed by subculturing another five times on five different media (Figure 3.2). These media were: nutrient-rich (PDA), nutrient-poor (water agar, Difco) and semi- synthetic (Czapek yeast extract agar, (CYA) Pitt & Hocking 1985 Appendix 2), MEA which supports prolific sporulation, and a medium selecting for hygromycin resistance (PDA+H). Hyphal transfers were carried out except for transfer onto water agar where hyphae were adherent to the agar so pieces of agar were transferred. Conidial transfers were made on malt extract agar. In the second transformation series co-transformants made with modified plasmids were

subjected to a series of ten successive subcultures on PDA and PDA+H (Figure 3.3).

In both transformation series each generation grown on non-selective agar was assessed for hygromycin resistance by hyphal inoculation and growth on PDA+H. These plates were incubated at 25 °C in darkness for 4-8 d and assessed for hyphal growth. The fifth and tenth generations from both sets of transformants were tested for GUS expression by incubation with 2 mM X-Gluc and 1 mM MUG (Couteaudier *et al.* 1993).

3.2.7.2 Molecular detection of gene integration in transformants

All transformants made with unmodified plasmids, from the fifth and tenth generation of the PDA+H line of subculturing underwent Southern analysis in a digested and undigested form. The DIG (digoxygenin) system was used to detect pNOM102. The presence of *gus*A was determined in ten isolates generated with modified plasmids from the first, fifth and tenth generations grown on PDA. Integration of the *hph* gene was also assessed by Southern analysis and ^{32}P detection in isolates from the fifth and tenth generations.

DNA was extracted from hyphae for polymerase chain reactions (PCR) and Southern analyses. Isolates were grown in Warcup's broth (Warcup 1955) at 25 °C with agitation. Cultures were then washed twice in sterile distilled water and blotted dry before being immersed in liquid nitrogen. Mycelia were ground to a fine powder and then extracted with 10 ml DNA extraction buffer. An equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) was added once the slurry had thawed; it was mixed for 20 min, then centrifuged (12 000 rpm 15 °C 12 min). The supernatant was retained and this step

repeated, first with phenol:chloroform:isoamyl alcohol and then with chloroform:isoamyl alcohol (24:1). The DNA was precipitated with 2 volumes of ice-cold ethanol and recovered by centrifugation (15 000 rpm 15 °C 15 min). After washing the pellet twice in 70 % ethanol it was dried and resuspended in tris EDTA pH 8 (TE8, P. Harvey personal communication 1994).

A PCR assay was used as a rapid test to determine the presence of the *gus*A gene in transformants made with unmodified plasmids from the fifth and tenth generations, and transformants from the first, fifth and tenth generations made with modified plasmids. Each 25 µl reaction included 2.5 mM $MgCl_2$, 0.2 mM deoxyribonucleoside triphosphates (dNTP), 32 pM of each of the primers and 2U *Taq* polymerase. The two primers (5'-CTG TAG AAA CCC CAA CCC CTG-3', 5'-CAT TAG GCT GCG ATG GAT CCC-3') used for amplification of the *gus*A gene yielded a fragment of 514 base pairs (van Wordragen *et al.* 1991) and were synthesised by Macromolecular Resources (Colorado State University) (Appendix 2). Forty cycles of a modification of the sequence outlined by van Wordragen *et al.* (1991) were found to amplify the *gus*A band sufficiently for detection (94 °C 1 min, 55 °C 2 min, 72 °C 2 min). PCR products were electrophoresed on 2 % agarose gels run in 0.5 x Tris Borate EDTA pH 8.3 (TBE) buffer. Gels were stained in ethidium bromide (2 µg/ml) for 45 min followed by destaining in water for 30 min. Southern analyses of the PCR gels were conducted to confirm that the amplified bands were the *gus*A gene. The ^{32}P detection system was used for all PCR membranes with a *gus*A probe (described in 3.2.7.2). Conditions for ^{32}P detection were as described below. Membranes from PCR gels were washed at 65 °C for 20 min in 2 x SSC, 0.1 % SDS followed by 1 x SSC, 0.1 % SDS and 0.5 x SSC, 0.1 % SDS

to remove unbound DNA. X-ray films were exposed for 15 to 60 min at room temperature.

DNA recovered from transformants and AST-1 (untransformed) was digested with *Hind*III (Boehringer Mannheim Australia) overnight at 37 °C according to the manufacturer's recommendations. DNA (0.5 to 1.0 µg) from transformants of *T. koningii* was used in hybridisation experiments to detect *gus*A and *hph* genes. Frequently the DNA did not digest fully. Purifying the DNA by reprecipitation and washing in 70 % ethanol between one and three times solved this problem. DNA was fractionated by gel electrophoresis (0.8 % agarose gel using Tris-acetate-EDTA (TAE) as the running buffer). Gels were then soaked in denaturing solution for 30 to 40 min followed by neutralising solution for 30 min on a rotating shaker. DNA was transferred to a positively charged nylon membrane (Boehringer Mannheim - DIG detections, Hybond N+, Amersham - ^{32}P detections) in 20 x SSC overnight (Southern 1975).

DNA from transformants made in the first transformation series was fixed to the membrane by UV cross linkage. The plasmid pNOM102 which had been linearised and DIG labelled according to the manufacturer's instructions (Boehringer Mannheim, Australia) was used as a probe. Exposure of the membrane to Cronex X-ray film varied from 5 to 18 h.

^{32}P labelling was used to reduce the high background frequently found with DIG detection. The ^{32}P hybridisation with *gus*A and *hph* probes is outlined below. The prehybridisation solution contained 6 rather than 4 ml of dextran sulphate. Only two washes were necessary for these membranes (2 x SSC 0.1 % SDS 20 min, 1 x SSC 0.1 % SDS 10 min). X-ray film was exposed to the membranes at -80 °C for 3 to 7 d depending on signal strength.

For ^{32}P detection of *gus*A or the *hph* gene DNA after PCR amplification of transformants from the second transformation series was fixed to the membrane by baking at 80 °C for 20 to 30 min and the alkali fixation protocol outlined by Amersham (UK). Membranes were prehybridised in a solution of 2 ml sterile water, 3 ml 5 x HSB, 1 ml 10 x Denhardts III solution, 4 ml 25 % dextran sulphate and 0.2 ml denatured salmon sperm DNA (5 µg/ml) at 65 °C, rotating overnight in a hybridisation oven. The radiolabelled probe was denatured with 200 µl salmon sperm DNA before use. Hybridisation was conducted at 65 °C rotating overnight. Following the washes, which are outlined above, the membranes were sealed in plastic and exposed to Cronex X-ray film in intensifying screens (Dupont).

The *gus*A probe used for ^{32}P detection was made by isolation of the *gus*A gene after *Nco*I digestion of pNOM102. The 1.9 kb fragment was extracted from the gel (1 % agarose in TAE buffer) using Bresa-Clean (Bresatec, Australia). The *hph* probe used for ^{32}P detection was generated by isolation of the *hph* gene from pAN7-1 after digestion with *Sca*I (Boehringer Mannheim Australia). The 1.3 kb fragment was extracted from a gel using Bresa-Clean. The probes (50-100 µg) were denatured, then labelled with [α-^{32}P] dCTP using random oligonucleotides as a primer (Feinber & Vogelstein 1983). The specific activity of the probes was approximately 2 x 10^9 dpm/µg. Labelled DNA was separated from unincorporated nucleotides via a Sephadex G-100 column (Sambrook *et al.* 1989).

3.2.7.3 Antibiotic production

Production of antibiotics by transformants of *T. koningii* with inhibitory effects on the growth of *Gaeumannomyces graminis* var. *tritici* was assessed using the tenth generation grown on PDA+H (unmodified plasmids) or PDA (modified plasmids), using the dialysis membrane overlay technique (Gibbs 1967, Whipps 1987, Simon & Sivasithamparam 1988 d) on CYA at 25 °C.

Figure 3.2. A schematic representation of the protocol followed to assess the stability of transformants made with unmodified plasmids pAN7-1 and pNOM102.

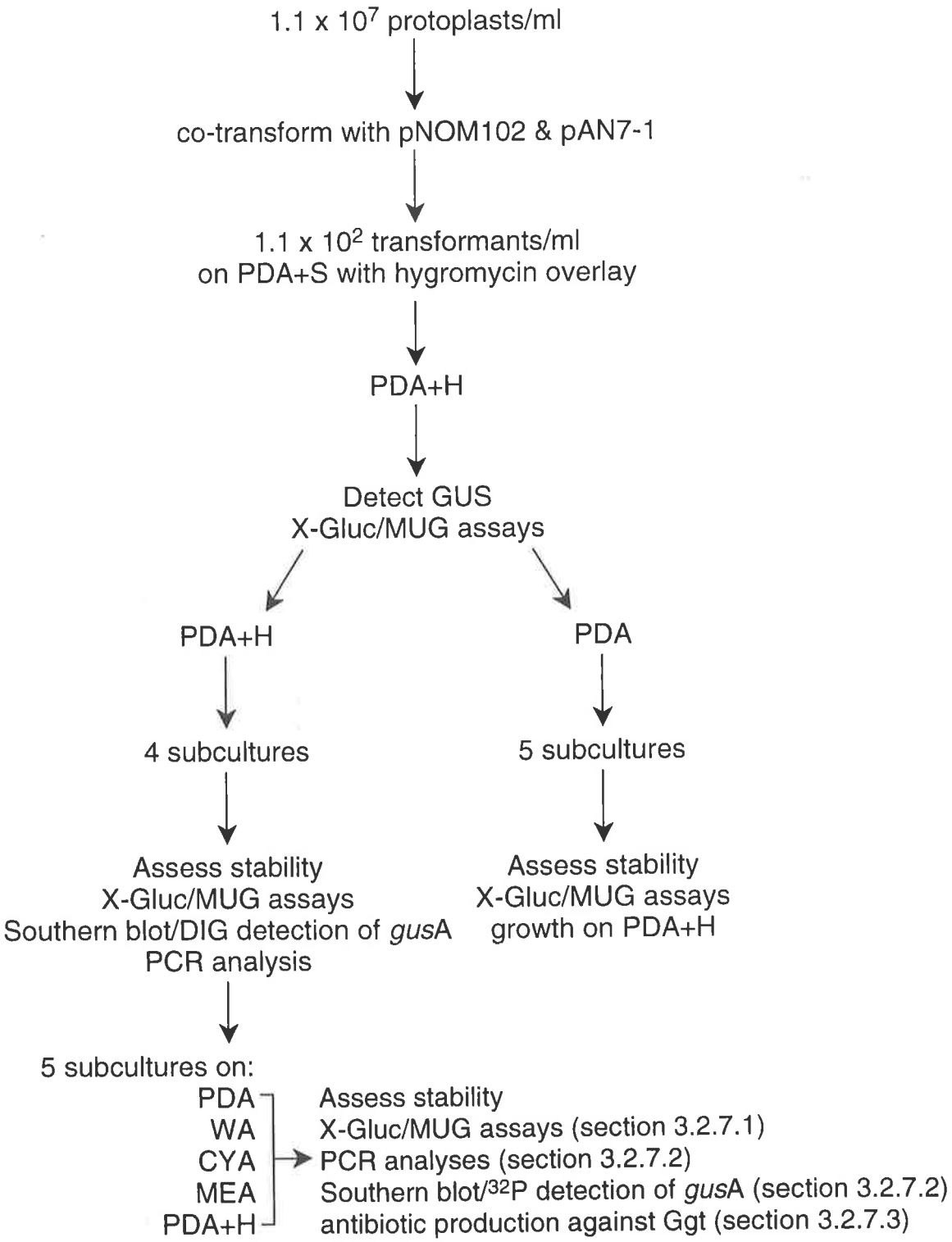
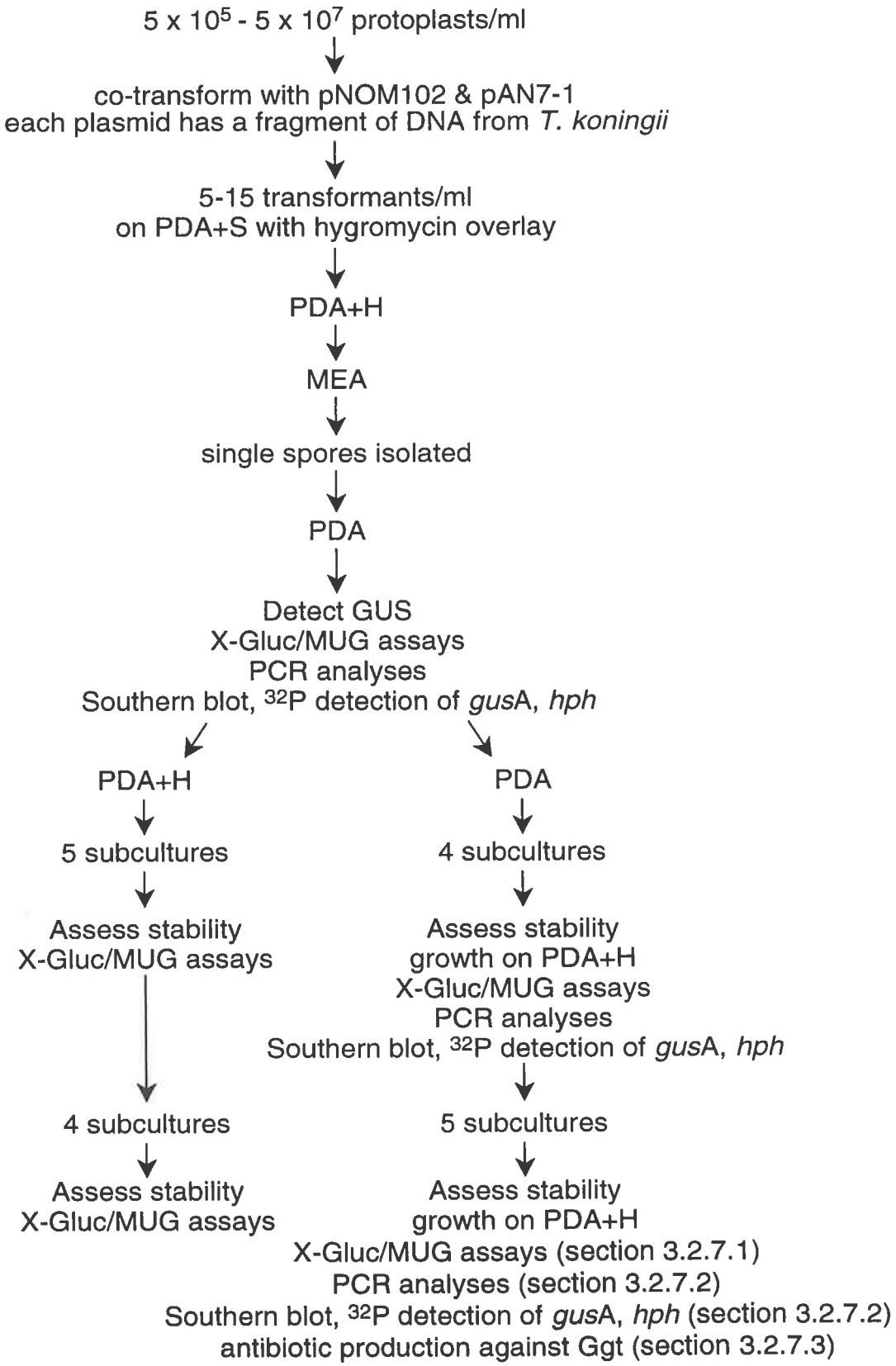


Figure 3.3. A schematic representation of the protocol followed to assess the stability of transformants made with modified plasmids pAN7-1 and pNOM102.



3.3 RESULTS

3.3.1 Growth of Isolates on Media Containing Hygromycin

All three isolates of *T. koningii* were able to grow in the presence of up to 50 µg/ml hygromycin B in PDA with sorbitol, but growth was completely inhibited (up to 14 d) by 100 µg/ml hygromycin B (Table 3.1). Hence hygromycin B at a concentration of 200 µg/ml was used in initial selection of hygromycin positive transformants and 100 µg/ml was used in PDA to assess if transformants retained this trait.

Table 3.1 Growth by *T. koningii* in response to hygromycin contained in PDA with sorbitol (section 3.2.2).

Days from inoculation	Hygromycin B concentration (µg/ml)	Maximum colony diameter (mm) Isolate		
		7a	7c	AST-1
4	15	39-44	40-45	31-37
	50	8-11	15-27	4-5
	100	0	0	0
	200	0	0	0
8	15	>60	>60	>60
	50	10-25	54-60	4-5
	100	0	0	0
	200	0	0	0
14	15	>60	>60	>60
	50	31-54	>60	5-10
	100	0	0	0
	200	0	0	0

3.3.2 Generation of Modified Plasmids

Complete restriction digests of DNA from *T. koningii* were used for transformation as partial restriction digests resulted in DNA fragments greater than 2 kb. Approximately 75 clones in *E. coli* were screened to find 2 kb inserts of DNA from *T. koningii* in pNOM102 and pAN7-1. Two clones of pNOM102 and two clones of pAN7-1 each containing an insert of DNA from *T. koningii* were selected for transformation into protoplasts of *T. koningii* (section 3.3.4.2).

3.3.3 Protoplast Isolation

After incubation with cell wall degrading enzymes the conidial solution was examined microscopically. Almost all the conidia had weakened cell walls and had swollen to form protoplasts. Approximately 10^7 protoplasts per ml were produced using this method. No colonies grew when the protoplast solution was spread on PDA. Colonies were regenerated on PDA+S from protoplasts made with buffer and protoplasts made with plasmids.

3.3.4 Transformation of *T. koningii* AST-1

3.3.4.1 Transformants made with unmodified plasmids

When co-transformed with pAN7-1 and pNOM102 100 colonies per ml were recovered on PDA+S+H from 10^7 protoplasts per ml. The control (protoplasts with buffer) did not grow on this medium. The time taken to form detectable colonies by the transformed protoplasts was slower on the selective medium containing hygromycin (3 d) than on PDA alone (1 d). 84 % of the transformants had been co-transformed as assessed by their ability to grow on media containing hygromycin and form a blue precipitate in the presence of X-

Gluc.

3.3.4.2 Transformants made with modified plasmids

When transformations were performed with plasmids containing inserts of DNA from *T. koningii* the results were similar to those obtained with unmodified plasmids. However only 5-15 transformants per ml transformation solution grew on PDA+S+H. When transferred to PDA+H to confirm hygromycin resistance, variation was seen among the transformants generated from the four modified plasmid combinations. All of the transformants from two of the combinations survived, but only 10 and 35 % of transformants from the other plasmid combinations withstood hyphal transfer. After confirming that the transformants were resistant to hygromycin, those generated with modified plasmids were grown on malt extract agar (MEA) and recultured from single spores as they were not well dispersed on the PDA+S+H plates. No colonies were lost during the transfer from PDA+H to MEA or single spore isolation on PDA. Of these first generation isolates 98 % were able to cleave GUS substrates (Table 3.4).

3.3.5 Stability of Transformants:

3.3.5.1 Transformants made with unmodified plasmids

The first five generations

All transformants survived five transfers on PDA (Table 3.2) and 81 % of these colonies were hygromycin resistant. However, none of these isolates

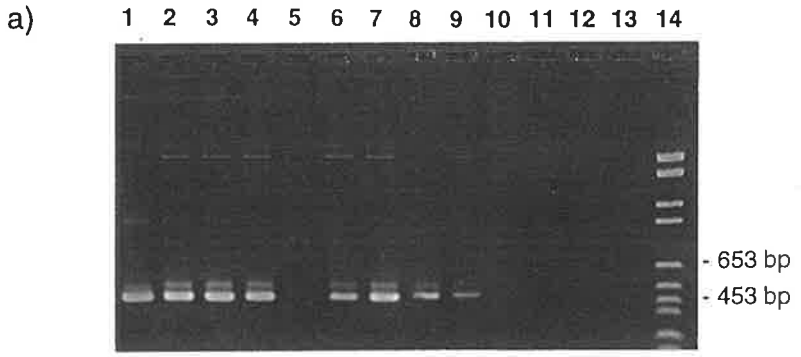
expressed the *gus*A gene in either X-Gluc or MUG. In contrast, only 24 % of the transformants subcultured on PDA+H survived five transfers (Table 3.2). All of these colonies were both hygromycin resistant and expressed the GUS-positive phenotype in the fifth generation.

Table 3.2. Phenotypic assessment of *gus*A and *hph* integration into *Trichoderma koningii* following five generations of the transformants on selective and non-selective media (section 3.2.7.1).

		Number of colonies after 5 generations		
	Survival	hygromycin resistant	blue on X-Gluc	fluoresced with MUG
PDA	80/80	65/80	0/80	0/80
PDA+H	19/80	19/19	19/19	19/19

The *gus*A gene was clearly detected by PCR analysis in the fifth generation of transformants (Figure 3.4). The *gus*A product was about 514 bp as seen in pNOM102. The presence of the *gus*A gene was confirmed with Southern hybridisation of the membrane (Figure 3.4). All 19 transformants from the fifth generation of the PDA+H line of subculturing underwent Southern analysis. Positive hybridisation of pNOM102 was observed to undigested DNA of 17 of the 19 transformants and digested DNA of 18 of the 19 transformants. Representative data from five isolates are presented (Figure 3.5. When Southern analyses of genomic DNA was performed, the *gus*A vector pNOM102 hybridised only to high molecular weight genomic DNA of the undigested transformants. There was no evidence of hybridisation to unintegrated

plasmids in the undigested samples. There was no detectable homology between pNOM102 and DNA from the untransformed isolate (Figure 3.5). Digested DNA from many of these transformants showed at least two bands approximately the same size as digested pNOM102. Since *Hin*dIII cuts once in pNOM102 there are two hybridising bands when the plasmid is integrated as a single copy and three hybridising bands when the plasmid is integrated in tandem. Some transformants contained a single integrated copy of *gus*A and some contained a tandem repeat, while others had complex hybridisation patterns indicating multiple sites of integration (Figure 3.5).



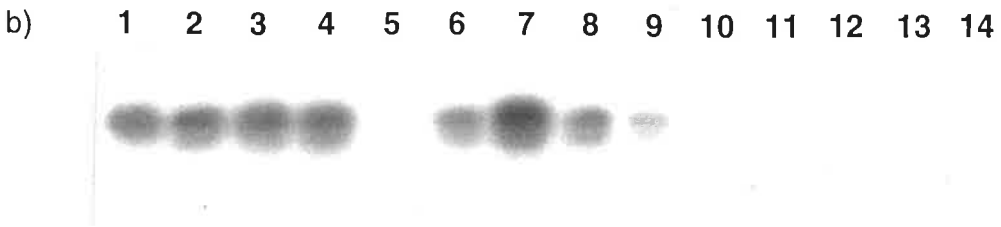


Figure 3.4 DNA from isolates of *T. koningii*, transformed with the *gus*A and *hph* genes, was amplified by PCR using *gus*A specific primers (section 3.2.7.2). PCR products were fractionated in a 2 % agarose gel (Figure 3.4a). DNA amplified by *gus*A specific primers hybridised to the β-glucuronidase (*gus*A) gene excised from pNOM102 by digestion with *Nco*I and labelled with ^{32}P (Figure 3.4b). Key: lane 1: pNOM102, lanes 2-6: transformants of *T. koningii* from the fifth generation, lanes 7-11: transformants of *T. koningii* from the tenth generation, lane 12: sterile water, lane 13: *T. koningii* AST-1 (untransformed), lane 14: DNA molecular marker six (Boehringer Mannheim, top to bottom 2.18, 1.76, 1.23, 1.03, 0.65, 0.52, 0.45, 0.39, 0.30, 0.23, 0.15, kbp) (Figure 3.4b).

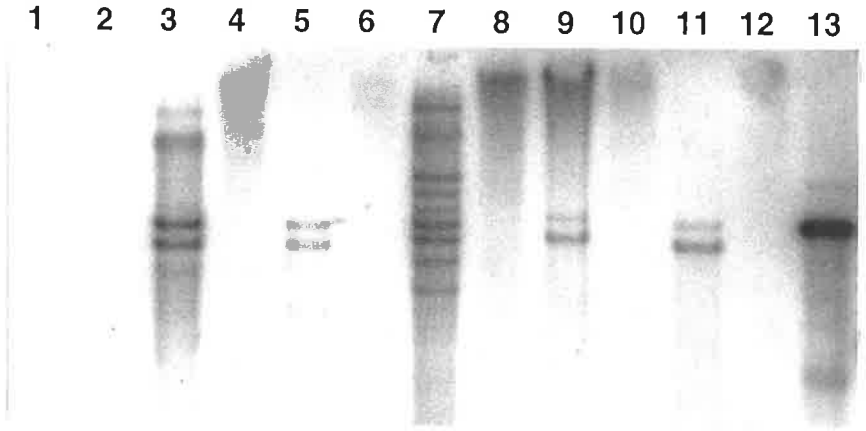


Figure 3.5 Southern blot hybridisation of genomic DNA from *T. koningii* transformed with *gus*A and *hph*. The hybridisation bands indicate the presence of *gus*A. Each DNA sample was left undigested or digested with *Hin*dIII, Southern blotted and hybridised with digoxigenin labelled pNOM102 (*gus*A). Key: lane 1: *T. koningii* AST-1 (untransformed) digested, lane 2: *T. koningii* AST-1 undigested, lanes 3-12: transformants of *T. koningii* from the fifth generation digested and undigested respectively in alternate lanes, lane 13: pNOM102 digested.

Generations six to ten

When subcultured on the four non-selective media (3.2.5.1), all but one transformant grew (Table 3.3). However, when transferred on PDA+H a decline in the number of viable isolates was observed as the number of generations increased. Hygromycin resistance amongst isolates grown on non-selective media from the sixth to the tenth generation was similar irrespective of whether the transformants were grown on a nutritionally weak or rich medium, or whether they were transferred as spores or hyphae. None of the tenth generation transformants from any of the selective or non-selective media were positive when assessed histochemically for GUS expression. However, when amplified with PCR the *gus*A sequence was present in some isolates (Figure 3.4). Southern analysis of the same membrane confirmed that it was the *gus*A gene that had been amplified (Figure 3.4). However when genomic DNA underwent Southern analysis the *gus*A gene could not be detected in any of the transformants.

Table 3.3. Phenotypic assessment of *gus*A and *hph* integration into *Trichoderma koningii* following ten passages of the transformants on selective and non-selective media (section 3.2.7.1).

Medium	Survival	Hygromycin resistant	GUS expression
PDA	19/19	18/19	0/19
CYA	19/19	16/19	0/19
WA	19/19	16/19	0/19
MEA	18/19	18/19	0/19
PDA+H	12/19	12/19	0/19

3.3.5.2 Transformants made with modified plasmids

The number of viable transformants decreased with each successive generation when maintained on PDA+H (Table 3.4). Whereas these isolates were resistant to hygromycin, they did not cleave X-Gluc. However two isolates cleaved MUG. After ten generations subcultured on PDA all transformants survived. 58 to 100 % of the colonies maintained on PDA retained their ability to grow on PDA+H, especially if permitted to grow for 8 d (Table 3.4). After ten generations on PDA, 42/50 of these isolates also retained their ability to cleave GUS substrates (Table 3.4). Transformed isolates had a similar growth rate and morphology to the non-transformed isolate of *T. koningii*.

Table 3.4. Phenotypic assessment of *gus*A and *hph* activity in transformants of *Trichoderma koningii* made with modified plasmids and maintained on selective and non-selective media (section 3.2.7.1).

Medium	Generation	Survival (%)	Number of transformants		
			hygromycin resistant	blue on X-Gluc	fluoresced with MUG
PDA	1	100	50/50	49/50	50/50
	5	100	41/50	44/50	42/50
	10	100	50/50	42/50	50/50
PDA+H	1	54	27/50	0/27	20/27
	5	6	3/50	0/3	0/3
	10	4	2/50	0/2	2/2

PCR amplification of the *gus*A gene in the first generation of transformants made with modified plasmids was unsuccessful despite attempts to optimise the PCR conditions. However, when a Southern transfer of the PCR reactions was hybridised to *gus*A, the *gus*A gene was clearly present in the transformants and not in the untransformed isolate (AST-1) (Figure 3.6). PCR analyses showed the *gus*A gene was present in transformants from the fifth (data not shown) and tenth generation (Figure 3.7). Southern analyses confirmed that the PCR amplification products were the *gus*A gene (Figure 3.8). Probing genomic DNA with *gus*A also showed at least one copy of the *gus*A gene was present in each isolate illustrated from the fifth generation except isolate A16 (Figure 3.9 lane 6). The *gus*A gene was detected in genomic DNA from at least eight of the ten isolates from the tenth generation grown on PDA, although the hybridisation was weak (data not shown).

The *hph* gene was detected in transformants made with modified plasmids from both the fifth and tenth generations (Figure 3.10). Nine of the ten isolates from the fifth generation contained at least one copy of the *hph* gene (Figure 3.10). No homology was seen between the *hph* probe and the non-transformed isolate of *T. koningii*.

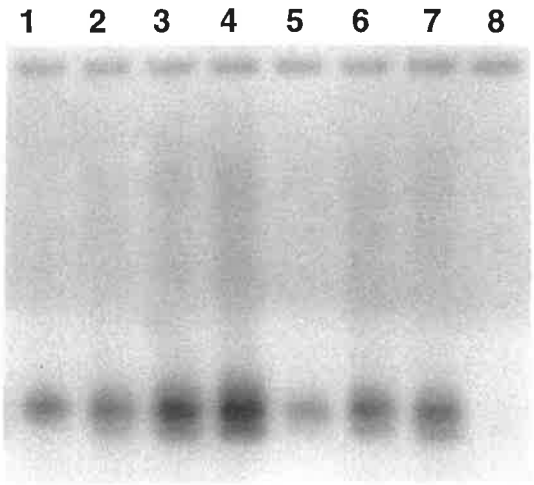


Figure 3.6 Southern blot hybridisation of DNA from the first generation of isolates of *T. koningii* transformed with *gus*A and *hph*. The plasmids carrying these genes had been modified by the addition of a 2 kb segment of DNA from *T. koningii*. DNA samples were diluted, amplified by *gus*A specific primers (section 3.2.7.2) and hybridised to the β-glucuronidase (*gus*A) gene excised from pNOM102 by digestion with *Nco*I and labelled with ^{32}P. Key: lanes 1-4: *T. koningii* transformant B2 (undiluted, 1:10, 1:100, 1:1000), lanes 5-7: *T. koningii* transformant A14 (1:10, 1:100, 1:1000), lane 8: *T. koningii* AST-1 (untransformed).

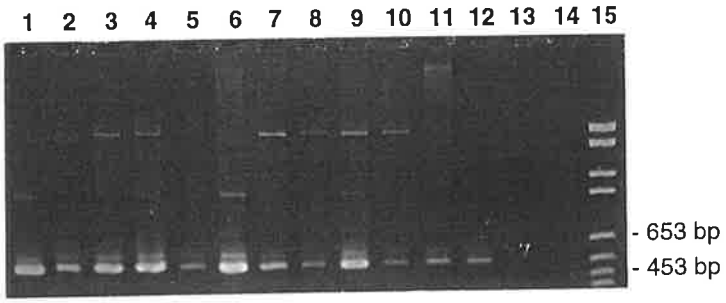


Figure 3.7 DNA from tenth generation transformants of *T. koningii* which had been transformed with the *gus*A and *hph* genes carried in pNOM102 and pAN7-1 respectively. Both plasmids contained a 2 kb segment of DNA from *T. koningii*. PCR products were generated with *gus*A specific primers (section 3.2.7.2) and fractionated in a 2 % agarose gel. Key: lane 1: pNOM102, lanes 2-12: transformants of *T. koningii*, lane 13: *T. koningii* AST-1 (untransformed), lane 14: sterile water, lane 15: DNA molecular marker six (Boehringer Mannheim, top to bottom 2.18, 1.76, 1.23, 1.03, 0.65, 0.52, 0.45, 0.39, 0.30, 0.23, 0.16 kbp).

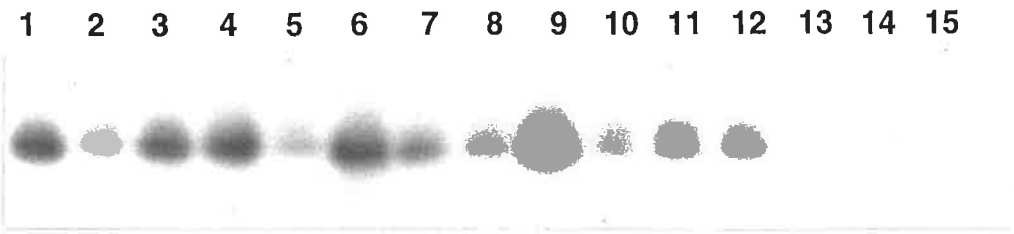


Figure 3.8 Southern blot hybridisation of DNA from tenth generation isolates of *T. koningii* transformed with *gus*A and *hph*. The plasmids carrying these genes had been modified by the addition of a 2 kb segment of DNA from *T. koningii*. DNA samples were amplified by PCR, Southern blotted and hybridised with the β-glucuronidase (*gus*A) gene excised from pNOM102 by digestion with *Nco*I and labelled with ^{32}P. Key: lane 1: pNOM102, lanes 2-12: transformants of *T. koningii*, lane 13: *T. koningii* AST-1 (untransformed), lane 14: sterile water, lane 15: DNA molecular marker six (Boehringer Mannheim, top to bottom 2.18, 1.76, 1.23, 1.03, 0.65, 0.52, 0.45, 0.39, 0.30, 0.23, 0.16 kbp).

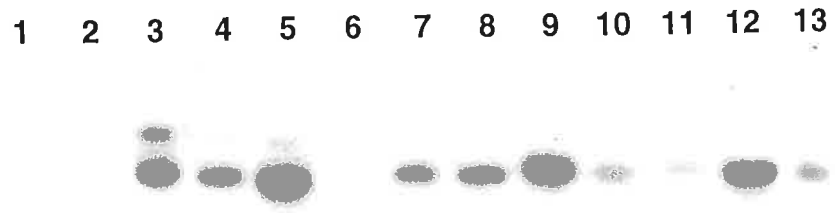


Figure 3.9 Southern blot hybridisation of genomic DNA from fifth generation isolates of *T. koningii* transformed with *gus*A and *hph*. The hybridisation bands indicate the presence of *gus*A. The plasmids carrying these genes had been modified by the addition of a 2 kb segment of DNA from *T. koningii*. DNA samples were digested with *Hin*dIII, Southern blotted and hybridised with the β-glucuronidase (*gus*A) gene excised from pNOM102 by digestion with *Nco*I and labelled with ^{32}P. Key: lane 1: *T. koningii* AST-1 (untransformed) undigested, lane 2: *T. koningii* AST-1 digested, lanes 3-12: transformants of *T. koningii*, lane 13: pNOM102 digested.

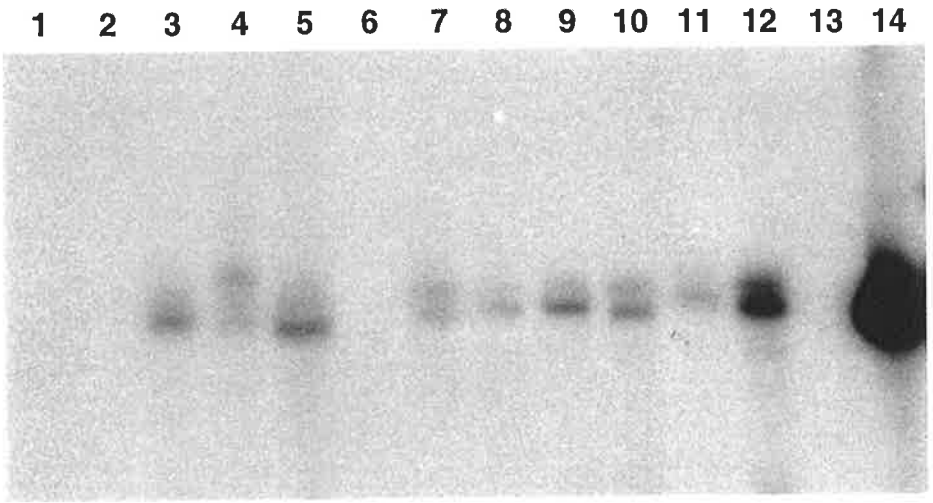


Figure 3.10 Southern blot hybridisation of genomic DNA from fifth generation isolates of *T. koningii* transformed with *gus*A and *hph*. The plasmids carrying these genes had been modified by the addition of a 2 kb segment of DNA from *T. koningii*. DNA samples were digested with *Hin*dIII, Southern blotted and hybridised with the *hph* gene excised from pAN7-1 by digestion with *Sca*I and labelled with ^{32}P. Key: lane 1: *T. koningii* AST-1 (untransformed), lane 2: blank, lanes 3-12: transformants of *T. koningii*, lane 13: blank, lane 14: pAN7-1.

3.3.6 Inhibition of Ggt *in vitro* by Transformants of *T. koningii*

Fifth generation transformants made with unmodified plasmids and untransformed isolates of *T. koningii* completely inhibited growth of Ggt when plates were assessed after 7 d. Colonies of Ggt not exposed to *Trichoderma* had diameters of 64-68 mm. All tenth generation transformants made with modified plasmids inhibited Ggt in dialysis membrane tests, as did the untransformed isolates. Many of the transformants (18/50) completely inhibited growth of Ggt while the remainder allowed growth by the pathogen only on top of the agar plug as was the case with the untransformed isolate. Colony diameters of Ggt on plates inoculated with a blank agar plug were 30 to 33 mm.

3.4 DISCUSSION

Transformation of *T. koningii* was successful using both modified and unmodified plasmids. The co-transformation rate was 98 and 84 % for the two types of plasmid respectively. Expression of the *gus*A gene was not maintained when *T. koningii* was transformed with the unmodified plasmids pNOM102 and pAN7-1. However incorporation of a 2 kb segment of DNA from *T. koningii* into each plasmid resulted in the production of stable transformants *in vitro*. Modification of the pNOM102 and pAN7-1 plasmids resulted in a ten-fold decrease in the number of transformants (5-15/ml) in comparison to the number of transformants made with unmodified plasmids (1.1×10^2/ml). This contrasts with the results in yeast (*Saccharomyces cerevisiae*) where the inclusion of homologous DNA in a transformation vector increased the rate of transformation of a single gene (Orr-Weaver *et al.* 1981). Herrera-Estrella *et al.* (1990) found a similar range in transformation frequency of *Trichoderma* species with and without homologous DNA.

The reported frequency of co-transformation of selectable markers with the *gus*A gene has ranged from 25-92 % (de Ruiter-Jacobs *et al.* 1989, Bunkers 1991). The co-transformation frequency of *hph* and *gus*A in modified plasmids in this study was high by these standards (98 %). For transformants made with modified plasmids the survival rate was strongly linked to the clone combinations used. In contrast, Judelson (1993) found that the presence of homologous DNA did not raise the co-transformation rate of *gus*A and *npt* or *hpt* in *Phytophthora infestans*. Co-transformation frequencies of 50-85 % have been reported for *T. reesei* (Penttila *et al.* 1987, Harkki *et al.* 1989), *T. harzianum* (Thrane *et al.* 1995) and *T. viride* (Herrera-Estrella *et al.* 1990,

Goldman *et al.* 1991).

Polyethylene glycol is known to stimulate protoplast fusion (Murray *et al.* 1992) so it is possible some of the transformants may have arisen as heterokaryons. *Trichoderma* species are reported to have one to 12 nuclei per hyphal cell (Sivan *et al.* 1990). However during conidial formation only one nucleus is deposited per conidium. Once in a conidium a nucleus may replicate itself by mitosis. Using conidia to make protoplasts meant that each conidium was homokaryotic, however if some conidia were multinucleate the plasmids may have integrated into one nucleus, different nuclei or more than one nucleus. For this reason a single spore was isolated from each transformant grown on MEA in the second transformation series.

The *gus*A gene appeared to be stably integrated up to the fifth generation of transformants made with unmodified plasmids and maintained on the selective medium. However, the *gus*A gene was not detected in the tenth generation by genomic Southern analysis or histochemical assays. These results were not consistent with those from PCR analysis. There may be several explanations why transformants were GUS-positive when amplified by PCR but were GUS-negative when assessed histochemically and by direct hybridisation. It is possible that the *gus*A gene is present, but not transcribed or that very low levels of free plasmid are present. Southern detection after PCR amplification was the most sensitive method used to detect the *gus*A gene both in this case and in the first generation of transformants made with modified plasmids (section 3.3.5.2). Hybridisation to amplifiates were not seen in control reaction indicating this was a genuine result.

Unlike transformants made from unmodified plasmids, those made with

modified plasmids probably contained the *gus*A gene integrated at single sites more frequently than multiple sites. The *gus*A gene has been reported to integrate at a single site in *Paxillus involutus* (Bills *et al.* 1995) and multiple sites in *Bipolaris sorokiniana, Pseudocercosporella herpotrichoides* and *T. harzianum* (Bunkers 1991, Liljeroth *et al.* 1993, Thrane *et al.* 1995). Results from histochemical tests, PCR and Southern analyses indicated that when the *gus*A gene was carried in modified plasmids it remained stable after ten generations. Differing intensity of the hybridising bands may indicate that the genomic DNA preparation may contain something which inhibits amplification. Alternatively the varying intensity of the hybridising bands may reflect unequal amounts of DNA used for each sample or the number of copies of pNOM102/*gus*A in the transformants. The number of copies of the *gus*A gene may (Bunkers 1991) or may not (Roberts *et al.* 1989) influence expression of the gene and the site of integration may also be important (Couteaudier *et al.* 1993). At this stage there is no evidence to distinguish between these possibilities in *T. koningii*.

Nine of the ten isolates from the fifth generation made with modified plasmids maintained on PDA contained at least one copy of the *hph* gene. A high proportion of these isolates appear to have the gene integrated in similar sites.

The nutritional composition of the four media tested did not influence survival of transformants or their resistance to hygromycin or ability to express the *gus*A gene. Conidiation on MEA also had no influence on expression of the gene. Conidiation did not influence expression of the *gus*A gene in *Fusarium oxysporum*, *B. sorokiniana*, *P. herpotrichoides* or *Cladosporium fulvum* (Oliver *et al.* 1987, Bunkers 1991, Couteaudier *et al.* 1993, Liljeroth *et al.* 1993,

Wubben *et al.* 1994). So, in the second series hyphal transfer on PDA and PDA+H was performed for all ten generations.

Transfer of transformants to PDA containing hygromycin (PDA+H) often resulted in death. Survival on PDA+H was poor in comparison to PDA, with only 4 % survival of isolates made with modified plasmids after ten transfers. Scoring growth on PDA+H was somewhat subjective. In these experiments any visible hyphal growth was considered positive. Transformants that initially express resistance to hygromycin, but then fail to grow when replated on the selective medium have been reported by other workers (Oliver *et al.* 1987, Cooley *et al.* 1988, Bunkers 1991, Murray *et al.* 1992, Monke & Schafer 1993). As hygromycin resistance is a dominant allele, expression of this gene would be consistently expected unless the gene is cut out of the fungal genome. Although they survived poorly on PDA+H, all of the transformants made with unmodified plasmids that survived five generations were able to cleave GUS substrates. However, this was not the case after the tenth generation, or for transformants made with modified plasmids. It was not clear why transformation was unstable in *T. koningii*. When *Paxillus involutus* (transformed with *gus*A and *hph* by particle bombardment) was transferred ten times on modified Melin-Norkran's nutrient medium containing 50 µg/ml hygromycin over six months it remained genetically stable (Bills *et al.* 1995). Similarly, transformants of *B. sorokiniana* retained their stability and expression of the *gus*A gene best on a hygromycin selective medium (Liljeroth *et al.* 1993).

Improved stability of transformants generated with vectors containing homologous DNA with the host has been reported (Tilburn *et al.* 1983, Herrera Estrella *et al.* 1990) and was observed *in vitro* in this study. Transformants

made with modified plasmids were more stable on non-selective media. At least 80 % of isolates cultivated on media not containing hygromycin retained their ability to grow on the selective medium and cleave GUS substrates. Similarly a single isolate of *T. harzianum* cultivated for eight months on media without hygromycin showed no reversion to wild-type and remained able to express *gus*A (Thrane *et al*, 1995). An approach which may improve stability is transformation of *gus*A with a selective marker located in the same plasmid (Judelson & Michelmore 1991, Richard *et al.* 1992, Li *et al.* 1993, Tanpo *et al.* 1994). Although this approach has generally been reported to increase stability it is not always improved (Liljeroth *et al.* 1993). Furthermore there is some evidence for more stable co-transformation by linearising the plasmids before transformation. Most work with the *gus*A gene has used circular plasmids, however improved transformation frequencies and stability have been reported with linear plasmids (Murray *et al.* 1992, Judelson 1993).

Transformants made in the second transformation series with modified plasmids survived ten generations, remained histochemically active and appeared to have the *gus*A and *hph* genes integrated. In addition their ability to inhibit growth of Ggt on CYA was retained. They therefore appear to have potential for tracking but the stability of these transformants in soil and wheat roots needed to be determined and work on this aspect is described in chapter 4.

CHAPTER 4. TESTING STABILITY OF THE TRANSFORMANTS

4.1 INTRODUCTION

The stability of the *gus*A gene in *T. koningii* in soil and roots was assessed before the transformed isolates of *T. koningii* carrying the *gus*A and *hph* genes could be used for environmental studies. Instability *in vitro* was observed in transformants made with unmodified plasmids carrying the *gus*A and *hph* genes, but when the plasmids were modified with a 2 kb segment of DNA from *T. koningii* the transformants remained stable on agar. Loss of activity by the *gus*A gene when *T. koningii* is added to soil would make interpretation of results impossible. Although the *gus*A gene has been successfully transformed into a number of fungi data on GUS expression following reisolation from roots and soil has not been published.

Most studies have focussed on tracking a fungus transformed with the *gus*A gene on media, artificial growth systems or excised leaves or fruits rather than tracking a fungus transformed with the *gus*A gene in soil (Murray *et al.* 1992, Oliver *et al.* 1993, Ashfield *et al.* 1994). In non-sterile soil the possible occurrence of organisms containing the *gus*A gene which are naturally present needs to be taken into account.

Root colonisation by *Fusarium oxysporum* and growth in soil and on roots by *Trichoderma harzianum* carrying the *gus*A gene have been studied in steamed or autoclaved soil (Eparvier & Alabouvette 1994, Green & Funck Jensen 1995). The transformed isolates of both fungi acted similarly in soil to the wild types when distinguished using the *gus*A gene. Alteration in the

behaviour of *T. koningii in vitro* and in soil as a result of transformation with the *gus*A gene also needed to be assessed.

The aim of the work described in this chapter was to determine if the transformed isolates of *T. koningii* were stable in soil and wheat roots and if transformants colonised wheat roots in the same way as wild-type *T. koningii*.

4.2 METHODS

The stability of the transformants, generated with modified plasmids, expressing the *gus*A gene was tested by placing the transformants on PDA (potato dextrose agar, Difco) and in sterile soil containing pregerminated wheat seeds. GUS expression was assessed in the wheat roots directly and in isolates recovered from the soil surface and wheat roots. The influence of fungal nutrition on expression of the *gus*A gene was examined on agar media and in soil.

4.2.1 Fungi

Transformants made with modified plasmids (section 3.2.6) were assessed for stability on media and in sterile soil. All isolates were GUS-positive when tested histochemically at the start of the experiment. Transformed isolates from the fifth rather than the tenth generation were used, in order to avoid problems that can arise from excessive subculturing.

4.2.2 Surface Disinfestation of Wheat Seeds

Wheat seeds (cv. `Spear') were surface disinfested by immersion in 95 % ethanol for 1 min followed by 0.5 % NaOCl for 3 min and two rinses in

sterile distilled water (2 min/rinse). The seeds were germinated on sterile wet paper towels at 25 °C in darkness for 20-24 h.

4.2.3 GUS Expression in Roots Grown *in vitro*

Wheat seedlings were placed on PDA with each of 15 transformants to determine if GUS expression could be detected in young roots grown *in vitro*. Pregerminated surface disinfested wheat seeds (section 4.2.2) were placed on one side of a plate of PDA (Difco) and a needle point of hyphae from a transformant on the opposite side. The plates were incubated for 5 d at 25 °C in darkness. Assays for β-glucuronidase using X-Gluc (5-bromo-4-chloro-3-indolyl β-D-glucuronide) and MUG (4-methylumbelliferyl β-D-glucuronide) as the substrates were then conducted (section 4.2.4) with and without preincubation and using a phosphate, histochemical or extraction buffer. The positive control used in these tests was a piece of GUS-transformed tobacco leaf, while the negative control was spores from AST-1.

4.2.4 X-Gluc and MUG Assays

MUG assays were performed essentially as outlined by Jefferson (1987), and Jefferson *et al.* (1987). Half the samples were cut into 0.5 cm lengths and incubated in Trichoderma Minimal Medium (TMM) (Gruber *et al.* 1990a) for 24 h at 25 °C in darkness prior to the MUG assay. Roots were ground in liquid nitrogen, extraction buffer (50 mM $NaPO_4$ 10 mM B-mercaptoethanol, 10 mM Na_2EDTA, 0.1 % SDS, 0.1 % Triton X) was added and the extract incubated with 1 mM MUG (4-methylumbelliferyl β-D-glucuronide) dissolved in extraction buffer or 0.05 M phosphate buffer pH 7 at 37 °C in darkness. To optimise

fluorescence, 0.9 ml of 0.2 M Na_2CO_3 was added to each 100 µl sample in an Eppendorf tube. These samples were examined for fluorescence by exposure to long wave ultra violet light (366 nm) (Jefferson *et al.* 1987).

X-Gluc assays were conducted on root segments (0.5 cm long) placed in ELISA trays, using the method of Couteaudier *et al.* (1993). Half the samples were incubated in TMM for 24 h at 37 °C prior to the addition of X-Gluc (5-bromo-4-chloro-3-indolyl β-D-glucuronide). Two buffers were tested - 0.05 M phosphate buffer pH 7 (Jefferson 1987) and a histochemical buffer (0.05 M $NaPO_4$, 0.5 mM potassium ferrocyanide, 0.5 mM potassium ferric cyanide, 10 mM EDTA) (Stummer 1993). Both contained 2 mM X-Gluc and were vacuum infiltrated for 2 min. The tray was sealed with plastic wrap prior to incubation at 37 °C in darkness for up to 7 d.

4.2.5 Adding Fungi to Soil

Kapunda soil (140 g) in a polycarbonate jar (500 ml) was autoclaved (121 °C, 20 min) on 3 consecutive days. Water (13 % w/w) was added to the base layer of soil (140 g) and each jar shaken to ensure even distribution. Four agar plugs of a transformant were added and the orientation of the jar noted. All isolates were grown on PDA for 3 d in darkness. Agar plugs (10 mm diam) were taken from the actively growing edge of each colony. The untransformed isolate of *T. koningii* AST-1 was included to determine if transformation influenced root and rhizoplane colonisation. Sterilised soil (30 g) overlayed the agar plugs and water was added (13 % w/w). Surface disinfested seeds (section 4.2.2) were then placed over the agar plugs and another 30 g sterilised soil and water (13 % w/w) added. Soil adhering to sides of the jars was

removed. The sealed jars were placed in a temperature controlled tank at 15 ^{o}C for 7 d. The soil surface was level with the wooden supports ensuring the soil and roots were in darkness. Each transformant was tested in two replicate jars. Jars with uninoculated PDA plugs served as negative controls.

4.2.6 Detecting GUS Activity in Roots Grown in Soil

To detect GUS expression in roots of wheat plants grown in soil 17 transformants were added to soil. In the first experiment two plants per jar were removed and tested directly for activity in the X-Gluc assay (section 4.2.4) and the other two plants were tested for MUG activity (section 4.2.4). As the highest level of root infection was previously found close to the seed (section 2.3.3) only the top 2.5 cm of seminal roots were tested. In the enzyme assays, controls of untransformed *T. koningii* (GUS-negative) and *E. coli* (JM109 GUS-positive) were included. Chloramphenicol (100 µg/ml) was included in both TMM and the buffer carrying MUG (K. Wilson, personal communication) to reduce background GUS activity due to bacterial contamination. As the optimum time for fluorescence was unknown for GUS in this system, fluorescence was observed after 15 min, 30 min, 1, 5, 20, 24, 28, 44, 48, 52, 68, 72, and 78 h.

PCR amplification was used to detect the *gus*A gene in root pieces from three jars inoculated with transformants as well as one jar inoculated with *T. koningii* isolate AST-1 and one jar inoculated with blank agar plugs. A positive control of hyphae from a transformed isolate of *T. koningii* grown on PDA was included. The root pieces and hyphal control were boiled in phosphate buffer for 20 min, then plunged into ice. A dilution series of the extract was made (undiluted, 10^{-1}, 10^{-2}, 10^{-3}) and used as the PCR template.

Each 25 µl PCR reaction included 1 µl root extract or diluted root extract, 2.5 mM $MgCl_2$, 0.2 mM deoxyribonucleoside triphosphates, 32 pM primers (Van Wordragen *et al.* 1991) and 2U *Taq* polymerase (Promega, Australia). An initial amplification of 40 cycles was performed (1 min 94 °C, 2 min 55 °C, 2 min 72 °C). One, 2 or 4 µl of the PCR product was amplified in another 40 cycles. Products from both PCR runs were electrophoresed for 2.5 h (50 V) in a 2 % agarose gel with Tris borate EDTA buffer. The gel was stained in 2 µg/ml ethidium bromide for 1 h.

4.2.7 Detecting GUS Activity and the *gus*A Gene in Isolates Recovered from Roots and Soil

17 transformants on agar plugs were added to sterile soil in which wheat seedlings were grown for 7 days as described previously (section 4.2.5). *Trichoderma* was reisolated from roots and soil, the isolates were then assessed for GUS expression. *Trichoderma* was reisolated from five places on the soil surface of each jar onto PDA with hygromycin 100 µg/ml (PDA+H). These plates were incubated at 25 °C for 8 d. *Trichoderma* isolates were also recovered from roots grown in this soil. Seminal roots were cut into 2.5 cm segments and plated on STSM (section 2.2.2). Half of the roots were surface disinfested (0.5 % NaOCl, 1µl/ml Tween 80, for 2 min) before plating. After incubation at 25 °C in darkness for 4 d roots were scored for colonisation by *Trichoderma*. A X^2 test of association with one degree of freedom was conducted on the total number of roots to compare the level of root infection by each transformant with the nontransformed isolate. Each *Trichoderma* colony recovered from root pieces was subcultured onto PDA+H. These plates were

incubated at 25 °C in darkness for 8 d. Two replicate colonies from both the soil and roots from each jar were tested for GUS activity using both the X-Gluc and MUG assays (Couteaudier *et al.* 1993), after incubation in TMM . Both GUS substrates were dissolved in 0.05 M phosphate buffer pH7.

Isolates from soil and roots were analysed by PCR to determine if the *gus*A gene had been retained. Three isolates not expressing the *gus*A gene recovered from roots and soil and three isolates expressing the *gus*A gene from soil were tested. Two isolates expressing the *gus*A gene from the second transformation series were included as positive controls. The method used was described in section 3.2.7.2. The PCR gel was also examined by Southern analysis and the *gus*A gene detected with a ^{32}P labelled *gus*A probe.

Methylation has been proposed as a means by which the *gus*A gene may be present but not expressed (Judelson & Whittaker 1995). Methylated genes are inactivated leading to poor expression of gene products. The methylation status can be examined by testing with methylation-sensitive restriction enzymes. Digestion of the *gus*A gene with these methylation sensitive enzymes would indicate that methylation has not occurred. Isolates from roots and soil which contained the *gus*A gene (confirmed by PCR amplification followed by Southern hybridisation) but which were histochemically GUS-positive or GUS-negative were grown in culture. In addition two GUS-positive (histochemically) isolates from the second transformation series were included. Genomic DNA was extracted and digested with *Msp*I and *Hpa*II (Boehringer Mannheim, Australia) according to the manufacturer's instructions and run into a 2 % TBE gel. After Southern blotting a ^{32}P labelled *gus*A probe was used to detect the *gus*A gene (section 3.2.7.2). The *gus*A gene used as

a probe was excised from pNOM102 by digestion with *Nco*I.

4.2.8 The Influence of Nutrition on GUS Expression

In order to determine if nutrient status of the fungus influenced stability of the *gus*A gene the transformants were grown on a range of media. Each of the 20 transformed isolates were inoculated onto PDA (Difco), water agar (WA, Difco), Czapek yeast extract agar (CYA) (Klich & Pitt 1988) and malt extract agar (MEA) (Klich & Pitt 1988). The MEA plates were incubated at room temperature for 7 d in the presence of diffuse natural light to allow conidiation. Cultures on other media were incubated at 25 °C in darkness for 5 d. A needlepoint of hyphae/conidia was then incubated on TMM and assessed for GUS expression by the addition of X-Gluc and MUG in 0.05 M phosphate buffer pH7 (Couteaudier *et al.* 1993).

An experiment was conducted to examine GUS expression in relation to the nutrient status of soil. Three transformants were added to sterile soil (as outlined in section 4.2.5) to which water or Hoagland's solution (Hoagland & Arnon 1950) and 30 g/L sucrose (13 % w/w) was added. In this experiment isolations were made from the soil surface of each jar and plants were plated with and without surface disinfestation as previously outlined (section 4.2.7). Each colony of *Trichoderma* was assessed for growth on PDA+H. X-Gluc and MUG assays were conducted on two replicate colonies from the soil and roots of each jar (section 4.2.7). All samples were preincubated in TMM and the GUS substrates were dissolved in 0.05 M phosphate-buffer.

4.3 RESULTS

4.3.1 GUS Expression in Roots Grown *in vitro*

Preincubation in TMM was essential when examining GUS expression to assess whether X-Gluc had been cleaved (Table 4.1). The histochemical and phosphate-based buffers were both effective for GUS expression. In contrast when undertaking MUG assays the buffer in which the MUG was dissolved was the critical factor, but preincubation in TMM was not necessary. Only the phosphate-based buffer gave positive results (Table 4.1). The pieces of GUS-transformed tobacco leaf went blue or fluoresced under all conditions.

Table 4.1 GUS expression determined by X-Gluc and MUG assays in wheat roots grown on PDA with *gus*A transformed isolates of *T. koningii*, showing the effects of preincubation in TMM and buffer composition (section 4.2.3).

Preincubation:	Buffer in which the GUS substrate was dissolved	Number of transformants expressing *gus*A
X-Gluc		
none	PO_4	0/15
TMM	PO_4	15/15
none	histochemical	0/15
TMM	histochemical	12/15
MUG		
none	PO_4	11/15
TMM	PO_4	13/15
none	extraction	0/15
TMM	extraction	0/15

4.3.2 GUS Expression in Roots

Almost every piece of root yielded a colony of *Trichoderma* when root pieces were plated directly onto STSM regardless of whether roots were grown in soil with a transformed or untransformed isolate (Table 4.2). No colonies of *Trichoderma* were recovered from roots inoculated with blank agar plugs. The mean level of infection of surface disinfested roots was not significantly different between treatments to which transformed or an untransformed isolate was added. The expected decrease in colonisation down the root was not seen in plants inoculated with the untransformed isolate. This may be because the data is based on four plants (2 jars) in contrast to the data for the transformants which is based on sixty eight plants (34 jars).

Root segments incubated in TMM supported denser hyphal growth on their surface than roots not incubated in TMM. However no fluorescence indicating cleavage of MUG was seen in any root extract. The positive control (JM109) fluoresced strongly. No blue hyphae were observed in or on any root segment when stained with X-Gluc. When examined microscopically hyphae were seen on the root surface and in the outer two to three cortical layers as well as root hairs and lateral roots. Sporulating conidiophores typical of *Trichoderma* were also frequently seen on the root surface. The positive controls without roots were consistently blue and the negative controls remained colourless. PCR amplification failed to detect the *gus*A gene in any of the root segments tested. However the *gus*A gene was detected when hyphae from colonies expressing the *gus*A gene in histochemical tests were ground and treated in the same way.

4.3.3 GUS Expression in Isolates from Soil and Roots

At harvest *Trichoderma* was visibly growing on the surface of the soil and in many cases was also sporulating prolifically. Each of the transformed isolates was expressed the *gus*A gene and grew on PDA+H before being added to soil. When reisolated from the soil surface 62 % of the isolates grew on PDA+H (Table 4.3). However only 15 % of these isolates were GUS-positive on the basis of X-Gluc and MUG assays. A trend similar to that observed for the isolates from soil was seen for isolates recovered from the rhizoplane. When isolates of *T. koningii* transformed with *gus*A were added to soil 54 % of *Trichoderma* isolates from the root surface were resistant to hygromycin however only 26 % of these isolates were able to cleave GUS substrates. In contrast only 30 % of the isolates recovered from surface disinfested roots were hygromycin resistant, but 67 % of these isolates expressed the *gus*A gene. When this experiment was repeated with another five transformants which expressed the *gus*A gene and had been made with modified plasmids similar results were obtained.

PCR amplification followed by Southern analysis detected the *gus*A gene in the histochemically GUS-positive isolates recovered from soil, two of the three histochemically GUS-negative isolates recovered from soil and each of the histochemically GUS-negative isolates recovered from roots except AST-1 (Figure 4.1).

When cytosine methylation of transformed isolates and an untransformed isolate was assessed there were no apparent changes in the hybridisation profile (Figure 4.2). This indicates that methylation did not play a role in lack of expression of the *gus*A gene before the isolates were added to

soil.

Table 4.2 Colonisation of roots and the rhizoplane at different distances from the seed by transformed and an untransformed isolate of *T. koningii*, with or without surface disinfestation of the root segments (section 4.2.7).

Distance from seed (cm)	Roots from which *Trichoderma* was isolated (%)					
	Inoculum:					
	blank[a]		untransformed[a]		transformed[b]	
	surface disinfestation:					
	+	-	+	-	+	-
0-2	0	0	11	100	34	100
2-4	0	0	14	100	6	99
4-6	-	0	20	100	3	98
mean	0	0	14	100	16	99

[a] mean of 4 plants (2 jars)

[b] mean of 68 plants (34 jars)

Table 4.3 The ability of transformed isolates reisolated from the soil surface, and wheat roots to grow on PDA+H and cleave GUS substrates. The isolates were added to sterile soil in which wheat plants were grown (section 4.2.7).

Isolates from	% Transformed isolates growing on PDA+H	% Transformed isolates expressing GUS
surface disinfested roots	30	67
non surface disinfested roots	54	26
soil	62	15

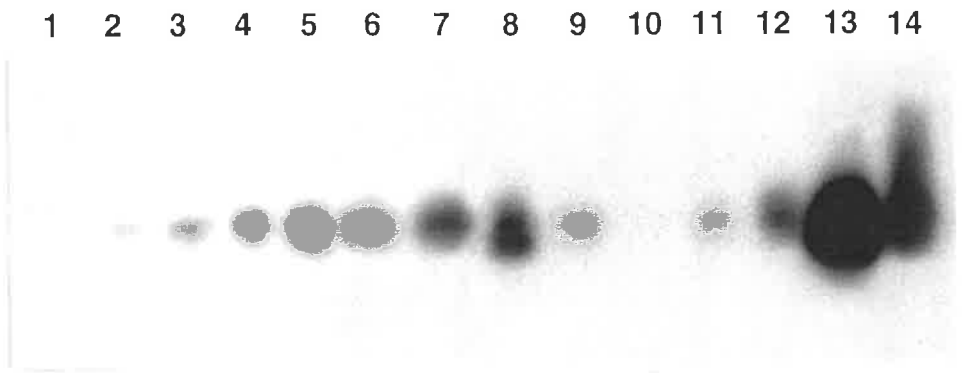


Figure 4.1 Southern blot hybridisation of DNA from isolates of *T. koningii* transformed with *gus*A and *hph*. The transformants were added to sterile soil and recovered from the soil surface and wheat roots. DNA samples were amplified by PCR, Southern blotted and hybridised with the β-glucuronidase (*gus*A) excised from pNOM102 by digestion with *Nco*I and labelled with ^{32}P. Key: Lane 1: *T. koningii* AST-1 (untransformed), lanes 2-4: fifth generation transformants of *T. koningii* that were histochemically GUS-positive, lanes 5-7: histochemically GUS-positive isolates recovered from soil, lanes 8-10: histochemically GUS-negative isolates recovered from soil, lanes 11-13: histochemically GUS-negative isolates recovered from wheat roots, lane 14: pNOM102.

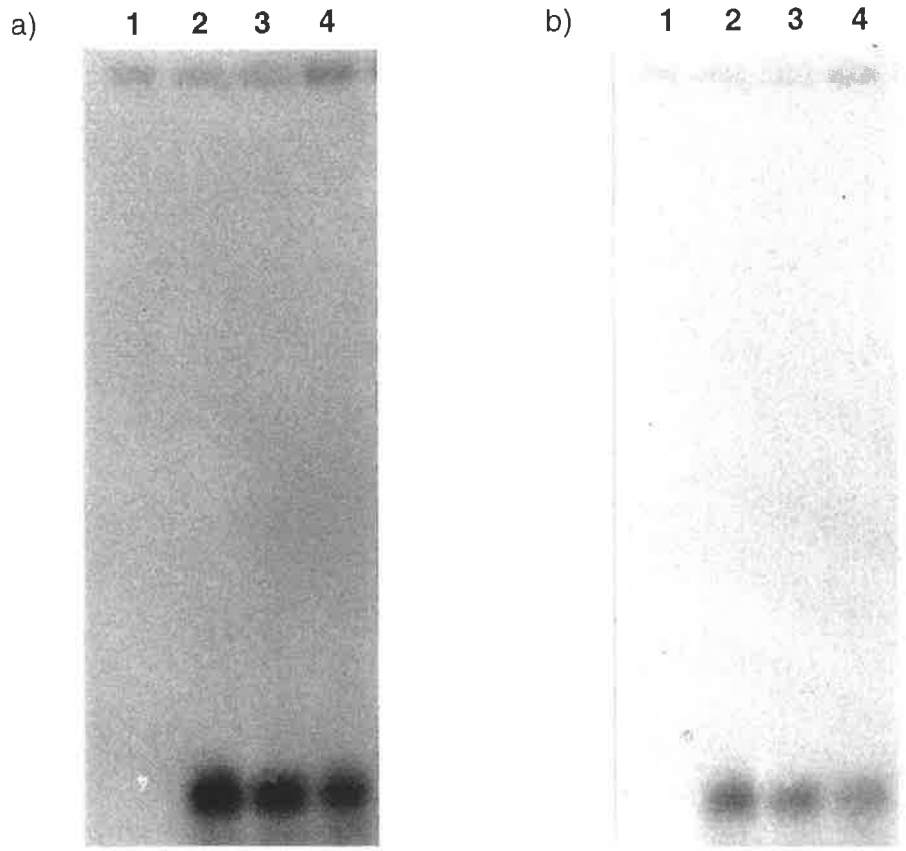


Figure 4.2 Southern blot hybridisation of genomic DNA from isolates of *T. koningii* transformed with *gus*A and *hph*. Each DNA sample was digested with *Msp*I (a) and *Hpa*II (b), Southern blotted and hybridised with the β-glucuronidase (*gus*A) gene excised from pNOM102 by digestion with *Nco*I and labelled with ^{32}P. Positive hybridisation after digestion with both methylation enzymes indicated methylation of the *gus*A gene had not occurred. Key: lane 1: *T. koningii* AST-1 (untransformed), lanes 2-3: transformants of *T. koningii*, lane 4: pNOM102.

4.3.4 The Influence of Nutrients

The medium on which transformants were grown influenced the expression of the *gus*A gene (Table 4.4). Cleavage of X-Gluc was greatest when fungi were grown on PDA or CYA. In contrast, when fungi were grown on WA only 2/20 isolates cleaved X-Gluc after 6 d and a similar trend was observed when MUG was used as the substrate (Table 4.4). After 3 d incubation with MUG all of the wells containing transformed hyphae grown on PDA or CYA were fluorescing. Approximately twice as many isolates grown on MEA cleaved MUG than X-Gluc. Culturing on WA produced only one isolate that fluoresced when incubated with MUG.

Table 4.4 Proportion of transformants (out of 20) expressing the *gus*A gene at different times of incubation on four different media (section 4.2.8).

Medium	Number of GUS-positive transformants (/20)					
	Time (d)					
	2	4	6	1	2	3
	X-Gluc			MUG		
Potato dextrose agar	6	8	8	3	15	20
Czapek yeast extract agar	2	7	8	4	16	20
Malt extract agar	0	1	6	1	11	15
Water agar	0	0	2	0	1	1

Addition of nutrient solution to soil did not impair rhizoplane colonisation by *T. koningii* (Table 4.5). Internal root infection was significantly increased by the addition of plant nutrient solution ($P<0.05$) as assessed by the sign test (Conover 1971)). The level of internal root colonisation was increased from 44

to 72 % in the presence of Hoagland's solution and sucrose. When Hoagland's solution with sucrose was added to soil 76 % of the transformed isolates which were recovered from surface disinfested roots grew on media containing hygromycin (Table 4.6). Only half of these isolates could cleave GUS substrates. In contrast only 27 % of isolates recovered from inside roots grown in unamended soil could grow on PDA+H. However 83 % of these isolates expressed the *gus*A gene in histochemical tests. Addition of nutrient solution to soil made little difference to the isolates recovered from soil, either in their ability to grow on PDA+H or to cleave GUS substrates (Table 4.6).

Table 4.5 The influence of nutrient solution in soil on the colonisation of wheat roots and the rhizoplane by transformed and an untransformed isolate of *T. koningii*, with or without surface disinfestation of the root segments (section 4.2.8).

Liquid added to soil	Percentage of root segments (2 cm long) from which *Trichoderma* was isolated					
	Inoculum:					
	blank		untransformed		transformed	
	surface disinfestation:					
	+	-	+	-	+	-
water	0	0	14	100	8	100
Hoagland's solution + sucrose	0	0	87	100	52	100

Table 4.6 The ability of transformed isolates to grow on PDA+H and cleave GUS substrates after reisolation from wheat roots and soil. Water was added to the soil in which the wheat plants were grown in half the treatments and Hoagland's solution with sucrose to the other half (section 4.2.8).

Isolates from	% Isolates growing on PDA+H	% Isolates expressing GUS
Water		
surface disinfested roots	27	83
non-surface disinfested roots	74	29
soil	87	12
Hoagland's Solution + Sucrose		
surface disinfested roots	76	54
non-surface disinfested roots	100	17
soil	70	17

4.4 DISCUSSION

Stability of transformed isolates of *T. koningii* carrying the *gus*A gene was examined in order to establish whether they remained stable in roots and soil. When transformants were added to soil, colonisation of wheat roots could not be detected when assayed by cleavage of the GUS substrates X-Gluc and MUG or by PCR. However when roots and soil were plated (on STSM and PDA+H respectively) some of the isolates recovered were found to be GUS-positive by histochemical tests and by hybridisation to the *gus*A gene. Hence, in at least some transformants reisolated from soil and roots the *gus*A gene was present but inactive.

There are a number of possible explanations why *gus*A may be inactive when the fungus is in the soil environment. Nutrition may influence gene expression. When transformants of *T. koningii* were added to soil, roots of wheat grown in the soil showed no GUS expression. In contrast, GUS expression was found in wheat roots which had been grown on a nutrient-rich medium (PDA) and extensively colonised by transformants. A higher proportion of isolates expressed the *gus*A gene when recovered from inside roots than when isolated from the rhizoplane. When tested histochemically. the lowest proportion of GUS-positive isolates came from the soil surface, indicating that GUS expression was greatest in the environment with the highest total nutrient concentration. This interpretation was supported by tests in which hyphae grown on nutrient-rich media expressed the *gus*A gene in histochemical tests, unlike those cultured on nutrient-poor media. Nutrient-rich media were densely covered with hyphae in contrast with nutrient-poor media where the hyphal density was sparse. Needlepoints of hyphae from nutrient-rich and

nutrient-poor media were unequal, so the GUS results may have been a consequence of differences in hyphal density rather than nutrient status. When inorganic nutrients and a carbon substrate were added to soil root colonisation increased, but GUS expression was still higher in transformants recovered from roots than those from soil. Although the nutritional status of the soil increased, the nutritional status inside wheat roots may have remained constant.

Methylation has been proposed as a mechanism by which the *gus*A gene may be present but not functional (Goyon & Faugeron 1989, Scheid *et al.* 1991, Freedham & Pukkila 1993, Selker *et al.* 1993). According to Judelson & Whittaker (1995) transformed sequences are particularly prone to inactivation when multiple copies of DNA integrate into chromosomes, as seen in some of the transformed isolates of *T. koningii*. There was no evidence of methylation in the transformants before they were added to soil. However, methylation may have occurred once the isolates were introduced to soil which is low in both organic and inorganic nutrients. Similarly neither methylation nor deletion could explain the silencing of the *gus*A gene over time in *Phytophthora infestans* (Judelson & Whittaker 1995). *P. infestans* was maintained on rye agar and subcultured monthly. After five transfers the *gus*A gene was expressed in 76 to 87 % of the 250 of strains of *P. infestans* tested. Twenty eight months later only 50 to 64 % of the 47 strains still in culture expressed the *gus*A gene. When assessed by Southern analysis the *gus*A gene was present in the histochemically GUS-negative strains. Although expression of the *gus*A gene declined growth and sporulation by the transformants was not impaired.

Rhizoplane or root colonisation by *T. koningii* was not impeded by the presence of the *gus*A gene, but *gus*A activity was not stable. These results are

in contrast to those found when *Fusarium oxysporum* and *T. harzianum* transformed with *gus*A were added to soil. Colonisation by both fungi was not affected and they could be quantified and/or accurately located by the use of GUS substrates (Eparvier & Alabouvette 1994, Green & Funck Jensen 1995). There is one report in the literature showing a fungus, *Arthrobotrys oligospora*, carrying the *gus*A gene that could not be tracked in soil (Persmark *et al.* 1995). In this case the background GUS activity was high and the lowest detectable limit of the fungus was 10^5 spores or 3 mg hyphae per gram of soil when assessed spectrofluorometrically.

As expression of the *gus*A gene could not be detected in wheat roots grown in sterile soil the transformed isolates cannot be used for identifying or measuring the active biomass of *T. koningii*. Further work is required to determine how or why the *gus*A gene is dropped or inactivated.

CHAPTER 5. GENERAL DISCUSSION AND CONCLUSIONS

The aim of this research was to track *Trichoderma koningii* in soil, the rhizoplane and wheat roots. The extent of rhizoplane and root colonisation by *T. koningii* was assessed to determine whether these factors play a significant role in the control of take-all disease caused by *Gaeumannomyces graminis* var. *tritici* (Ggt).

Trichoderma species can be differentiated from other soil fungi using a selective medium (chapter 2). Plating methods have limitations in indicating the abundance of a fungus in a soil or plant sample. When a piece of plant or soil sample is plated on a selective medium a colony may develop from a hypha, single conidium, chlamydospore, clump of conidia or a mass of mycelium. These structures may have been metabolically active or inactive, and do not reflect the biomass of the fungus that was functioning in the soil sample or a piece of plant. A means of tracking is required that indicates the location and biomass of active cells of *T. koningii*. This could be used to gain a better understanding of the ecology of *T. koningii*.

This study helped unravel some of the problems associated with devising a selective medium for *T. koningii*. Captan is no longer readily available and some isolates of *T. koningii* are sensitive to PCNB. The best media currently available for isolating *T. koningii* are Modified Trichoderma Selective Medium (Smith *et al.* 1990) without Captan and a medium developed in this study (STSM). Both media could be further improved to reduce the growth of *Penicillium* species, colonies of which initially may be mistaken as *Trichoderma* (section 2.3.1).

Most members of the genus *Trichoderma* colonise fruit and leaf surfaces. In contrast *T. koningii* commonly inhabits acidic soils. Previous studies indicated that species of *Trichoderma* in their wild state are not competent inhabitants of the rhizosphere (Papavizas 1981). The isolate of *T. koningii* used in this study (AST-1) was selected as it reduced growth of Ggt in a soil-sandwich bioassay (Simon & Sivasithamparam 1988c).

One aim of this study was to determine if *T. koningii* colonised the rhizoplane and roots of wheat plants (chapter 2). Both indigenous species of *Trichoderma* and *T. koningii* colonised the rhizoplane in soil tests. The untransformed isolate of *T. koningii* (AST-1) and transformed derivatives of this isolate could also be isolated from within wheat roots, unlike the native population of *Trichoderma* from Kapunda soil. When surface disinfested roots were plated *T. koningii* was found in up to 30 % of the seminal roots of wheat. This may be a critical factor in the success of AST-1 in biocontrol.

Recovery from roots with and without surface disinfestation is used to differentiate cortical and stellar colonisation, however this distinction is somewhat arbitrary and results depend on the concentration of chlorine, time of application and the age and thickness of the root. As the method used influences the assessment of the degree of root colonisation, an alternative approach such as use of a gene to track specific isolates is required. The extent of root and rhizoplane colonisation by the native population of *Trichoderma*, *T. koningii* AST-1 and the transformed isolates was higher than that recorded in the literature (section 2.3.3). This may be related to isolate of *T. koningii*, the soil type or the large number of plants sampled in this study.

Field trials, in which wheat was grown in cylinders to contain each

treatment, have confirmed that *T. koningii* is effective in reducing take-all disease caused by Ggt (Ryder, Stone & Simon 1992). However disease control was inconsistent over the four years of trials (Rovira *et al.* 1992). *T. koningii* is reported to reduce the growth of other fungi by antibiotic and volatile production and mycoparasitism (section 1.2.3). Alternatively, with its rapid rate of growth, *T. koningii* may outcompete other fungi for nutrients and space.

The site of biocontrol activity by *T. koningii* against Ggt was examined in this study (chapter 2). Although *T. koningii* colonises wheat roots, its primary action against Ggt appears to occur in the bulk soil. This result supports the earlier work of Simon (1989) who observed suppression of take-all disease by the action of *T. koningii* against Ggt in its saprotrophic phase. Data from experiments in this study show that the antagonist is most effective against regenerating hyphae and growth of new hyphae, rather than an established mycelial network of Ggt (section 2.3.3).

Further work is required to determine why root and rhizoplane colonisation by *T. koningii* could not be related to reduction of take-all disease. A higher proportion of wheat root colonisation by *T. koningii* was found towards the crown than the root tip (section 2.3.3) which is similar to the pattern of root colonisation by Ggt. Competition in the root may be insignificant in comparison to competition in bulk soil. Unlike Ggt, *T. koningii* may not grow into the root beyond the endodermis. Thus the two fungi may have colonised different parts of the wheat roots. If *T. koningii* does colonise the stele Ggt may simply have a more rapid mode of entry. The action of *T. koningii* in the cortex of wheat roots needs to be clarified. The effectiveness of *T. koningii* in bulk soil implies that if it is to be used commercially it needs to be applied on a broad scale at

the end of summer before rain stimulates the growth of Ggt. Formulation of the biocontrol agent will be a critical factor determining the success of this option. It may be practical to apply *T. koningii* to soil at the end of summer and it could be combined with fertiliser application.

The *gus*A gene appeared to be suitable as a marker to track *T. koningii* at the time this work was begun. The gene had been transformed into other fungi and used to quantify their presence in excised or artificially grown plant material (Yourman *et al.* 1992, Liljeroth *et al.* 1993, Brown *et al.* 1995). It was also used to study fungal infection of plants in detail (Murray *et al.* 1992, Monke & Schafer 1993, Oliver *et al.* 1993). At the same time, Green & Funck Jensen (1995) were able to transform the *gus*A gene into *T. harzianum* and quantify expression of the gene in steamed sphagnum peat to show that the population of the transformed isolate was at a similar level to that of the wild- type. These authors were also able to use the *gus*A gene to locate *T. harzianum* on roots. Wound colonisation on cucumber (*Cucumis sativus*) roots was identified as being a typical location of the marked fungus when assessed by the β-glucuronidase assay (Green & Funck Jensen 1995).

When the *gus*A gene in unmodified plasmids was inserted into *T. koningii* it was present in multiple sites. However expression of the *gus*A gene in the transformants was not maintained, irrespective of the composition of the media on which they were cultured (section 3.3.5.1). When a 2 kb piece of DNA from *T. koningii* was incorporated into the plasmids carrying the *gus*A and *hph* genes there was a high rate of co-transformation. The transformants showed a similar rapid rate of growth, morphology and antibiotic production to the wild-type and were stable on agar. The integrated gene could be identified

using Southern hybridisation and multiple integration sites were rare (section 3.3.5.2).

Stability of a genetically modified microbe is a concern in the practical use of these organisms. The *gus*A gene was stable when the fungus was cultured *in vitro*. However when the transformed fungus was added to soil and wheat roots were assessed using MUG or X-Gluc assays no fluorescence or blue colour could be detected, indicating there was no GUS expression in the roots (section 4.3.2). Root infection was not significantly different from that seen by the untransformed isolate of *T. koningii* (AST-1). Some isolates recovered from the soil, rhizoplane and roots were found to be GUS-positive in histochemical assays (section 4.3.3). The proportion of GUS-positive (histochemically) isolates recovered from roots was greater than that recovered from soil, suggesting a nutritional effect on expression of the *gus*A gene. This suggestion was supported by data showing a higher proportion of transformed isolates grown on nutrient-rich media expressed the *gus*A gene than when grown on nutrient-poor media (section 4.3.4). It was interesting to note that composition of the medium had no influence on stability of the gene, but did effect expression. Transcription of the *gus*A gene may impose a metabolic load on the fungus such that it is not expressed in inhospitable conditions.

The main reason for using the *hph* gene to transform *T. koningii* was to select transformed colonies. Hygromycin resistance could have also been useful as a secondary tracking gene. Initial tests showed that three isolates of *T. koningii* from Newdegate (WA) were unable to grow on PDA with 100 µg/ml hygromycin (section 3.3.1). Similarly the *hph* gene has been used to transform and identify other members of the genus *Trichoderma* (Goldman *et al.* 1990,

Lorito *et al.* 1992, Ulhoa *et al.* 1992). However in soil from Kapunda the *Trichoderma* population had a high level of resistance to this antibiotic and grew easily on media containing 100 µg/ml hygromycin. Further work using the *hph* gene for antibiotic resistance in the genus *Trichoderma* is not advised.

In the course of this study alternative approaches to transforming the *gus*A gene into fungi were published but were not attempted due to time limitations. The restriction enzyme-mediated integrated transformation technique may be a quicker transformation method than that used in this study. This involves digesting the genomic DNA with a restriction enzyme prior to adding the marker gene. It is reported to result in high transformation rates and random integration of single copies of the transformed gene (Sweigard 1996). Integration of the *gus*A and *hph* genes at other sites in the genome of *T. koningii* could be assessed.

The inclusion of DNA from *T. koningii* into the plasmids carrying the marker genes had a stabilising effect on the transformants (chapter 3). A different piece of DNA from *T. koningii* may further improve the stability. This would involve screening which would be worthwhile to achieve a transformant that is stable in soil. Likewise the size of the inserted piece of DNA from *T. koningii* could be changed and might improve stability. A 2 kb segment was selected as it had been reported to overcome the problem of instability of the *hph* gene in *Trichoderma* species (Herrera-Estrella 1990). An alternative promoter could also be tested. The *gpd* promoter is a constitutive promoter which is unlikely to be influenced by nutritional conditions. The *pgk* (3-phosphoglycerate kinase) promoter was linked to *gus*A in *Rhizopus niveus*. Although this promoter comes from a highly-expressed gene, expression of the

*gus*A gene was induced by glucose but not by glycerol indicating the promoter was regulated by the carbon source (Takaya *et al.* 1994). Further, the *hph* gene and *gus*A gene could be transformed in the same plasmid, as opposed to the two plasmids used in these experiments. Having both genes in the same plasmid has been found to be a successful means of introducing the *gus*A gene into other fungi (Tada *et al.* 1991, Richard *et al.* 1992, Hwang & Kolattukudy 1995).

Plasmids were modified with DNA from *T. koningii* to assist integration into *T. koningii* through homologous recombination (Herrera Estrella *et al.*) The *gus*A gene appeared to be maintained whether the transformants were made with pAN7-1 and pNOM102 or these plasmids modified with a 2 kb piece of DNA from *T. koningii*. However, expression of the *gus*A gene was more stable when modified plasmids were used. It is not clear why insertion of sequences of DNA from *T koningii* in pNOM102 and pAN7-1 affected expression of the *gus*A gene in *T. koningii*. The occurrence of gene silencing in plants and other eukaryotes has been reviewed by Kooter *et al.* 1999. The position in which the plasmids integrated when amended with DNA from *T. koningii* may have influenced expression of *gus*A (Kooter *et al.* 1999). Epigenetic inactivation can arise when multiple copies of a gene are present in a genome (Kooter *et al.* 1999). Interactions between homologous nucleic acid sequences may result in silencing in contradiction to the generally accepted view that an increase in gene copy number will elevate expression. The higher frequency of multiple copies of the *gus*A gene in transformants made with pAN7-1 and pNOM102 than when the plasmids were modified with DNA from *T. koningii* may explain the difference in expression of the *gus*A gene. Methylation of cytosine may be another reason for the difference in expression of the *gus*A gene in the two

transformation series. Silencing of the *gus*A gene has been observed in *Phytophthora infestans* (Judelson & Whittaker 1995). Methylation was not detected in isolates transformed with modified plasmids and was not examined in the first transformation series.

A marker gene that could be used to quantify the presence and abundance of a metabolically active fungus has great potential to examine the inconsistency observed when using biocontrol agents. The *gfp* gene may be a useful alternative to *gus*A. The *gfp* gene, cloned from the jellyfish *Aequorea victoria* encodes a green fluorescent protein (Kolberg 1994). This fluorescent protein can be used to monitor gene expression in living organisms, as, unlike other light emitting proteins, it fluoresces when exposed to near UV or blue light without the requirement of exogenous substrates or cofactors (Chalfie *et al.* 1994, Haseloff *et al.* 1996). Seedlings grown in the presence of a fungus tagged with the *gfp* gene could be examined by confocal microscopy and infection monitored over time (Haseloff *et al.* 1996, Spellig *et al.* 1996, Vanden-Wymelenberg *et al.* 1997). Studies on plant infection by viruses tagged with the *gfp* gene have also been conducted (Baulcombe *et al.* 1995, Heinlein *et al.* 1995, Oparka *et al.* 1995). The small size of the *gfp* gene may increase the rate of transformation, decrease damage to the host genome into which it is transformed and reduce the nutritional load imposed.

The interaction between Ggt and *T. koningii* in soil with wheat plants was examined without using a marker gene (chapter 2). Extensive colonisation of the rhizoplane by native isolates of *Trichoderma* and the introduced isolate (AST-1) was observed. Internal root colonisation by the introduced isolate of *T. koningii* (AST-1) was higher than by native isolates indicating that this could be important in biocontrol. Addition of *T. koningii* after Ggt has begun to grow

reduces the chances of effective biocontrol.

A farmer can determine if there is a significant amount of Ggt present in his soil by wheat plant bioassay or a DNA assay of organic matter (Herdina *et al.* 1996). If required, *T. koningii* could be added to soil at the break of the season before Ggt can become established in soil so *T. koningii* would be present to inhibit hyphal regeneration of Ggt.

This research shows that inhibition of Ggt in soil by *T. koningii* is critical in biocontrol. The role of internal root and rhizosphere colonisation is still to be elucidated. This study suggested that the high level of root colonisation may also be an important factor in protecting wheat roots from take-all disease.

APPENDIX 1. Selective media

Medium	g/L (unless stated)
Davet's	
Davet 1979	
$CaNO_3$	1
KNO_3	0.25
$MgSO_4$	0.25
KH_2PO_4	0.125
$CaCl_2$	1
Citric acid	0.05
Sucrose	2
Agar	25
Streptomycin	0.03
Allyl alcohol	0.5 ml
Vinclozalin	0.0025

Medium	g/L
DRBC	
King *et al.* 1979	
$MgSO_4$	0.5
KH_2PO_4	1
Glucose	10
Peptone	5
Chloramphenicol	0.1
Rose bengal	0.025
Agar	15
Dicloran	0.002

TME-SA	
Papavizas & Lumsden 1982	
V8 juice	200 ml
Glucose	1
Agar	20
Neomycin sulphate	0.1
Bacitracin	0.1
Penicillin G	0.1
Chlortetracycline	0.025
Nystatin	0.02
Sodium propionate	0.5
Triton X	2 ml

TSMC	
Elad & Chet 1983	
$MgSO_4$	0.2
KH_2PO_4	0.9
KCl	0.15
NH_4NO_3	1
Glucose	3
Chloramphenicol	0.25
Fenaminosulf	0.3
PCNB	0.2
Rose bengal	0.15
Agar	20
Captan	0.02

C & H	
Chen *et al.* 1988	
$MgSO_4$	0.2
KH_2PO_4	0.9
KCl	0.15
NH_4NO_3	1
Glucose	3
Agar	20
Rose bengal	0.03
$FeSO_4$	0.02
$MnSO_4$	0.02
$ZnSO_4$	0.02
Chloramphenicol	0.05
Streptomycin-sulfate	0.05
PCNB	0.1
Metalaxyl	0.05

TSMC & Previcur	
Askew & Laing 1993	
TSMC as above	
left Captan out	
Previcur	1.2

Medium	g/L (unless stated)
MTSM	
Smith *et al.* 1990	
$Ca(NO_3)_2$	1
KNO_3	0.26
$MgSO_4$	0.26
KH_2PO_4	0.12
$CaCl_2$	1
Citric acid	0.05
Sucrose	2
Agar	20
Igepal	1
Chlortetracycline	0.05
Captan	0.04
Vinclozalin	0.0025

APPENDIX 2. Composition of Solutions and Media

A2.1. Media

Czapek Yeast Extract Agar (CYA) for 1 L: K_2HPO_4 1 g, Czapek concentrate 10 ml, yeast extract (Oxoid) 5 g, sucrose 30 g, agar 15 g, DI H_2O 1 L Klich & Pitt (1988)

Czapek concentrate (with trace metals) for 100 ml: $NaNO_3$ 30 g, KCl 5 g, $MgSO_4.7H_2O$ 5 g, $FeSO_4.7H_2O$ 0.1 g, $ZnSO_4.7H_2O$ 0.1 g, $CuSO_4.5H_2O$ 0.05 g, DI H_2O 100 ml (does not need to be sterilised) Klich & Pitt (1988).

Luria Broth (LB) for 1 L: tryptone 10 g, yeast extract 5 g, NaCl 10 g, 1 L H_2O Sambrook *et al.* (1989).

Malt Extract Agar (MEA) for 1 L: powdered malt extract (Oxoid) 20 g, peptone 1 g, glucose 20 g, agar 20 g, DI H_2O 1 L Klich & Pitt (1988).

NDY for 1 L: $NaNO_3$ 2 g, KH_2PO_4 1 g, $MgSO_4.7H_2O$ 0.5 g, KCl 0.5 g, yeast extract 0.5 g, sucrose 30 g 0.1% $FeSO_4$ 10 ml, DI H_2O 1 L Warcup (1955).

Potato Dextrose Broth (PDB) for 1 L: white skinned potatoes 250 g, glucose 20 g, DI H_2O 1 L. Wash potatoes, dice or slice unpeeled into 500 ml H_2O. Steam, boil or microwave till very soft. Strain potato through several layers of cheese cloth, include some potato pulp. Add the glucose, mix well and make up to 1 L with DI H_2O. Pitt & Hocking (1985).

Trichoderma Minimal Medium (TMM) for 1 L: glucose 10 g, KH_2PO_4 10 g, $(NH_4)_2SO_4$ 6 g, trisodium citrate.$2H_2O$ 3 g, mineral solution 10 ml pH 5.8. Autoclave all ingredients in 980 ml H_2O, filter sterilise glucose in 20 ml H_2O, combine when cool. Gruber *et al.* (1990a).

Trichoderma Minimal Medium Mineral Solution for 100 ml: $MgSO_2.7H_2O$ 10 g, $FeSO_2.7H_2O$ 0.05 g, $MnSO_2.7H_2O$ 0.016 g, $ZnSO_2.7H_2O$ 0.014 g, $CaCl_2.2H_2O$ 0.02 g, DI H_2O 100 ml Gruber *et al.* (1990a).

A2.2. Protoplast Preparation and Transformation

Polyethylene glycol 6000 25% PEG 6000 in 10 mM Tris HCl, 50 mM $CaCl_2$, pH 7.5

Solution I 50 mM glucose, 25 mM Tris-HCl, 10 mM EDTA, pH 8

Solution II 200 µl of 0.2 N NaOH, 1 % SDS

1.2 M ST 1.2 M sorbitol, 10 mM Tris-HCl, pH 7.5

1.2 M STC 1.2 M ST with 10 mM $CaCl_2$

A2.3. PCR

Oligonucleotide primers 5'-CTG TAG AAA CCC CAA CCC CTG-3'
5'-CAT TAG GCT GCG ATG GAT CCC-3'
van Wordragen *et al.* (1991)

A2.4. RFLP Analysis

Denaturing solution 0.4 M NaOH 0.6 M NaCl

10 x Denhardts III solution 2 % gelatine, 2 % Ficoll, 2 % polyvinyl pyrollidone, 10 % SDS, 5 % tetrasodium pyrophosphate

DNA extraction buffer 50 mM Tris, 50 mM NaCl, 0.5 % SDS (sodium dodecyl sulphate), 10 mM EDTA, 50 mM Na_2SO_3, pH 8

10 x gel loading buffer 100 mM Tris HCl, 200 mM EDTA, 0.25 % bromophenol blue, 0.25 % xylene cyanol, 30 % ficoll type 4000, pH 8

5 x HSB 3 M NaCl, 100 mM PIPES, 25 mM Na_2EDTA, pH 6.8

Neutralising solution 1.5 M NaCl, 0.5 M Tris HCl, pH 7.5

2 x SSC 0.3 M NaCl 30 mM trisodium citrate

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