REPORTER GENE TRANSFORMATION OF GRAPEVINE PATHOGENS *PHAEOMONIELLA CHLAMYDOSPORA* AND *PHOMOPSIS VITICOLA*, AND BIOCONTROL AGENT *TRICHODERMA HARZIANUM*

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Thesis presented in partial fulfillment of the requirements for the degree of Master of Science in Agriculture at the University of Stellenbosch

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March 2007

DECLARATION

I, the undersigned, hereby declare that the work contained in this thesis is my own original work and has not previously in its entirety or in part been submitted at any university for a degree.

Date: 01/03/07

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SUMMARY

Trunk diseases cause major economical losses in the grape growing industry due to decline and premature dieback, resulting in major reductions in quantity and quality in yield. Trunk diseases are caused by a complex of pathogens including basidiomycetes, species of Botryosphaeriaceae (including *Botryosphaeria* and aggregate genera, *Lasiodiplodia* and *Neofusicoccum*), *Eutypa lata*, *Phaeoacremonium* spp., *Phaeomoniella chlamydospora* and *Phomopsis viticola*. Limited knowledge is available on interactions between the grapevine host and these pathogens. Currently, there are no sustainable control measures available for management of trunk disease pathogens.

Grapevine pruning wounds serve as the main entry portals for trunk disease pathogens, and therefore require prolonged protection. The biological grapevine pruning wound protectant, *Trichoderma harzianum*, have demonstrated the ability to colonise pruning wound sites, thereby providing a sustainable living barrier against trunk disease pathogen invasion. Although *T. harzianum* has the potential for controlling trunk disease pathogens, limited knowledge is available on its host colonisation ability and methods of antagonism.

Knowledge on the interactions of trunk disease pathogens with the host, as well as with *T. harzianum* within grapevine host tissue will greatly aid the development of sustainable control strategies. However, host interaction studies have been hampered due to difficulty in observing and studying target fungi within the grapevine host, where several endophytic fungi are also present. The transformation of fungi with reporter genes, which convey detectable phenotypes such as green and red fluorescence to fungi, will greatly aid these studies. Therefore, the first aim of this study was to stably transform South African *Pa. chlamydospora*, *P. viticola* (two isolates) and *T. harzianum* isolates with the green (GFP) or red (DsRed-Express) reporter genes using a polyethylene glycol/calcium chloride transformation method and the selectable marker gene hygromycin phosphotransferase (*hph*). The second aim of the study was to determine whether the transformation process has altered biological characteristics [colony colour, spore

size, germination percentage, growth rate at different temperatures, virulence (*Pa. chlamydospora* and *P. viticola*) and host colonisation (*T. harzianum*)] of the transformants. Furthermore, in addition to characterisation of these transformants and wild type isolates, a *Pa. chlamydospora* GFP transformant (pCT74-P7) and its wild type isolate from New Zealand were also characterised.

Phaeomoniella chlamydospora (STE-U 7584) was stably transformed with the GFP or DsRed-Express protein reporter genes, yielding brightly fluorescing transformants. The presence of the transgenes was also confirmed through polymerase chain reaction amplifications. Characterisation of the colony colour, spore size, germination percentage and growth rate at different temperatures of two of the stable, highly fluorescent GFP (PcG1 and PcG10) and DsRed-Express (PcR1 and PcR2) transformants as well the *Pa. chlamydospora* GFP transformant (pCT74-P7), revealed no differences between the transformants and their respective wild type isolates. The only exception was the significant lower germination percentage of transformant PcG10, and the different colony colour of transformants PcG1 and PcR1, when compared with the wild type isolate. Characterisation of the virulence of three of the reporter gene transformants (PcG1, PcR1 and pCT74-P7) showed that they did not differ from their respective wild type isolates.

Two *P. viticola* isolates (STE-U 6048 and STE-U 6049) were stably transformed with the *GFP* and *DsRed-Express* reporter genes. However, brightly fluorescing transformants could not be obtained, limiting the usefulness of these isolates in host colonisation studies. All the GFP transformants exhibited low to intermediate levels of fluorescence in mycelia as well as in conidia over a 6-week growth period. Similarly, all the DsRed-Express transformants also showed low to intermediate levels of fluorescence in the mycelia of 1- to 3-week-old cultures, whereas no fluorescence was observed in 4- to 6-week-old cultures. Contrarily, the conidia of DsRed-Express transformants showed very bright fluorescence in 3- to 4-week-old cultures. The presence of the *hph*, *GFP* and *DsRed-Express* genes was shown through polymerase chain reaction amplifications. Biological characterisation of a subset of the isolates and their respective wild type isolates showed that they were similar to their respective wild type isolates.

Trichoderma harzianum strain T77 was stably transformed with the GFP or DsRed-Express reporter genes. However, transformation of *T. harzianum* strain T77 was difficult, since most transformants proved to be unstable, and did not retain the selectable marker gene (hygromycin phosphotransferase) or the reporter genes. Only four stably transformed isolates, two expressing *GFP* (TG1 and TG2) and two expressing *DsRed-Express* (TR1 and TR2) were

obtained. Characterisation of the growth rate, morphology, conidial size and germination percentage of these transformants showed that transformants TR1 and TG1 were altered in their growth rate and/or germination percentage. Characterisation of the host colonisation ability of transformants TR1 and TG1 on grapevine cuttings of cvs. Chenin blanc and Merlot, showed that both transformants were able to colonise grapevine cuttings in a similar manner than the wild type isolate, and that they retained their fluorescent phenotype following isolation from the host. All isolates, including the wild type, showed a significantly higher colonisation rate on Merlot than on Chenin blanc.

VERKLIKKERGEEN-TRANSFORMASIE VAN WINGERDPATOGENE *PHAEOMONIELLA CHLAMYDOSPORA* EN *PHOMOPSIS VITICOLA* EN BIOBEHEER-AGENT *TRICHODERMA HARZIANUM*

OPSOMMING

Stamsiektes veroorsaak groot ekonomiese verliese in die wingerdbedryf as gevolg van agteruitgang en terugsterwing van wingerde, wat 'n verlaging in kwaliteit en kwantiteit van oesopbrengste tot gevolg het. Stamsiektes word deur 'n kompleks van patogene veroorsaak, insluitend basidiomysete, Botryosphaeriaceae spesies (insluitende Botryosphaeria en die kompleks genera, *Lasiodiplodia* en *Neofusicoccum*), *Eutypa lata*, *Phaeoacremonium* spp., *Phaeomoniella chlamydospora* en *Phomopsis viticola*. Beperkte kennis is oor die interaksies tussen die wingerdgasheer en hierdie patogene beskikbaar, en daar is ook geen volhoubare beheermaatreëls beskikbaar vir die bestuur van stamsiektepatogene nie.

Stamsiektepatogene verkry hoofsaaklik toegang tot die wingerdgasheer via snoeiwonde, gevolglik is langdurige beskerming van die snoeiwonde nodig. Die biologiese beskermingsagent van wingerdsnoeiwonde, *Trichoderma harzianum*, is al bewys om snoeiwonde te koloniseer en daardeur 'n volhoubare, lewende versperring teen die indringing van stamsiektepatogene te verleen. Alhoewel *T. harzianum* die vermoë het om stamsiektepatogene te beheer, is beperkte inligting beskikbaar oor die vermoë van die swam om die gasheer te koloniseer, sowel as watter meganismes van antagonisme gebruik word.

Kennis oor die interaksies van stamsiektepatogene met die gasheer, asook met *T. harzianum* binne-in die wingerdgasheerweefsel, sal grootskaals tot die ontwikkeling van volhoubare beheerstrategieë bydra. Gasheer-interaksiestudies is egter in die verlede deur struikelblokke in die waarneming en bestudering van teikenswamme binne-in die wingerdgasheer belemmer, veral in die teenwoordigheid van verskeie ander endofitiese swamme. Die transformasie van swamme met verklikkergene, wat rapporteerbare

fenotipes soos groen en rooi fluoressensie na swamme oordra, sal grootskaals tot hierdie studies bydra. Die eerste doel van hierdie studie was dus om stabiele transformante van Suid-Afrikaanse *Pa. chlamydospora*, *P. viticola* (twee isolate) en *T. harzianum* isolate met die groen (*GFP*) of rooi (*DsRed-Express*) verklikkergene te verkry, deur van 'n poli-etileen glikol/kalsium chloried transformasie metode en die selektiewe merkergeen, hygromycin fosfotransferase (*hph*), gebruik te maak. Die tweede doel van die studie was om te bepaal of die transformasieproses die biologiese karakter-eienskappe [koloniekleur, spoorgrootte, ontkiemingspersentasie, groeitempo by verskillende temperature, virulensie (*Pa. chlamydospora* en *P. viticola*) en gasheerkolonisasie (*T. harzianum*)] van die transformante verander het. Bykomend tot die karakterisering van hierdie transformante en hul natuurlike isolate, is 'n *Pa. chlamydospora* GFP transformant (pCT74-P7) en sy wilde-tipe isolaat van Nieu-Seeland (Bradshaw *et al.*, 2005) ook gekarakteriseer.

Phaeomoniella chlamydospora (STE-U 7584) is stabiel met die GFP of DsRed-Express proteïen verklikkergene getransformeer, wat helder fluoresserende transformante opgelewer het. Die teenwoordigheid van die transgene is ook met polimerase-ketting-reaksie-amplifiserings bevestig. Karakterisering van die koloniekleur, spoorgrootte, ontkiemingspersentasie en groeitempo by verskillende temperature van twee van die stabiele, helder fluoresserende GFP (PcG1 en PcG10) en DsRed-Express (PcR1 en PcR2) transformante, asook die *Pa. chlamydospora* GFP transformant (pCT74-P7), het geen verskille tussen die transformante en hul onderskeie natuurlike isolate aangetoon nie. Die enigste uitsondering was transformant PcG10 wat 'n betekenisvolle laer ontkiemingspersentasie getoon het, asook die verskil in koloniekleur van transformante PcG1 en PcR1, in vergelyking met die wilde-tipe isolaat. Karakterisering van die virulensie van drie van die verklikkergeen transformante (PcG1, PcR1 en pCT74-P7) het aangetoon dat hulle nie van die onderskeie natuurlike isolate verskil het nie.

Twee *P. viticola* isolate (STE-U 6048 en STE-U 6049) is stabiel met die *GFP* en *DsRed-Express* verklikkergene getransformeer. Helder fluoresserende transformante kon egter nie verkry word nie, wat die gebruik van hierdie isolate in gasheerkolonisasie-studies beperk. Al die GFP transformante het lae tot intermediêre vlakke van fluoressensie in die miselia, asook in die konidia, oor 'n 6-week groeiperiode getoon. Soortgelyk het al die DsRed-Express transformante ook lae tot intermediêre vlakke van

fluoressensie in die miselia van 1-week-oue tot 3-week-oue kulture getoon, maar geen fluoressensie is in 4-week-oue tot 6-week-oue kulture waargeneem nie. Hierteenoor het die konidia van DsRed-Express transformante helder fluoressensie in 3-week-oue to 4-week-oue kulture getoon. Die teenwoordigheid van *hph*, *GFP* en *DsRed-Express* gene is deur die polimerase-ketting-reaksie-amplifisering bevestig. Biologiese karakterisering van geselekteerde isolate en hul onderskeie wilde-tipe isolate het getoon dat hulle nie van hul onderskeie wilde-tipe isolate verskil het nie.

Trichoderma harzianum ras T77 is stabiel met die *GFP* of *DsRed-Express* verklikkergene getransformeer. Die transformasie van *T. harzianum* ras T77 was egter moeilik aangesien meeste van die transformante onstabiel was, en hulle kon nie die selektiewe merkergeen (hygromycin fosfotransferase) of die verklikkergene behou het nie. Slegs vier stabiel getransformeerde isolate is verkry, twee wat *GFP* (TG1 en TG2) uitdruk en twee wat *DsRed-Express* (TR1 en TR2) uitdruk. Karakterisering van die groeitempo, morfologie, konidiagrootte en ontkiemingspersentasie van hierdie transformante het getoon dat transformante TR1 en TG1 se groeitempo en/of ontkiemingspersentasie verander is. Karakterisering van die gasheerkolonisasie-vermoë van transformante TR1 en TG1 op wingerdlote van cvs. Chenin blanc en Merlot, het getoon dat beide transformante die vermoë het om die wingerdlote op 'n soortgelyke manier as die wilde-tipe isolaat te koloniseer, en hulle het hulle fluoressensie fenotipe behou ná isolasie uit die gasheer. Al die isolate, insluitende die wilde-tipe isolaat, het 'n betekenisvolle hoër kolonisasie-persentasie op Merlot, in vergelyking met Chenin blanc, getoon.

ACKNOWLEDGEMENTS

I wish to convey my sincere thanks to the following:

Dr. Adele Mcleod for sharing her incredible knowledge and unlimited time, patience and kindness during the past two years;

Dr. Paul Fourie for his advice, amazing wisdom and contagious enthusiasm;

The technical staff at the Department of Plant Pathology, University of Stellenbosch, especially Jan-Cor Brink, Dr. Lizel Mostert and Sonja Coertze;

Frikkie Calitz (ARC-Infruitech Nietvoorbij) for statistical analysis;

J.M. Lorang (Oregon State University, Oregon, USA) and L. Mikkelsen (The Royal Veterinary and Agricultural University, Frederiksberg, Denmark) for providing the transformation vectors;

Deciduous Fruit Producer's Trust, The National Research Foundation, Winetech and the University of Stellenbosch for financial support;

My dear friends and loved ones for irreplaceable friendships and for always being there. Special thanks to Raphael Jensen, Tinake van Zyl and Elrie Bosch;

My parents for their unconditional love, encouragement and loyal support before and during my studies at Stellenbosch University;

Most importantly my Heavenly Father for making it all possible. I am eternally grateful for the opportunity and blessings bestowed upon me.

CONTENTS

1. THE USE OF REPORTER GENE TRANSFORMED FUNGI FOR STUDYING THE GRAPEVINE TRUNK DISEASE PATHOGEN-BIOCONTROL SYSTEMS

INTRODUCTION

Trunk diseases cause major economical losses in the grapevine industry due to decline and premature dieback, resulting in significant reductions in quantity and quality in yield (Ferreira *et al.*, 1989; Mugnai *et al.*, 1999; Chiarappa, 2000; Halleen and Fourie, 2005). Trunk diseases are caused by a complex of pathogens including basidiomycetes (Fischer, 2006), species of Botryosphaeriaceae (including *Botryosphaeria* and aggregate genera, *Lasiodiplodia* and *Neofusicoccum*) (Van Niekerk *et al.*, 2004; Crous *et al.*, 2006; Van Niekerk *et al.*, 2006), *Eutypa lata* (Munkvold and Marois, 1995), *Phaeoacremonium* (Mostert *et al.*, 2006a, b), *Phaeomoniella chlamydospora* (Mugnai *et al.*, 1999) and *Phomopsis viticola* (Van Niekerk *et al.*, 2005). These fungi exhibit a unique and complex interaction with their host of which little is yet understood, even though many years of intensive investigations, aimed at understanding and controlling these intricate pathogens, have been conducted.

Unprotected pruning wounds are major entry portals for trunk disease pathogens, even for several weeks after cuts have been made (John *et al.*, 2004). These pruning wounds are infected by trunk disease pathogen spores, which are released and dispersed by wind and water splash from other infected vines or pruning debris (Mugnai *et al.*, 1999; Van Niekerk *et al.*, 2004; Van Niekerk *et al.*, 2005; Crous *et al.*, 2006; Mostert *et al.*, 2006a, b; Van Niekerk *et al.*, 2006). Under favourable weather conditions and incorrect disease management practices, trunk disease pathogen spores will infect and flourish in established vineyards leading to a loss in yield and grape quality (Ferreira *et al.*, 1999).

The control of trunk disease pathogens is very difficult due to the fact that pruning wounds stay susceptible to pathogen infections for several weeks (John *et al.*, 2004; Van Niekerk *et al.*, 2006). Therefore, it is crucial to develop methods for

protecting grapevine wood for a prolonged period of time. *Trichoderma harzianum* is a biological control agent that has been registered commercially in certain countries for protection of exposed pruning wounds in grapevines, since it may provide better long-term protection than fungicides (John *et al.*, 2004; John *et al.*, 2005). However, substantial controversy still exists on recommendations made on the time of application of biocontrol agents for obtaining sustained protection under field conditions in grapevines (John *et al.*, 2005).

Little is known about the mode of infection and survival of trunk disease pathogens in and around their hosts, hampering the elucidation of their life cycle and the development of efficient management strategies. Similarly, limited knowledge exists on the colonisation, interaction and mode of action of biological control agents, such as *T. harzianum*, with trunk disease pathogens in and on grapevine surfaces (Di Marco *et al.*, 2004). Key aspects that need to be addressed include the identification of pathogen infection pathways, colonisation and spread, as well as interaction and spatial distribution of pathogens and biocontrol agents within host tissue. Furthermore, the identification of specific host responses to pathogen infection in resistant and susceptible cultivars also needs to be identified. Up until now, these studies have all been difficult since the target fungi can not be readily discriminated within host tissue, along with the presence of several endophytic fungi which make it difficult to follow pathogen infection and spread. Furthermore, the interaction of grapevine pathogens with biocontrol agents when co-inoculated is also not possible since the fungi cannot be discriminated from each other.

Only a few techniques are available for the detection of fungi within host tissue, as well as for discriminating between fungi that have been co-inoculated. Although fluorescent staining techniques can be used to detect fungi within the host, these techniques suffer from a few shortcomings. Firstly, the conidia or spores of most fungi do not stain, or only a small percentage will stain due to the very thick and rigid nature of fungal spore cell walls (Prigione and Marchisio, 2004). Secondly, all fungi will exhibit the same colour of fluorescence, precluding studies on co-inoculated fungi. Lastly, staining and fixing techniques are very time-consuming and do not readily allow the observation of live tissue within infected host tissue (Sexton and Howlett, 2001).

Transformation of pathogenic and biocontrol agent fungi with reporter genes have proven to be very useful for studying fungal infection, colonisation and ecological aspects, as well as interactions between co-inoculated fungi within host tissue (Nahalkova and Fatehi, 2003; Skadsen and Hohn, 2004). Reporter genes are genes of which the product conveys a detectable phenotype such as green or red fluorescence, once they are transformed and expressed in the organism of choice (Lemke and Peng, 1995). The reporter genes can be transformed into fungi using several different transformation methods, of which *Agrobacterium*-mediated transformation and the polyethylene glycol/calcium chloride-mediated transformation method are most commonly used (Lorang *et al.*, 2001; Fitzgerald *et al.*, 2003; Meyer *et al.*, 2003; Weld *et al.*, 2006).

REPORTER GENES USED TO LABEL FUNGI

In fungi, a reporter gene fused to an appropriate promoter that is incorporated into nuclear DNA, will convey a detectable phenotype, often a specific colour, to the fungus. The expression of a reporter gene on a continual basis under all fungal life cycle stages requires the fusion of the reporter gene to a constitutive promoter, which drives expression of the gene under all circumstances. Therefore, the fusion of the reporter gene to a constitutive promoter is required for the purpose of visualisation of fungi within host tissue (Lorang *et al.*, 2001). However, the promoter alone will not always determine the level and timing of reporter gene expression, which may vary from transformant to transformant, most likely due to varied integration sites and RNA silencing-related phenomena such as quelling and meiotic silencing by unpaired DNA (Selker, 1998; Nakayashiki, 2005).

Several reporter genes have been used in bacteria, mammalian cells and a variety of other organisms (Chalfie *et al.*, 1994; Cubitt *et al.*, 1995; Plautz *et al.*, 1996; Spellig *et al.*, 1996; Valdivia *et al.*, 1996; Maor *et al.*, 1998; Sessitsch *et al.*, 1998; Tsien, 1998; Cummings *et al.*, 1999; Dumas *et al.*, 1999; Lorang *et al.*, 2001; Jansson, 2003). The most widely used and promising reporter genes for use in fungi include the green fluorescent protein (GFP), GFP colour variants, reef coral fluorescent proteins and the β-glucuronidase (GUS) gene (Chalfie and Kain, 1998;

Maor *et al.*, 1998; Lorang *et al.*, 2001; Hakkila *et al.*, 2002; Jansson, 2003). However, not all of these reporter genes will yield a detectable phenotype in all fungal species. For example, there are reports where some of the *GFP* variants are not expressed in filamentous fungi, resulting in no detectable phenotype (Lorang *et al.*, 2001). This is due to the fact that the choice of *GFP* gene depends on the codon or other binding site preference of the transformed fungus, as well as the ability of the fungus to fold the protein (Lorang *et al.*, 2001). An important characteristic that all reporter genes must have is that they should not affect the growth and pathogenicity of the transformed fungus (Spellig *et al.*, 1996; Maor *et al.*, 1998; Lorang *et al.*, 2001; Mullins and Kang, 2001).

Green Fluorescent Protein (GFP). The well-known versatile GFP gene is a spontaneously fluorescent polypeptide of 27 kDa derived from the Pacific Northshore jellyfish, *Aequorea victoria* (Prasher *et al.*, 1992; Chalfie *et al.*, 1994; Prasher, 1995). The ability of GFP to generate a highly visible, efficiently emitting internal fluorophore (Tsien, 1998) is both fundamentally intriguing and very valuable. GFP owes its visible absorbance and fluorescence to a *p*-hydroxybenzylideneimidazolinone chromophore formed by the cyclisation of amino acids Ser 65, Tyr 66, Gly 67 and 1, 2 dehydrogenation of the tyrosine (Cubitt *et al.*, 1995). The originally cloned *GFP* gene has excitation maxima at 395 nm and 470 nm and emits green light with a maximum of 507 nm when exposed under UV or blue light (Cormack, 1998).

The *GFP* gene has several characteristics contributing to its frequent use as reporter gene in various studies. This unique chromophore does not rely on the addition of exogenous substrates or cofactors other than oxygen, which is required for fluorescence (Chalfie *et al.*, 1994; Chalfie, 1995; Prasher, 1995; Lorang *et al.*, 2001). Therefore, GFP can be monitored easily in single cells using epifluorescence microscopy or confocal laser scanning microscopy in intact tissue without any disturbance (Lu *et al.*, 2004). GFP is species independent and has a high stability under various conditions, which include resistance to proteases and denaturation, stable fluorescence in pH regimes of 3 to 12 (fluorescence optimal at pH 7.2 to 8.0) and in inorganic solvents such as 1% sodium dodecyl sulphate (SDS), 8 M urea, 6 M guanidium chloride, glutaraldehyde or formaldehyde (Cubitt *et al.*, 1995; Yang *et al.*, 1996b; Li *et al.*, 1997; Tsien, 1998; Lopes *et al.*, 2004).

Due to GFP's excellent properties, it has been developed to act as a reporter gene in a variety of organisms (Cormack, 1998; Bae and Knudsen, 2000; Howard, 2001; Bourett *et al.*, 2002). GFP was first expressed in *Escherichia coli* (Chalfie *et al.*, 1994) followed by expression in *Caenorhabolitis elegans* (Chalfie *et al.*, 1994) and *Saccharomyces cerevisiae* (Niedenthal *et al.*, 1996). It was also the first fluorescent genetic marker to be cloned and expressed in plant cells (Haselhoff and Amos, 1995; Sheen *et al.*, 1995). The first filamentous fungus to be successfully transformed with GFP was *Ustilago maydis* (Spellig *et al.*, 1996), followed by *Aspergillus nidulans* (Suelmann *et al.*, 1997) and *Aureobasidium pullulans* (Van den Wymelberg *et al.*, 1997). Subsequently, the combined efforts of several GFP studies have had a great impact on filamentous fungal molecular biology studies worldwide. GFP can be used for either labeling of fungi for detection purposes (Dumas *et al.*, 1999; Maor *et al.*, 1998; Visser *et al.*, 2004), or it can be used for the tagging of proteins for gene regulation analysis (Weld *et al.*, 2006), protein localisation (Lehmler *et al.*, 1997; Cormack, 1998), or specific organelle labeling (Suelmann, *et al.*, 1997; Tsien, 1998).

There are still a number of problems with the use of GFP in certain organisms such as uneven excitation of the cells or interference from background fluorescence (Leveau and Lindow, 2002). Furthermore, sometimes transformants containing the *GFP* gene do not fluoresce, which may be the result of misfolding of the fluorescent protein, insufficient translation, failure of chromophore formation, gene silencing or integration of the plasmid into heterochromatin chromosomal sites (Selker, 1998; Lorang *et al.*, 2001; Lagopodi *et al.*, 2002; Visser *et al.*, 2004). In some fungi it has been found that transformants containing a single *GFP* copy will only display low levels of visualisation, whereas multicopy transformants are required for obtaining high levels of fluorescence (Lopes *et al.*, 2004).

Since the first discovery and cloning of the *GFP* gene from *A. victoria* (Prasher *et al.*, 1992; Chalfie *et al.*, 1994; Prasher, 1995), several GFP variants have been developed for yeasts, fungi, plants and mammals which have increased sensitivity, optimised codon usages for specific organisms, faster folding of the protein or altered spectral properties for better macroscopic visualisation (Lorang *et al.*, 2001). These variants have been obtained by creating mutations in the

chromophore region of the originally cloned *GFP* gene. Most of the currently used *GFP* genes have a serine-to-threonine substitution at amino acid 65 (S65T), which increases fluorescence levels but also causes a shift from excitation maxima of 395 and 470 nm to a maximum of 488 nm (Lorang *et al.*, 2001). For example EGFP has a human optimised codon usage as well as a S65T mutation in the chromophore, which results in enhanced green fluorescence (Lorang *et al.*, 2001; Clontech, 2003). GFPuv was optimised by introducing point mutations that replaced three amino acid codons in the native GFP DNA sequence (Chalfie *et al.*, 1994; Crameri *et al.*, 1996). This GFP variant is 18 times brighter than the wild type GFP when expressed in *E. coli*, and can therefore been seen at a macroscopic level when excited by standard UV light (Crameri *et al.*, 1996).

Specifically in plants and fungi, SGFP have been widely used. *SGFP* is a *GFP* variant with a plant-optimised codon usage, also containing a mutation that removed a cryptic intron splice site that abolished GFP detection in *Arabidopsis* (Chiu *et al.*, 1996; Lorang *et al.*, 2001). Fernández-Ábalos *et al.* (1998) compared SGFP to three other GFP variants (driven by the *A. nidulans* promoter) and found that SGFP conferred the highest GFP concentration and fluorescence in transformants. Similarly, Spellig *et al.* (1996) and Maor *et al.* (1998) could only obtain GFP fluorescent transformants when using SGFP and not wild type GFP in *U. maydis* and *Cochliobolus heterostrophus.* GFP-enhanced variants have been developed for specific use in fungi and other organisms, resulting in increased fluorescence levels in *Acremonium chrysogenum* (Pöggeler *et al.*, 2003), *Aspergillus flavus* (Du *et al.*, 1999), *C. heterostrophus* (Maor *et al.*, 1998), *Colletotrichum gloeosporiodes* (Robinson and Sharon, 1999), *E. lata* (Bradshaw *et al.*, 2005), *Magnaporthe grisea* (Kershaw *et al.*, 1998), *Pa. chlamydospora* (Bradshaw *et al.*, 2005), *Podospora anserina* (Berteaux-Lecellier *et al.*, 1998), *Sordaria macrospore* (Pöggeler *et al.*, 2003) and *T. harzianum* (Inglis *et al.*, 1999; Bae and Knudsen, 2000).

GFP colour variants and reef coral fluorescent proteins. Although GFP is currently the most widely used reporter gene, the use of GFP colour variants is rapidly increasing. These colour variants have been obtained by creating mutations in certain positions in chromophore of the *GFP* gene originally cloned from *A. victoria*, yielding GFP mutants with blue, cyan and yellowish green emissions (Cubitt *et al.*, 1995;

Tsien, 1998). Some examples of these include EYFP (Ormo *et al.*, 1996), EBFP (Heim and Tsien, 1996; Yang *et al.*, 1996a; Yang *et al.*, 1998) and ECFP (Heim and Tsien, 1996), which refer to the yellow, blue and cyan fluorescent proteins, respectively.

Recently, new fluorescent protein genes have been discovered and cloned from a group of non-bioluminescent reef corals (Matz *et al.*, 1999; Lukyanov *et al.*, 2000). The reef coral proteins (RCFPs), like GFP, do not necessitate exogenous cofactors or substrates to fluoresce and have unique excitation and emission patterns within the visible spectrum (Bourett *et al.*, 2002). These RCFPs include AmCyan1, AsRed1 and AsRed2, DsRed1 and DsRed2, ZsGreen1 and ZsYellow derived from *Anemonia majona*, *Anemonia sulcat*, *Discosoma* spp. and *Zoanthus* spp. and are now commercially available (Clontech, 2003). However, the effectiveness of some of these reporter proteins is limited due to the slow maturation time of the proteins and obligate oligomerisation (Andrie *et al.*, 2005; Larrainzar *et al.*, 2005).

One of the RCFPs that are quickly gaining more interest is the DsRed protein. The DsRed protein is a 28 kDa red fluorescent protein that can be used as a reporter gene similar to GFP (Matz *et al.*, 1999; Jansson, 2003; Mikkelsen *et al.*, 2003). According to Davey and Kell (1996), DsRed could offer better sensitivity than GFP, because auto-fluorescence is lower at longer wavelengths. The originally cloned DsRed gene has an excitation maximum of 558 nm and emission maximum 583 nm with negligible sensitivity to pH changes (Matz *et al.*, 1999; Baird *et al.*, 2000; Stewart, 2001; Hakkila *et al.*, 2002). However, unlike GFP, DsRed is sensitive to mild denaturants such as sodium dodecyl sulfate (Clontech, 2003).

The originally cloned DsRed protein sometimes displays slow maturation of the chromophore and may create large amounts of aggregates in transformed cells (Baird *et al.*, 2000). Therefore, mutations have been made in the originally cloned DsRed gene in order to yield proteins that are more soluble and folded in a shorter period of time (Mikkelsen *et al.*, 2003). DsRed2 has been developed through a combination of random and site-directed mutagenesis of the wildtype DsRed (DsRed1), resulting in moderately enhanced fluorescence, improved solubility and speed of maturation (Bevis and Glick, 2002; Clontech, 2003; Larrainzar *et al.*, 2005). DsRed2 has an excitation maximum of 563 nm and emission maximum of 582 nm

(Clontech, 2003). The DsRed-Express gene is also a variant of DsRed1 containing nine amino acid substitutions, which has yielded a protein with improved maturation and solubility, as well as a reduction in the tendency of the protein to form aggregates, when compared to DsRed2 (Bevis and Glick, 2002; Clontech, 2003; Mikkelsen *et al.*, 2003; Larrainzar *et al.*, 2005). DsRed-Express has an excitation maximum at 557 nm and emission maximum at 579 nm (Bevis and Glick, 2002; Mikkelsen *et al.*, 2003). Recently, mRFP1 has been engineered from DsRed, yielding a faster maturing, brighter and monomeric protein, which will be more useful (Campbell *et al.*, 2002). Due to the large differences between the excitation and emission wavelengths of the DsRed protein variants and GFP, they are ideal for dual-colour experiments when appropriate filter settings are used (Mikkelsen *et al.*, 2003). Variants of the DsRed protein have been transformed and expressed in several fungi including *A. nidulans*, *Fusarium oxysporum* f. sp. *lycopersici*, *Penicillium paxilli* and a *Trichoderma* sp. (Dou *et al.*, 2003; Mikkelsen *et al.*, 2003; Nahalkova and Fatehi, 2003).

β-D-glucuronidase (GUS). The *E. coli* GUS reporter gene is widely used as a histochemical marker in transformed organisms. Detection of the *GUS* gene fusion system requires the addition of a substrate, as well as active functioning cells. The *GUS* gene codes for an enzyme that is able to hydrolyse the colourless substrate 5-bromo-4-chloro-3-indolyl-ß-D-glucuronide (X-gluc) to an insoluble dark blue product once cells are incubated at 37°C with the substrate (Jefferson *et al.*, 1987). Therefore, fungi that are able to successfully express the GUS gene is visualised as dark blue cells, following incubation with the substrate.

The first report on the construction and testing of a plasmid expressing GUS in filamentous fungi suggested that the system could be developed for studies on disease spread, fungal biomass quantification, fungicide efficacy studies and monitoring gene activity in fungi for the development of analysis systems (Jefferson *et al.*, 1987; Couteaudier *et al.*, 1993; Green and Jensen, 1995; Doohan *et al.*, 1998; Pamphile *et al.*, 2004). The *GUS* gene has facilitated visualisation of *Bipolaris sorokiniana* (Liljeroth *et al.*, 1993), *Clonostachys rosea* (Lübeck *et al.*, 2002), *Fusarium culmorum* (Doohan *et al.*, 1998), *Fusarium moniliforme* (Yates *et al.*, 1999), *F. oxysporum* (Couteaudier *et al.*, 1993), *F. oxysporum* f. sp. *lycopersici* (Mes *et al.*, 1999), *Pseudocercosporella herpotrichoides* (Bunkers, 1991), *Pyrenopeziza brassicae*

(Ashby and Johnston, 1993) and *T. harzianum* (Green and Jensen, 1995; Lo *et al.*, 1998). Thrane *et al.* (1995) and Bae and Knudsen (2000) concluded that GUS was a promising tool for ecological studies of biocontrol agents, due to its low background activity in fungi and plants, the easiness and sensitivity of detection and the apparent lack of influence of GUS expression on biocontrol efficacy.

The usefulness of *GUS* can be limiting, due to the fact that it is a destructive assay, a few organisms including some plants and microbes have endogenous GUS activity and because the staining product can be leached (Maor *et al.*, 1998; Elliot *et al.*, 1999). Lübeck *et al.* (2002) compared *GUS* and *GFP* marker genes and concluded that *GFP* was superior to the *GUS* gene. Although *GUS* was able to cause staining of both spores and mycelia, the intensity was less than what could be visualised with *GFP* fluorescence. Thomma *et al.* (1999) also reported that *GUS* is not always the most suitable reporter gene for studying plant-pathogen interactions, particularly when the *GUS* marker is used to quantify fungal biomass. Therefore, *GUS* is most likely to be replaced by GFP and other fluorescent reporter genes due to their higher stability, sensitivity and precision (Cormack, 1998).

METHODS USED FOR THE TRANSFORMATION OF FUNGI

The creation of organisms expressing reporter genes requires a transformation technique for introduction of the gene into the organism of choice. Transformation techniques have been developed for many prokaryotes as well as eukaryotes. The first transformation techniques were developed for bacteria (prokaryotes) and have been well documented (Goosen *et al.*, 1991). The transformation of higher and lower eukaryotes were developed much later, leading to new ways of altering biological and ecological characteristics as well the ability to study gene function (McBride and Ozer, 1973; Hinnen *et al.*, 1978). The first eukaryotic deoxyribonucleic acid (DNA)-mediated transformation method was described in *S. cerevisiae* in 1978 (Beggs, 1978; Hinnen *et al.*, 1978) and shortly afterwards in *Neurospora crassa* (Case *et al.*, 1979) and *A. nidulans* (Balance *et al.*, 1983; Tilburn *et al.*, 1983; Yelton *et al.*, 1984). Subsequently, the transformation system was modified and adopted for the transformation of various filamentous fungi (Peberdy, 1989; Goosen *et al.*, 1991;

Lorang *et al.*, 2001). The first transformation method was based on a polyethylene glycol and calcium chloride ($PEG/CaCl_2$) protoplast protocol, which was followed by the development of *Agrobacterium tumefaciens*-mediated and biolistic transformation methods (Fitzgerald *et al.*, 2003).

The aim of all transformation systems is to introduce DNA into cells and to subsequently select those cells containing the chromosomal inserted exogenous DNA. In general, the production of DNA-mediated transformed fungal cells requires several critical components and steps, including a) vectors which normally consist of *E. coli* plasmid DNA containing an appropriate selectable marker gene as well as the DNA fragment of interest under control of the appropriate promoter and terminator, b) fungal cells that are competent to take up DNA (protoplasts), c) incubation of competent fungal cells with vector DNA, d) regeneration of fungal cells that received the exogenous DNA into colony forming units, and e) selection of those cells that have stably incorporated the exogenous DNA, usually through the use of antibiotics for which a gene conveying resistance was introduced (Van den Hondel and Punt, 1991; Lemke and Peng, 1995).

The polyethyleneglycol/calcium chloride ($PEG/CaCl_2$) protoplast mediated transformation method. The $PEG/CaCl_2$ protoplast-mediated transformation method, along with *Agrobacterium tumefaciens*-mediated transformation (ATMT) are currently the two most widely used fungal transformation methods (Peberdy, 1989; Goosen *et al.*, 1991; Lemke and Peng, 1995; Herzog *et al.*, 1996; Hynes 1996; Riach and Kinghorn, 1996; De Groot *et al.*, 1998; Fitzgerald *et al.*, 2003; Meyer *et al.*, 2003). The $PEG/CaCl_2$ method was developed prior to ATMT, and has thus been in use for a longer time period that ATMT (Goosen *et al*, 1991). Therefore, one of the advantages of the $PEG/CaCl_2$ method is that more vectors, especially those containing fungal reporter genes, are available for transformation with the $PEG/CaCl_2$ method than for the ATMT method (Mullins and Kang, 2001; Fitzgerald *et al.*, 2003).

The $PEG/CaCl_2$ method consists of two main steps that include the production of fungal protoplasts followed by the transformation of exogenous DNA into the protoplasts through treatment with PEG and $CaCl_2$ (Goosen *et al.*, 1991). Generally the components of the $PEG/CaCl_2$ transformation mixture will include protoplasts at a density of approximately 10^8 to 10^9/ml, PEG, ± 5 µl/ml DNA, 10 or 50 mM calcium

chloride and a buffer (Lemke and Peng, 1995; Mullins and Kang, 2001; Ruiz-Díez, 2002). Successful transformation of fungi using the PEG/$CaCl_2$ method requires a certain amount of skill and optimisation since the production of viable protoplasts, which are competent to take up exogenous DNA, can be difficult. Consequently, a thorough understanding of the steps and components used in this method is required to obtain stable transformants. Some of the most critical components that will ultimately lead to success include the vectors used, cell wall digesting enzymes, as well as the specific wash buffers used for protoplast washing (Mullins and Kang, 2001). Many filamentous fungi have been transformed using the PEG/$CaCl_2$ method (Hynes, 1996), but the efficacy of the procedure may be low in some cases, which may limit the use of this system (Blakemore *et al.*, 1989; Daboussi *et al.*, 1989; Durand *et al.*, 1991).

Transformation vectors and promoters used in the PEG/$CaCl_2$ method. Generally, vectors used for fungal transformation experiments are comprised of *E. coli* plasmid DNA, which becomes integrated into the genome of the host after the transformation procedure (Van den Hondel and Punt, 1991). In order to select and obtain transformants, the vector must contain a gene that will convey a selective advantage to transformed cells that is absent in wild type cells. Therefore, transformants can be identified and selected by the specific gene, often referred to as a selectable marker gene, that is included in the vector and conveys a selection advantage to the transformed cells (Mullins and Kang, 2001; Miki and McHugh, 2004). In general, the selectable marker gene on the vector enables the transformed cells to grow in the presence of a specific antibiotic that is otherwise toxic to the wild type strain or non-transformed cells (Mullins and Kang, 2001; Miki and McHugh, 2004). In reporter gene transformations, the gene encoding the reporter gene can be present along with the selectable marker gene on the same vector, or the reporter gene can be present on a different vector than the selectable marker gene.

The specific promoter used within transformation vectors for expressing the gene of interest plays an important part in ultimately obtaining transformants, as well as obtaining high levels of reporter gene expression in transformants. A promoter is that part of a gene located in front, or upstream, at the 5'end of the DNA gene sequence, which drives expression of the gene and therefore determines when, where

and how much the gene is transcribed (Peberdy, 1989; Jansson, 2003). The promoters used in transformation vectors must be recognised by the specific fungal species into which it has been introduced. Additionally, the promoter is also often required to be constitutive and drive high levels of expression of the gene, especially when reporter genes are being expressed (Lorang *et al.*, 2001). The stronger the promoter driving transcription of a specific gene, the more protein will be made per cell (Tsien, 1998). However, regarding fungal transformation, the selection of an appropriate promoter often pose a problem since a limited number of fungal promoters have been characterised and most importantly, the strength of these promoters can vary considerably in heterologous fungi (Peberdy, 1989; Lorang *et al.*, 2001). Although higher levels of gene expression can sometimes be obtained by introducing more copies of the vector into the fungal genome, this is often difficult and can not substitute for a promoter being expressed at low levels or for not being recognised in specific fungal species (Tsien, 1998; Lorang *et al.*, 2001; Andrie *et al.*, 2005).

The use of a strong promoter for expression of reporter genes in vectors is very important, since this will yield transformants with an easily detectable phenotype. Currently, one of the most widely used promoters for driving expression of reporter genes in fungi is the *ToxA* promoter of *Pyrenophora tritici-repentis* (Maor *et al.*, 1998; Lorang *et al.*, 2001). Lorang *et al.* (2001) showed that this promoter is able to yield strong expression of the green fluorescent protein (GFP) reporter gene in eight different genera of plant pathogenic fungi. Recently, vectors have also been constructed showing that the *ToxA* promoter can drive high expression levels of the *EYFP*, *ECFP* and *mRFP1* fluorescent reporter genes in *P. tritici-repentis* and *Verticillium dahliae* (Andrie *et al.*, 2005).

The glyceraldehyde 3-phosphate gene (*gpd*) promoter is another promoter that has been widely used for the expression of GFP in filamentous fungi, proving to be strong and widely functional (Lemke and Peng, 1995). Maor *et al.* (1998) found that when the *gpd* promoter of *A. nidulans* drove expression of *SGFP*, fluorescence was considerably stronger than when using the *trpC* promoter of *A. nidulans*. Green fluorescence has been obtained in a wide range of fungi where GFP expression was driven by the *gpd* promoter, including *Aspergillus giganteus* (Meyer *et al.*, 2003), *A. nidulans* (Toews *et al.*, 2004), *C. rosea* (Lübeck *et al.*, 2002), *C. heterostrophus* (Maor *et al.*, 1998), *Colletotrichum destructivum* (Chen *et al.*, 2003), *Colletotrichum*

orbiculare (Chen *et al.*, 2003), *E. lata* (Bradshaw *et al.*, 2005), *F. oxysporum* f. sp. l*ycopersici* (Mes *et al.*, 1999; Nahalkova and Fatehi, 2003), *F. oxysporum* f. sp. *radicis-lycopersici* (Lagopodi *et al.*, 2002), *Fusarium verticilliodes* (Oren *et al.*, 2003), *Leptosphaeria maculans* (Sexton and Howlett, 2001), *Mycosphaerella* sp. (Balint-Kurti *et al.*, 2001), *Ophiostoma* sp. (Lee *et al.*, 2002), *Penicillium griseoroseum* (Lopes *et al.*, 2004), *Phanerochaete chrysosporium* (Ma *et al.*, 2001), *Pa. chlamydospora* (Bradshaw *et al.*, 2005), *Taloromyces* sp. (Jain *et al.*, 1992), *Trichoderma virens* (Sarrocco *et al.*, 2006) and *Venturia inaequalis* (Fitzgerald *et al.*, 2003). The *gpd* promoter has also been used to obtain successful red fluorescence in *Fusarium*, *Penicillium* and *Trichoderma* when driving expression of the DsRed-Express or DsRed2 genes (Mikkelsen *et al.*, 2003; Nahalkova and Fatehi, 2003). Recently, Bolwerk *et al.* (2005) also used the *A. nidulans gpd* promoter to drive expression of ECFP and YCFP in a *F. oxysporum* f.sp. *radicis-lycopersici* and a saprophytic *F. oxysporum* isolate. However, the fluorescence of YCFP was lower than GFP in *F. oxysporum* when viewed using confocal laser scanning microscopy, and was not used in further studies (Bolwerk *et al.*, 2005).

Various promoters have been used for driving expression of selectable marker genes, such as the hygromycin (*hph*) and phleomycin (*ble*) resistance genes, in fungal transformation vectors (Timberlake and Marshall, 1989). The *trpC* promoter of *A. nidulans* has been used to select *A. nidulans* transformants expressing the *hph* gene, and has also proved useful in other fungi (Cullen *et al.*, 1987). The *gpd* promoter of *Trichoderma reesei* was used to drive expression of the *ble* gene in a *Talaromyces* species (Jain *et al.*, 1992). *Rhynochosporium secalis* was successfully transformed using the *hph* gene from *E. coli* and the *ble* gene from *Streptoalloteichus hindustanus* under the control of *A. nidulans* promoter and terminator sequences (Rohe *et al.*, 1996). Mitotically stable *Ophiostoma piceae* and *Ophiostoma quercus* transformants were obtained by transforming the protoplasts with the *hph* gene under control of the *trpC* promoter of *A. nidulans* (Wang *et al.*, 1999). De Guido *et al.* (2003) were able to transform *Phomopsis viticola* with the *hph* gene driven by the *A. nidulans gpd* and *OliC* promoter. However, it was found that the *OliC* promoter was more effective than the *gpd* promoter, since more transformants were obtained with the *OliC* promoter (De Guido *et al.*, 2003).

Cell wall digestion enzymes. The use of the correct cell wall digesting enzymes is one of the most important components required for successful protoplasts production. Cell wall digesting enzymes perforate and degrade fungal cell walls yielding osmotically sensitive protoplasts that are able to take up DNA (Peberdy, 1989; Timberlake and Marshall, 1989; Goosen *et al.*, 1991).

Filamentous fungal protoplasts can be produced from various different tissue types, including germinating macro-conidia, micro-conidia, fragmented hyphal cells or whole mycelium (Rossier *et al.*, 1985; Parsons *et al.*, 1987; Wang *et al.*, 1988). Usually, young mycelium produced from germinating spores in liquid cultures or on plates with cellophane discs are used, since protoplasts released from hyphae by enzyme treatment can easily be separated from hyphal debris (Tilburn *et al.*, 1983; Yelton *et al.*, 1984; Feher *et al.*, 1986; Pentilla *et al.*, 1987; Timberlake and Marshall, 1989; Ruiz-Díez, 2002). The choice of cell type used for transformation is a matter of convenience. However the physiological state of the fungal cells used to produce protoplasts is important, and needs to be performed under consistent conditions (Fincham, 1989; Peberdy, 1989). The production of protoplasts from specific fungal tissue must be monitored constantly and the optimum timing and concentration have to be determined for each batch of enzyme, since different batches of the same enzyme can vary (Fincham, 1989; Ruiz-Díez, 2002).

The correct choice of enzyme combinations is a key factor in the transformation protocol, which must be determined experimentally for each fungal species and enzyme batch (Ruiz-Díez, 2002). A large set of commercially available enzymes is used in combination or alone for production of fungal protoplasts. The first cell wall digestion enzyme that was used for fungal protoplast production was a commercial snail stomach preparation (Helicase and Glusulase), which was used for the production of *S. cerevisiae* protoplasts (Beggs, 1978; Hinnen *et al.*, 1978). Subsequently, several other enzymes have been used including β-glucuronidase (Picknett *et al.*, 1987; Turgeon *et al.*, 1987), cellulases (Peberdy, 1985; Henson *et al.*, 1988), chitinase (Feher *et al.*, 1986; Binninger *et al.*, 1987), chitosanase (Revuelta and Jayaram, 1986), driselase (Picataggio *et al.*, 1983; Turgeon *et al.*, 1987), helicase (Tilburn *et al.*, 1983; Peberdy, 1985), lysing enzyme (Balance *et al.*, 1983), Novozym 234 (Hamlyn *et al.*, 1981; Cantoral *et al.*, 1987; Parsons *et al.*, 1987; Sanchez *et al.*,

1987), Streptozyme (Revuelta and Jayaram, 1986), Zymolyase (Parsons *et al.*, 1987) and in several cases an extracellular enzyme preparation from *T. harzianum* (Wernars *et al.*, 1985). Novozym 234, a hydrolytic enzyme mixture containing chitinase and 1, 3-glucanases secreted by *Trichoderma viridae* (Binninger *et al.*, 1987; Riach and Kinghorn, 1996), has been one of the most widely used enzymes for protoplast production since its introduction (Goosen *et al.*, 1991). This enzyme was very effective in consistently producing high quality protoplasts from many different filamentous fungal species (Timberlake and Marshall, 1989). However, the production of Novozyme 234 was discontinued in the late 1900's, leaving somewhat of a void in reliable and consistent cell wall digestion enzymes for production of fungal protoplasts.

Osmotic stabilisers used for preparation and washing of protoplasts. The correct osmotic stabiliser (buffer) used for production and washing of protoplasts is an important aspect of successful protoplast production and transformation (Peberdy, 1989). All protoplast preparations have to be protected by the presence of an osmotic stabiliser, since it stabilises the plasma membrane that remains after cell walls have been removed by cell-wall degrading enzymes (Fincham, 1989; Goosen *et al.*, 1991). Osmotic stabilisers are also used to wash enzyme solutions away from protoplasts, thus removing contaminating enzymes that may interfere with the transformation or regeneration procedures (Goosen *et al.*, 1991).

The first osmotic stabiliser used for stabilising protoplasts was 1 M sorbitol, which was used for the production of *S. cerevisiae* protoplasts (Fincham, 1989). Currently, sorbitol, at a concentration between 0.8 and 1.2 M, is most often used as an osmotic stabiliser for the production of protoplasts from most filamentous fungi (Fincham, 1989; May, 1992). Furthermore, mannitol, sucrose, magnesium sulphate and sodium chloride have also been used (Fincham, 1989; Goosen *et al.*, 1991; Ruiz-Díez, 2002).

DNA uptake through PEG and calcium chloride addition. The universal compound for transformation of fungi, apart from the DNA, is the calcium ion, except in non-protoplast-forming transformation procedures such as the lithium acetate and certain biolistic protocols (Fincham, 1989). The PEG/$CaCl_2$ transformation method involves incubating competent protoplasts for a short period of time (15-30 min) with

the vector DNA in the presence of Ca^{2+}, followed by the addition of PEG, which induces fusion (Ruiz-Díez, 2002). The DNA molecules are apparently internalised during fusion, as no transformation occurs when PEG is omitted (Timberlake and Marshall, 1989). PEG treatment causes the protoplasts to clump, thus possibly facilitating the trapping of DNA (Fincham, 1989; Ruiz-Díez, 2002). The concentration, amount and type of PEG used is critical and needs to be determined experimentally (Goosen *et al.*, 1991).

Protoplast regeneration and selection of transformed cells. The transformed protoplasts need to regenerate their cell walls once PEG has been added and the vector DNA molecules have been internalised in the protoplasts. Therefore, the transformed protoplasts are plated onto an osmotically balanced regeneration medium that selects for cells expressing the function supplied by the selectable marker gene on the added vector DNA molecules (Timberlake and Marshall, 1989; Ruiz-Díez, 2002). The essential requirement for obtaining growing colonies from protoplasts is the maintenance of the osmotic stabiliser in the growth medium until the cell wall has been regenerated (Fincham, 1989).

In general, the transformed cells are selected on the regeneration medium by means of their ability to grow in the presence of specific antibiotics or fungicides to which the wild type cells (untransformed cells or non-mutated organisms) are sensitive (Fincham, 1989; Ruiz-Díez, 2002). The specific antibiotic or fungicide on which the transformed cells can grow depends on the selectable marker gene with which the protoplasts were transformed (Miki and McHugh, 2004). Several antibiotics and fungicides, for which resistance genes have been cloned, are commercially available and have proved successful in the transformation of filamentous fungi including benlate, benomyl, β-Lactamase, carbendazim, geneticin, hygromycin B, kanamycin, neomycin, oligomycin, phleomycin and phosphinothricin (Banks, 1983; Kaster *et al.*, 1984; Turgeon *et al.*, 1985; Orbach *et al.*, 1986; Punt *et al.*, 1987; Skatrud *et al.*, 1987; Dickman, 1988; Wang *et al.*, 1988; Ward *et al.*, 1988; Timberlake and Marshall, 1989; Drocourt *et al.*, 1990; Lemke and Peng, 1995; Kim *et al.*, 1999; Mullins and Kang, 2001; Miki and McHugh, 2004). Hygromycin B, kanamycin and phosphinothricin have been used extensively and have been successful in a wide variety of fungal species (Lemke and Peng, 1995; Miki and McHugh, 2004;

Weld *et al.*, 2006). However, certain fungal species such as *Aspergillus* have showed resistance to these antibiotics and can therefore not be used for transformation of these fungi (Thomas and Kenerley, 1989; May, 1992). Hygromycin B is an amino glycoside antibiotic produced by *Streptomyces hygroscopicus*, which kills fungi, bacteria and higher eukaryotic cells by inhibiting the organisms' protein synthesis and it may also interfere with translocation, causing a mistranslation at the 70S ribosome (Singh *et al.*, 1979; Cabanas *et al.*, 1987; Gonzales *et al.*, 1987; Miki and McHugh, 2004).

Transformants will start growing on the selective regeneration media as single colonies after a few days (Goosen *et al.*, 1991). The appearance of so-called "abortive" colonies on the primary plates is the result of transient-transformants (transformants that did not integrate the reporter gene in the fungal genome) or unstable plasmid transcription (Cullen *et al.*, 1987; Punt *et al.*, 1987; Timberlake and Marshall, 1989; Goosen *et al.*, 1991; Hamada *et al.*, 1994; Sánchez-Torres *et al.*, 1994; Riach and Kinghorn, 1996). The initial transformant colonies will most likely be heterokaryons if the transformed cells were multinucleate conidia, or if protoplasts were made from multinucleate mycelial compartments (Fincham, 1989). Heterokaryotic transformants are transformants where some nuclei contain the transgenes and others don't, or where different nuclei were transformed in different ways (Lübeck *et al.*, 2002). The presence of heterokaryotic transformants are not desirable since nuclei that do not carry the plasmid with the selectable marker gene, will continue to divide and may dominate once the selection pressure has been removed, resulting in the loss of any transformed cells (Wiebe, 2003). The transformants obtained from multinucleate cells will often exhibit fluorescence in only some fungal cells that continued to grow (Lorang *et al.*, 2001). Therefore, purification of all transformants is usually required, which can be achieved laboriously by a few rounds of single sporing and sub-culturing (Wiebe, 2003).

***Agrobacterium tumefaciens*-mediated transformation.** *Agrobacterium tumefaciens*-mediated transformation (ATMT) is based on the principle that the plant pathogenic bacterium *A. tumefaciens* can transfer fragments of DNA into host genomic DNA. *Agrobacterium tumefaciens* harbours a Ti-plasmid that contains all the genes encoding the enzymes and proteins required for the introduction of a

fragment (T-DNA) of the Ti-plasmid into the genomic DNA of the host (Goosen *et al.*, 1991; Hooykaas and Beijersbergen, 1994; Bundock *et al.*, 1995; Piers *et al.*, 1996; De Groot *et al.*, 1998; Covert *et al.*, 2001; Fitzgerald *et al.*, 2003; Gelvin, 2003; Michielse *et al.*, 2005). Exogenous genes of interest can be introduced into the organism of choice by removing the "tumour inducing" genes encoded on the Ti-plasmid between the left and right border repeats, and replacing them with the exogenous genes of interest (De La Riva *et al.*, 1998). ATMT has been successfully applied in plants, yeast and filamentous fungi (Goosen *et al.*, 1991; Hooykaas and Beijersbergen, 1994; Bundock *et al.*, 1995; Piers *et al.*, 1996; De Groot *et al.*, 1998; Covert *et al.*, 2001).

ATMT is regarded as a highly efficient alternative to other conventional fungal transformation methods and has several advantages (Goosen *et al.*, 1991; De Groot *et al.*, 1998; Fitzgerald *et al.*, 2003; Meyer *et al.*, 2003). One of the main advantages is that it provides versatility in the starting material to transform, which can include protoplasts, spores, blocks of mycelial tissue and fruiting bodies (Timberlake and Marshall, 1989; Fitzgerald *et al.*, 2003; Michielse *et al.*, 2005). Other advantages include an easy to follow protocol and low set-up cost, no requirements for protoplasts, higher transformation efficiencies, large segments of DNA can be transferred with very little rearrangement of genomic DNA and single site or low gene copy number integration (Ishida *et al.*, 1996; Tinland, 1996; Covert *et al.*, 2001; Mullins *et al.*, 2001; Fitzgerald *et al.*, 2003; Michielse *et al.*, 2005). However, the ATMT protocol may take longer compared to other transformation protocols and the preparation of the bacterial cells can be time consuming (Gelvin, 2003).

Several studies have found that ATMT is a more efficient transformation method when compared to other transformation methods used for filamentous fungi. Meyer *et al.* (2003) attempted transformation of *Aspergillus giganteus* using four different transformation methods including PEG/$CaCl_2$, biolistic, electroporation and ATMT. The biolistic and electroporation method proved unsuccessful, whereas the ATMT method yielded higher transformation frequencies than the protoplast-based method and was therefore considered a more promising approach. Similarly, Fitzgerald *et al.* (2003) found that the PEG-method was superseded by ATMT when transforming *V. inaequalis*.

Biolistic transformation. Biolistic transformation was introduced in 1987 by Klein *et al.* (1987) and has been developed as a method for incorporation of plasmid DNA into intact, thick-walled fungi. Armaleo *et al.* (1990) was the first to prove that this transformation method could lead to stably inherited modifications of the nuclear genome of the yeast *S. cerevisiae*. Subsequently, biolistic transformation has been used for the transformation of several filamentous fungi including *A. nidulans* (Fungaro *et al.*, 1995; Gomes-Barcellos *et al.*, 1998), *Botryotinia fuckeliana* (Hilber *et al.*, 1994), *Cercospora caricis* (Aly *et al.*, 2001), *Gliocladium virens* (Lorito *et al.*, 1993), *M. grisea* (Riach and Kinghorn, 1996), *N. crassa* (Riach and Kinghorn, 1996), *T. harzianum* (Lorito *et al.*, 1993; Riach and Kinghorn, 1996), *T. reesei* (Hazell *et al.*, 2000; Te'o *et al.*, 2002), *Uncinula necator* (Smith *et al.*, 1992) and *V. inaequalis* (Parker *et al.*, 1995), yielding relatively high transformation efficiencies. Biolistic transformation has been especially important for transforming fungi for which protoplasting methods have not been developed due to a lack of knowledge on the fungal cell-wall components, or for fungi that do not grow sufficiently enough in laboratory cultures (Lemke and Peng, 1995; Durand *et al.*, 1997).

Biolistic transformation refers to a transformation protocol where tungsten, gold or platinum micro-projectiles, coated with nucleic acid (DNA), are shot into cells (Armaleo *et al.*, 1990; Hazell *et al.*, 2000; Tian and Seguin, 2004). The fungal tissue used for shooting DNA into, can include intact conidia and mycelium (Lorito *et al.*, 1993; Fungaro *et al.*, 1995; Aly *et al.* 2001; Robinson and Deacon, 2001; Meyer *et al.*, 2003). The micro-projectile particles, of which the size, type and density are of great importance, serve as vehicles (vectors) for DNA delivery (Tian and Seguin, 2004). The micro-projectiles should be accelerated to specific velocities in order to penetrate the target cells without too much disruption of the biological integrity of the cell. The vacuum used in the bombardment chamber as well as the distance the micro-particles have to travel before reaching the target cells also have a dramatic effect on the transformation efficiency (Te'o *et al.*, 2002). After the DNA has entered the cells, the DNA is integrated into the genome while the cells are repairing themselves after being damaged by the micro-projectiles (Rasmussen *et al.*, 1994).

The use of the biolistic transformation protocol has a few drawbacks, with one of the most important being the high setup cost. Furthermore, it is difficult to

measure the efficiency of the transformation, transformation parameters have to be optimised and preparation of the projectiles may be time-consuming (Fitzgerald *et al.*, 2003; Meyer *et al.*, 2003; Evans, 2006). Similar to other transformation methods, biolistic transformation may also yield mitotically unstable transformants, as has been found for *Aspergillus nidulans* (Gomes-Barcellos *et al.*, 1998).

Electroporation-mediated gene transformation. Electroporation-mediated gene transfer has been documented as being very effective for bacterial, lower eukaryotic as well as plant cells (Peberdy, 1989; Goosen *et al.*, 1991). Electroporation is a technique where cells (protoplasts or germinating conidia) are mixed with DNA and exposed to pulses of high electrical voltage (Hashimoto *et al.*, 1985). Prior to exposure to electrical voltages, conidial preparations are pre-treated with a cell wall weakening agent (Chakraborty *et al.*, 1991; Ozeki *et al*, 1994; Weld *et al.*, 2006). The pulses of high electrical voltage make the cell membranes permeable, allowing the DNA of interest to enter the fungal cells and express the gene of interest (Weld *et al.*, 2006). The specific pulses used should be correct, since pulses of the wrong length or intensity can cause some pores to become too large, or the pores may fail to close after membrane breakage, causing cell damage or rupture (Weaver, 1995).

Electroporation-mediated gene transformation has a high equipment setup cost and requires the optimisation of several parameters (Peberdy, 1989). This transformation method has successfully been used for the transformation of a few filamentous fungi, including *Aspergillus fumigatus* (Kwon-Chung *et al.*, 1998), *A. nidulans* (Sánchez and Aguirre, 1996), *A. niger* (Ozeki *et al.*, 1994), *Fusarium solani phaseoli* (Marek *et al.*, 1987), *N. crassa* (Chakraborty *et al.*, 1991), *Penicillium urticae* (Chakraborty *et al.*, 1991), *Scedosporium prolificans* (Ruiz-Díez and Martìnez-Suárez, 1999) and *U. maydis* (Bej and Perlin, 1988). The electroporation-method might be a good alternative method to the PEG/$CaCl_2$ method, if protoplast production is difficult, since swollen or geminated conidiospores can be used (Goosen *et al.*, 1991).

Lithium-acetate transformation. The lithium-acetate method (based on yeast transformation) was developed as an alternative transformation system for *N. crassa* (Ito *et al.*, 1983; Dhawale *et al.*, 1984; Timberlake and Marshall, 1989). The

main advantage of this system is that it avoids the laborious preparation of protoplasts (Van den Hondel and Punt, 1991). The principle on which the transformation method is based is that germinated *N. crassa* conidiospores can develop a "competence" when treated with lithium acetate, allowing uptake of exogenous DNA through permealised cell walls when the cells are incubated with exogenous DNA and PEG (Timberlake and Marshall, 1989; Goosen *et al.*, 1991). Short heat-shock exposures at 37°C before plating cells on plates containing selective medium proved to be essential for successful transformation of *N. crassa.* Although the procedure has proved successful for a few other filamentous fungi, it has not been widely adopted for other fungi (Dhawale *et al.*, 1984; Goosen *et al.*, 1991; Lemke and Peng, 1995). Moreover, in general no improvement with respect to the transformation frequencies of some fungi could be obtained (Goosen *et al.*, 1991).

ECOLOGICAL AND PATHOGENICITY STUDIES OF REPORTER GENE LABELED FILAMENTOUS FUNGI

Reporter genes have been used to investigate the ecology of a few biocontrol *T. harzianum* isolates, revealing important aspects of the conditions required for effective biocontrol. Bae and Knudsen (2000) and Orr and Knudsen (2004) respectively monitored and quantified the hyphal growth of a GUS- and GFP-tagged *T. harzianum* isolate (ThzIDI) in non-sterile soil. Their studies showed that the reporter gene-labeled biocontrol agent could be used to distinguish actively growing hyphal biomass from other inactive conidia or chlamydospores in non-sterile soil. Consequently, the important conclusion was made that failure in the biocontrol ability of *T. harzianum* in some natural field soils is due to the formation of mainly resting chlamydospores, with no active hyphae available for attacking the pathogen (Orr and Knudsen, 2004). *Trichoderma harzianum* ThzIDI labeled with GFP was also used to show that the increase of soil microbial biomass reduces the radial growth of the biocontrol agent in soil as well as its biocontrol efficiency, explaining why some natural soils are often resistant to introduced biocontrol agents (Bae and Knudsen, 2005). This specific *T. harzianum* transformant was also used to show that the presence of fungus-eating nematodes in soil can also influence the efficacy of *T.*

harzianum ThzIDI in non-sterile soil (Bae and Knudsen, 2001). *Trichoderma harzianum* strain T3 labeled with GUS was used to show that germination and hyphal growth of the biocontrol agent was only supported by pea and cucumber seed coats, decaying roots, and wounds, but not by exudates from healthy roots of various ages in natural soil (Green *et al.*, 2001).

Fluorescently labeled *Trichoderma* species have also been used to study the direct interaction of biocontrol agents with plant pathogens. *Trichoderma atroviride* labeled with GFP allowed studies on the interaction of this biocontrol agent with *Pythium ultimum* directly on cucumber seeds and roots in sterile soil (Lu *et al.*, 2004). The biocontrol agent was able to colonise the seed surface as well as the roots, with its growth being stimulated by the presence of *P. ultimum*. The hyphae of *T. atroviride* initially grew alongside pathogen hyphae, followed by the formation of papilla-like swellings at hyphal tips just prior to and when contacting pathogen hyphae on cucumber seeds. The study was able to show that a direct mycoparasitic interaction of *T. atroviride* with *P. ultimum* on cucumber seed surfaces was an important biocontrol mechanism, but that other mechanisms might also be involved. Sarrocco *et al.* (2006) investigated the mycoparasitic capability of a GFP labeled *T. virens* isolate against sclerotia of *Sclerotinia minor*, *Sclerotinia sclerotiorum* and *Sclerotium rolfsii*, and were able to show internal growth of the biocontrol agent in pathogen survival structures.

Clonostachys rosea (syn. *Gliocladium roseum*), a common saprophyte, has been shown to control a wide range of phytopathogenic fungi (Whipps, 1987; Sutton *et al.*, 1997; Lübeck *et al.*, 2002). Lübeck *et al.* (2002) used a GFP transformed *C. rosea* IK726 isolate to investigate the biocontrol agent's ecology (i) in field soil with and without inorganic and organic supplements; (ii) in vermiculite; (iii) on carrot seeds and roots; and (iv) on barley leaves. The study showed that *C. rosea* is able to thrive in different niches, including intact healthy root tissue and leaves. However, the biocontrol agent showed limited mycelial growth when inoculated into field soils, with most of the biomass being present as conidia unless an organic substrate was available as an exogenous nutrient (Lübeck *et al.*, 2002). Their study furthermore clearly showed that laser scanning confocal microscopy (LSCM), that allows optical

rather than mechanical sectioning of specimens, was superior for use in ecological studies when compared to epi-fluorescence microscopy.

Several studies have shown that reporter gene-labeled pathogenic fungi are very useful for studying fungal infection and colonisation of host tissue, as well as host responses. Doohan *et al.* (1998) was able to investigate micro- and macroscopic localisation of GUS-labeled *Fusarium culmorum* isolates within wheat tissue, as well as measuring pathogen biomass within the host tissue. *Fusarium subglutinans* GUS transformants were used to prove unequivocally that mango malformation disease was caused by this pathogen, and that it was most likely disseminated through pollen (Freeman *et al.*, 1999). A GFP-labeled isolate of the tomato foot and root rot causing pathogen, *F. oxysporum* f. sp. *radicis-lycopersici*, was used to show that there is a close interaction between pathogen hyphae and root hairs that are initially colonised, followed by colonisation of the main root, after which the pathogen grows along the intercellular junctions and colonises the crown (Lagopodi *et al.*, 2002). Oren *et al.* (2003) found similar results when studying the infection of maize seedlings by a GFP labeled *F. verticillioides* isolate in soil. They were also able to confirm that the pathogen is capable of moving from the soil into seedlings, and that it can spread to aboveground parts, although in such low amounts that it could not be observed microscopically, only through isolations onto marker gene selective media (Oren *et al.*, 2003). A GFP-labeled *Fusarium graminearum* isolate was used to reveal that the pathogen is very effective in colonising pollen and anthers of wheat. Furthermore, the study enabled the visualisation of slower and less extensive colonisation of the fluorescent pathogen through the parenchyma and vascular tissues of the rachis in a resistant versus a susceptible cultivar (Miller *et al.*, 2004).

Reporter gene-labeled pathogenic fungi have also been used to investigate infection and colonisation of leave and other above ground host tissue. Du *et al.* (1999) was able to show that *Aspergillus flavus*, labeled with a *GFP* gene and driven by the promoter of an aflatoxin biosynthetic regulatory gene, could be used for the fast and economical screening of the resistance of maize genotypes to aflatoxin accumulation (Du *et al.*, 1999). The infection of a GFP-labeled *C. heterostropus* transformant was used to show that pathogen hyphae inside a leaf form a unique parallel growth pattern, which is apparently mediated by the anatomy of the leaf. The

fungal biomass as well as disease levels could further be calculated using PMIS image analysis software (Photometrics, Tucson, Arizona) (Maor *et al.*, 1998).

Differentially dual labeled fluorescent pathogenic fungi and biocontrol agents have also been used to study their interactions within host tissue and in soil. This is possible due to the exceptional spectral properties of some fluorescent proteins that allow their simultaneous visualisation. Olivain *et al.* (2006) studied the simultaneous colonisation of DsRed2-labeled pathogenic and GFP-labeled non-pathogenic *F. oxysporum* strains at the tomato root surface in soil using confocal laser scanning microscopy. It was found that when roots were in soil, the pathogen and biocontrol agent colonised the entire root surface, except the apical zone. These results were markedly different from results found in previous colonisation studies conducted in hydroponic systems (Olivain and Alabouvette, 1997; Olivain and Alabouvette, 1999). Furthermore, it became evident that there is competition for nutrients, rather than for infection sites since the biocontrol agent was not able to exclude the pathogen from the root surface (Olivain *et al.*, 2006). Bolwerk *et al.* (2005) also studied the interaction of pathogenic and non-pathogenic *Fusarium* isolates in tomato using EGFP and ECFP as fluorescent labels. Their study concluded that competition for nutrients as well as attachment sites were important. However, this was only true when the concentration of the biocontrol strain was 50-fold higher than that of the pathogen, since the biocontrol agent was a poorer root coloniser than the pathogen. Therefore, systemic induced resistance is probably also an important mechanism of biocontrol (Bolwerk *et al.*, 2005).

The information presented here clearly indicates that fluorescently labeled pathogenic and biocontrol fungi can be used effectively to study the ecology of biocontrol agents, as well as the interaction of biocontrol and pathogenic fungi with each other and the host. However, it is clear that although there have been substantial advances in the use of fluorescent labels for studying fungal pathogens and biocontrol agents within soilborne and foliage systems (Tegli *et al.*, 2000; Larrainzar *et al.*, 2005), similar studies on trunk disease and canker causing pathogens are limited. The only reporter gene-labeled fungus that has been used to study fungi within aboveground wood tissue is the sapstain fungus, *Ophiostoma piceae*, and a potential biocontrol agent, *Ophiostoma piliferium*, which were transformed with GFP (Lee *et*

al., 2002). Therefore, more studies need to be initiated in wood pathogen-host-biocontrol systems. Ultimately, this methodology will improve our understanding of the ecology and host-pathogen-biocontrol interactions of wood colonising fungi.

TRUNK DISEASE PATHOGENS

Trunk disease pathogens cause a slow decline and loss of yield in grapevines at all stages of growth (Ferreira *et al.*, 1989; Halleen and Fourie, 2005). Vine decline caused by these pathogens has been given various names, including black measles, esca, Petri disease, apoplexy and black goo disease (Mugnai *et al.*, 1999). The disease is caused by a complex of pathogens including basidiomycetes, species of Botryosphaeriaceae (including *Botryosphaeria* and aggregate genera, *Lasiodiplodia* and *Neofusicoccum*), *E. lata*, *Phaeoacremonium* spp., *Pa. chlamydospora*, and *P. viticola* (Munkvold and Marois, 1995; Mugnai *et al.*, 1999; Van Niekerk *et al.*, 2004; Van Niekerk *et al.*, 2005; Crous *et al.*, 2006; Fischer, 2006; Mostert *et al.*, 2006a, b; Van Niekerk *et al.*, 2006). In the following section emphasis will only be placed on two trunk disease pathogens, *Pa. chlamydospora* and *P. viticola.*

Phaeomoniella chlamydospora. Decline and dieback of young (1-5 year old) grapevines have been reported in many grape-growing countries (Fourie and Halleen, 2002). One of the main causal agents is *Pa. chlamydospora* that primarily causes Petri grapevine decline, which was formerly also known as Black goo and slow dieback (Mugnai *et al.*, 1999; Crous and Gams, 2000; Fourie *et al.*, 2000). The pathogen is further also implicated along with *Phaeoacremonium* spp. in the Esca disease complex (Crous *et al.*, 1996; Mugnai *et al.*, 1999; Crous and Gams, 2000; Mostert *et al.*, 2006b). *Phaeomoniella chlamydospora* predisposes the grapevine tissue to infection by other wood-rotting fungi, i.e. *Fomitiporia punctata*, as the plant matures resulting in the cause of Esca disease (Mugnai *et al.*, 1999). Altogether these pathogens cause a severe loss in the grapevine industry due to reduction in quantity and quality in yield as well as a reduced lifetime of grapevines.

Typical external symptoms of Petri disease include stunted growth, interveinal chlorosis, leaf necrosis, leaf roll, small leaves, shorter internodes, smaller trunks and

branches with a general decline of young vines, which eventually results in dieback (Morton, 1995; Bertelli *et al.*, 1998; Fourie *et al.*, 2000; Fourie and Halleen, 2002). These slow dieback symptoms have often been incorrectly attributed to many other abiotic factors. Internal wood symptoms are more characteristic to *Pa. chlamydospora*. These symptoms include vascular streaking with brown to black discoloration which is caused by the formation and accumulation of tyloses, gums and phenolic compounds in xylem vessels as part of a defense response of the grapevine host to pathogen infection (Mugnai *et al.*, 1999; Fourie *et al.*, 2000).

Several important inoculum sources of *Pa. chlamydospora* have been identified. Numerous studies have shown that *Pa. chlamydospora* is mainly distributed through infected propagation material (Bertelli *et al.*, 1998; Groenewald *et al.*, 2001; Halleen *et al.*, 2003). Symptom development in the propagation material only develops later, possibly as a result of stress-induced stimulation of the asymptomatic latent infections (Gubler *et al.*, 2004). Airborne spores are also thought to be an important inoculum source, since *Pa. chlamydospora* has been shown as being a very aggressive coloniser of grapevine pruning wounds, which is the main entry portals for the pathogen (Crous and Gams, 2000; Larignon and Dubos, 2000; Whiteman *et al.*, 2002). Another potential inoculum source is chlamydospores that might be able to survive for long periods in plant debris. However, little information is available on the survival rate of these structures in the soil, and whether the pathogen can be considered as having a soil-borne phase (Fourie *et al.*, 2000; Ridgway *et al.*, 2005).

Limited knowledge exists on the disease cycle of Petri disease, the actual mode of infection, infection pathways as well as host response mechanisms. This might be attributed to the slow growing characteristic of *Pa. chlamydospora* in the host and on artificial media, as well as the fact that grapevines are often simultaneously colonised by several other fungi, including non-pathogenic endophytes. Comprehensive knowledge of the *in vivo* interactions of *Pa. chlamydospora* and the grapevine host could ultimately lead to improved and sustainable management strategies.

Phomopsis viticola. *Phomopsis viticola* is the causal agent of a disease called Phomopsis cane and leaf spot (Pearson and Goheen, 1994; Mostert *et al.*, 2001). This disease occurs in most of the grape-growing areas where it can cause reduced yields

in susceptible cultivars. Symptoms that are generally associated with cane and leaf spot include dark fissure-like lesions on green shoots (which crack and scar as the canes swell and harden), as well as bleaching and lesions on canes. The shoot lesions eventually result in the formation of mechanically weakened shoots that may break off during strong winds (Melanson *et al.*, 2002). Small necrotic spots form on leaves that are usually surrounded by yellow halos (Melanson *et al.*, 2002). These spots may fall out of the leave and cause a "shot hole" appearance. Eventually some leaves may become distorted and fall off (Pearson and Goheen, 1994).

Phomopsis viticola has been implicated in the trunk disease complex in grapevines since it has been isolated from young asymptomatic nursery vines (Halleen *et al.*, 2003), and has also been frequently isolated from trunk disease symptoms in grapevine pruning wounds (Bester, 2006). Van Niekerk *et al.* (2005) described several *Phomopsis* spp. associated with grapevines, but demonstrated that *Phomopsis amygdali* (a pathogen of peaches in the USA) and *P. viticola* proved to be the most virulent in a detached shoot assay.

Phomopsis viticola generally overwinters as mycelium and pycnidia inside infected grapevine canes, spurs, pruning wounds and dormant buds (Pearson and Goheen, 1994). In the case of cane and leaf spot, *P. viticola* can also penetrate uninjured young shoots or leaves directly (Pearson and Goheen, 1994). However, within the trunk disease complex, the role of *P. viticola* as a primary pathogen, as opposed to a secondary wound invader, as well as its mode of infection and survival, still needs to be elucidated.

TRICHODERMA SPP. AS BIOLOGICAL CONTROL AGENTS

The potential of *Trichoderma* spp. as biological control agents was first recognised in the early 1930s. Since then it has received considerable attention as a potential biological control agent of a wide range of root, seed, foliar and storage rot diseases (Thrane *et al.*, 1995; Lo *et al.*, 1998; Harman, 2000; Hanson and Howell, 2004). *Trichoderma* spp. is considered to be opportunistic, fast-growing and avirulent

plant symbionts that are ideally suited as biocontrol agents (Harman, 2006; Woo *et al.*, 2006).

Trichoderma spp. currently comprise one-third of all the fungal biocontrol products produced and sold for the control of diseases of horticultural crops. Commercial products of biological control agents differ fundamentally from chemical fungicides, since these products must grow, multiply and establish in order to be effective in the applied environment. *Trichoderma* spp. achieve biological control of fungal plant pathogens through various mechanisms including mycoparasitism, antibiosis, production of cell-wall lytic enzymes, competition and induction of systemic acquired resistance (Lo *et al.*, 1998; Harman, 2000; Steyaert *et al.*, 2003; Harman *et al.*, 2004; Lu *et al.*, 2004; Carpenter *et al.*, 2005; Harman, 2006; Howell, 2006). These employed mechanisms vary according to the biological control isolate used, the pathogen and the host involved in the interaction (Howell, 2003).

In grapevines, *Trichoderma* spp. have been shown to form a living barrier in pruning wounds against pathogen invasion (Di Marco *et al.*, 2004; John *et al.*, 2004). Although this kind of biological strategy is gaining more ground and extensive studies have been done on *Trichoderma* as wound protectant (Fourie *et al.*, 2001; Hunt, 2003), controversy surrounds the recommendation of this agent as pruning wound protectant (John *et al.*, 2004). Studies still need to show that *Trichoderma* will provide better long-term protection than fungicides, and that the fungus can establish and colonise grapevine pruning wounds under a wide range of environmental conditions (John *et al.*, 2004). The implementation of *Trichoderma* spp. as commercial products against trunk disease pathogens will require a thorough understanding of their host colonisation, host interactions and ecology (Thrane *et al.*, 1995).

Future studies should determine whether *Trichoderma* spp., especially *T. harzianum*, can be considered as an aggressive coloniser of grapevine wound tissue, especially in the presence of several grapevine trunk disease pathogens and endophytic fungi. These studies can be facilitated through the use of reporter gene-labeled *T. harzianum* isolates, which will facilitate the visualisation of these interactions within the grapevine host.

CONCLUSION

Fungal trunk disease pathogens are an enormous threat to the grapevine industry. Currently, no cost effective management strategies are available for these pathogens. Therefore, management strategies need to be improved in order to positively influence the life-expectancy and physiology of the grapevine host. The use of biological control agents, such as *Trichoderma* spp., have the potential to provide a long-term sustainable control method of these pathogens. However, knowledge on the ecology, host interactions and host colonisation of the pathogens and biocontrol agent is required in order to establish a reliable management strategy.

The aim of this study was to develop transformation methods for obtaining stable *Pa. chlamydospora*, *P. viticola* and *T. harzianum* transformants expressing two fluorescent protein reporter genes. These transformants were characterised and compared to the respective untransformed wild type isolates in order to ensure that the transformation process has not altered their growth, morphology, pathogenicity or biocontrol efficiency. The transformed and characterised pathogens and biocontrol agents will be used in future research projects, which aim to investigate the mode of infection and survival of these fungi in and around the economically important grapevine host, as well as their interaction with each other. Ultimately this research will help to unravel some important aspects of the grapevine trunk disease complex and the potential biocontrol thereof.

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2. STABLE TRANSFORMATION OF *PHAEOMONIELLA CHLAMYDOSPORA* WITH THE GREEN (GFP) - AND RED (DSRED-EXPRESS) FLUORESCENT PROTEIN GENES

ABSTRACT

The trunk disease pathogen, *Phaeomoniella chlamydospora*, causes Petri disease in *Vitis vinifera* and is also associated with the Esca trunk disease complex. Little is known about the pathogen's epidemiology and interactions with the grapevine host, other pathogens in the trunk disease complex and biological control agents. The labeling of fungi with reporter genes has opened up new avenues for studying these aspects. Therefore, the aim of this study was to stably transform a South African *Pa. chlamydospora* isolate with the green fluorescent protein (GFP) and the red fluorescent (DsRed-Express) protein reporter genes, using a polyethylene glycol/calcium chloride transformation method and the selectable marker gene, hygromycin phosphotransferase (*hph*). The transformation method yielded a low transformation frequency of one transformant per microgram of vector DNA. A selected subset of the fluorescent transformants was shown to be stably transformed, since they retained their fluorescent phenotype after successive sub-culturing onto non-selective medium. The presence of the transgenes was also confirmed through polymerase chain reaction amplifications. Two of the stable, highly fluorescent GFP (PcG1 and PcG10) and DsRed-Express (PcR1 and PcR2) transformants as well as a *Pa. chlamydospora* GFP transformant (pCT74-P7) from New Zealand and their respective wild type isolates were characterised further. Comparative studies on the colony colour, spore size, germination percentage and growth rate at different temperatures revealed no differences between the transformants and their respective wild type isolates. The only exception was the significant lower germination percentage of transformant PcG10, and the different colony colour of transformants PcG1 and PcR1, when compared with the wild type isolate. The virulence of three of reporter gene transformants (PcG1, PcR1 and pCT74-P7) and their respective wild type isolates were determined on Chenin blanc grapevine cuttings, which showed that

lesion lengths caused by transformants did not differ from those caused by the wild type isolates.

INTRODUCTION

The fungus *Phaeomoniella chlamydospora* primarily causes Petri grapevine decline in young vines (Mugnai *et al.*, 1999; Crous and Gams, 2000; Graniti *et al.*, 2000; Ridgway *et al.*, 2002; Edwards and Pascoe, 2004; Gubler *et al.*, 2004). *Phaeomoniella chlamydospora* has furthermore been implicated along with *Phaeoacremonium* spp. in the grapevine trunk disease complex (Crous *et al.*, 1996; Mostert *et al.*, 2006). It is known that *Pa. chlamydospora* predisposes maturing grapevine tissues to infections by wood-rotting basidiomycetes, such as *Fomitipora mediterranea*, which eventually results in Esca disease (Fischer, 2002; Mugnai *et al.*, 1999). The etiology of this disease is complex, since other fungi, or combinations of them, have also been implicated in the grapevine decline syndrome (Munkvold and Marois, 1995; Graniti *et al.*, 2000; Groenewald *et al.*, 2001; Halleen *et al.*, 2003; Van Niekerk *et al.*, 2004; Van Niekerk *et al.*, 2005; Crous *et al.*, 2006; Fischer, 2006; Mostert *et al.*, 2006).

Petri (1912) first reported decline in young grafted vines and mother plants in Italy. The author was able to successfully reproduce the typical internal disease symptoms of brown/black wood-streaking, by injecting the xylem of healthy vines with two fungi that were later identified as *Phaeoacremonium chlamydosporum* and *Phaeoacremonium aleophilum* (Crous *et al.*, 1996). The taxonomic status of *Phaeoacremonium chlamydosporum* has subsequently been re-investigated, and it is now known as *Phaeomoniella chlamydospora* (Crous and Gams, 2000). This infamous decline and dieback disease has since been reported in Australia (Pascoe and Cottral, 2000), Italy (Mugnai *et al.*, 1999), New Zealand (Clearwater *et al.*, 2000), Portugal (Rego *et al.*, 2000), South Africa (Ferreira *et al.*, 1994), Spain (Armengol *et al.*, 2001) and the United States of America (Morton, 1995).

Numerous studies have identified a few conclusive inoculum sources of Petri disease, of which infected propagation material, i.e. mother vines, is most important

(Bertelli *et al.*, 1998; Mugnai *et al.*, 1999; Pascoe and Cottral, 2000; Groenewald *et al.*, 2001; Halleen *et al.*, 2003; Ridgway *et al.*, 2003; Edwards *et al.*, 2004; Retief *et al.*, 2006). Infections of mother vine cuttings increases substantially in nurseries, since pathogen inoculum is spread through pre-storage hydration and grafting water, as well as callusing medium (Whiteman *et al.*, 2003; Retief *et al.*, 2006). The disease is most likely expressed in grafted nursery plants as the result of stress-induced stimulation of asymptomatic latent infections, since *Pa. chlamydospora* is thought to behave as an endophyte or latent pathogen that takes advantage of host stress conditions (Ferreira *et al.*, 1999; Serra *et al.*, 2000; Gubler *et al.*, 2004). *Phaeomoniella chlamydospora* conidia are also considered as a source of inoculum. Larignon and Dubos (2000) suggested that conidia could be airborne, and can thus be dispersed from infected vineyards (Whiteman *et al.*, 2002). Airborne conidia will infect the host through pruning wounds, followed by aggressive wound colonisation (Crous *et al.*, 1996; Adalat *et al.*, 2000; Larignon and Dubos, 2000).

Soil is thought to be another potential inoculum source of *Pa. chlamydospora*, since the pathogen has been detected in vineyard soils through the use of polymerase chain reaction amplifications (Tegli *et al.*, 2000; Whiteman *et al.*, 2002; Whiteman *et al.*, 2003; Damm *et al.*, 2004; Retief *et al.*, 2006). Ridgway *et al* (2005) has found that pathogen conidia can survive for 17 months in soil. Furthermore, since chlamydospores might be able to survive for long periods in plant debris, these spores may also play an important role in the soil ecology of the pathogen (Fourie *et al.*, 2000). However, the significance of soilborne inoculum is still unknown and needs further investigation.

The exact mode of spread of *Pa. chlamydospora* within host tissue is uncertain, as well as how infections spread from old infected pruning wounds to new wounds. Edwards *et al.* (2003) detected spores and hyphal fragments along the full length of canes and therefore suggested that the spores are carried in the sap flow (xylem tissue) of infected mother vines. This pathogen invasion of the xylem will cause the grapevine host to produce balloon-like tyloses and gummosis, which can be seen as black discolouration and brown/black streaking (Mugnai *et al.*, 1999; Fourie *et al.*, 2000). The tyloses block the xylem vessels and eventually stop water and mineral flow (Mugnai *et al.*, 1999; Fourie *et al.*, 2000). Pascoe and Cottral (2000)

studied the colonisation of *Pa. chlamydospora* in grapevine tissue culture plants, following infection through a wound. The pathogen was found only in the xylem parenchyma neighbouring the vessels in the inoculated area, suggesting that these infected cells produce the tyloses (Pascoe and Cottral, 2000).

Reporter genes will allow investigations into *Pa. chlamydospora*'s inoculum sources and its mode of infection and colonisation and spread in susceptible and resistant host cultivars. The green fluorescent protein (GFP) derived from the jellyfish *Aequorea victoria* has been developed to function as a reporter gene for expression in fungi (Chalfie *et al.*, 1994). It has been successfully expressed in various organisms and proved to be a useful tool to monitor and detect fungal strains in their natural environment (Lorang *et al.*, 2001). Bradshaw *et al.* (2005) have successfully transformed *Pa. chlamydospora* with *GFP*, but they did not characterise the transformants, nor used them in any infection studies. The *DsRed-Express* reporter gene, derived from the reef coral *Discosoma* sp. (Matz *et al.*, 1999) is suited for dual labeling of fungi with *GFP*, allowing the discrimination of two different fungi within the same environment (Baird *et al.*, 2000; Mikkelsen *et al.*, 2003; Nahalkova and Fatehi, 2003).

The purpose of this study was to stably transform a South African *Pa. chlamydospora* isolate with the *GFP* or *DsRed-Express* reporter genes. Transformants expressing these reporter genes, along with a GFP-labeled isolate from New Zealand (Bradshaw *et al.*, 2005) and the wild type isolate from which it was derived, were characterised further to establish whether the transformation process has altered their morphology, physiology and virulence. The characteristics that were studied included colony colour, conidial size, germination percentage, growth rate at different temperatures and virulence.

MATERIAL AND METHODS

Growth and culturing of isolates. The *Pa. chlamydospora* isolate (STE-U 5784) used in the transformation studies was collected from a vineyard in Vredendal in the Western Cape province of South Africa, and will hereafter be referred to as PcWSA. A *Pa. chlamydospora* culture isolated from a grapevine in New Zealand,

hereafter referred to as PcWNZ, as well as a GFP-expressing transformant (pCT74-P7) derived from this isolate, was kindly provided by R.E. Bradshaw (National Centre for Advanced Bio-Protection Technologies, Institute of Molecular BioSciences, Palmerston North, New Zealand). The isolates were routinely cultured on potato dextrose agar (PDA) (Difco Laboratories, MI, USA) and incubated at 25°C in the dark. The wild type cultures as well as the transformed isolates were stored in sterile distilled water as mycelial plugs, as well as in glycerol at -80°C.

Screening for hygromycin B sensitivity. The specific hygromycin B concentration required to inhibit the wild type (non-transformed) PcWSA isolate was determined by first amending 90-mm PDA plates with different hygromycin B concentrations, which included concentration of 20 µg/ml, 40 µg/ml and 80 µg/ml. A mycelial plug (5-mm-diameter) from a 10-day-old culture of PcWSA was plated onto each of these plates, as well as a control plate containing no hygromycin B, in triplicate. The Petri dishes were incubated in the dark at 25°C for 7 days, whereafter the presence or absence of mycelial growth was evaluated.

Plasmid vectors. Vector pCT74 was kindly provided by J.M. Lorang (Oregon State University, Oregon, USA). The vector contains the *SGFP* gene, that is a variant of the *GFP* gene from the jellyfish *Aequoria victoria*, fused to the *Pyrenophora tritici-repentis Tox A* promoter, as well as the hygromycin phosphotransferase (*hph*) gene fused to the *trp-C* promoter of *Aspergillus nidulans* (Lorang *et al.*, 2001). The plasmid pPgpd-DsRed was kindly provided by L. Mikkelsen (The Royal Veterinary and Agricultural University, Frederiksberg, Denmark). Vector pPgpd-DsRed contains the *DsRed-Express* gene under control of the constitutive *A. nidulans glyceraldehyde 3-phophate* promoter (*gpdA*), with expression being terminated by the *A. nidulans trpC* transcriptional terminator (Mikkelsen *et al.*, 2003). Since vector pPgpd-DsRed does not contain the selectable marker gene, co-transformation with vector pHyg8 (provided by A. McLeod, Stellenbosch University, South Africa) was conducted. Vector pHyg8 was constructed by cloning the blunt-ended *Sal*I fragment from pCT74, containing the *hph* gene driven by the *A. nidulans trp*C promoter, into pBluescript (Stratagene, La Jolla, CA) digested with *Eco*RV. All plasmids used for the transformation of protoplasts were isolated using the PureYield Plasmid Midiprep

System (Promega Corporation, Madison, WI, USA), according to manufacturer's instructions.

Transformation of Pa. chlamydospora

Protoplast preparation. The transformation procedure was based on that of Lu *et al.* (1994). PcWSA was grown for 14 days on PDA at 25°C in the dark. Spores were harvested from the plates by flooding the plates with sterilised distilled water, followed by filtration through a layer of Miracloth (Calbiochem, La Jalla, CA, USA). The spore concentration was adjusted to 2×10^6 spores/ml, and combined in a 1:1 ratio with 2 × 2YEG (0.8 g Yeast extract, 4 g glucose and 200 ml distilled water). The spore suspension was incubated for 36 hours at 28°C at 140 rpm to allow germination.

Protoplasts were produced from the germinated spores using cell wall digesting enzymes. Several enzyme concentrations and combinations were tested (unpublished data), of which the best combination consisted of 100 mg/ml lysing enzyme from *Trichoderma harzianum* (Sigma-Aldrich, Aston Manor, South Africa), 40 mg/ml driselase (Sigma-Aldrich) and 2 mg/ml chitinase (Sigma-Aldrich). Prior to dissolving the enzymes in 0.7 M NaCl, the starch carrier was removed from the driselase by incubating the driselase mixed with 20 ml of 0.7 M NaCl on ice for 15 min, followed by centrifugation at 652 g for 5 min. The decanted driselase-NaCl suspension was then added to the other enzymes. Hundred millilitres of the germinated spores were pelleted through centrifugation at 805 g for 10 min (Eppendorf centrifuge 5810R; Eppendorg, Hamburg, Germany). The pellet was washed with 30 ml 0.7 M NaCl using the same centrifugation settings. The washed pellet was re-suspended in 20 ml of the enzyme solution in a 50-ml Falcon tube, and incubated for 1 hour at 28°C with mild shaking at 50 rpm, until a sufficient number of protoplasts were released, after approximately 1 hour. The protoplasts were pelleted through centrifugation at 805 g for 10 min at 5°C, and were subsequently washed in 20 ml 0.7 M NaCl using the same centrifugation conditions. In the final two steps the pellet was washed twice in cold STC (1.2 M Sorbitol, 50 mM $CaCl_2$, 500 mM Tris-HCl pH 7.5), and the protoplasts adjusted to a final concentration ranging from 1.7×10^7/ml to 2×10^7/ml.

Transformation of protoplasts. GFP transformation was conducted using 10 µl of vector pCT74, whereas DsRed-Express transformation was conducted using a co-transformation with 10 µl of vector pHyg8 and 20 µl of vector pPgpd-DsRed for each 100 µl of protoplasts. A negative control consisting of protoplast receiving no vector DNA was also included to ensure that the wild type nuclei were suppressed. The protoplasts (100 µl) and plasmid suspension was incubated in 12 ml centrifuge tubes on ice for 10 min, whereafter a sterile polyethylene glycol calcium chloride solution [12 g Fluka PEG 4000 (Fluka, Taufkirchen, Germany), 400 µl mM Tris-HCl (500 mM) pH 7.5, 1 ml $CaCl_2$ (1 M) and 6.6 ml distilled water] was added in three aliquots of 200 µl, 200 µl and 800 µl with gentle mixing between each addition at room temperature. The suspension was incubated for another 10 min at room temperature followed by the addition of 2 ml STC to each tube. Two millilitres or 500 µl of this solution, depending on the protoplast concentration, were added to 20 ml molten (55°C) regeneration media [three part medium consisting of solution A (250 mg yeast extract, 250 mg casein hydrolysate, 12.5 ml water), solution B (85.5 g sucrose, 12 ml distilled water, and solution C (4 g agar, 112.5 ml water) that were autoclaved separately before being mixed], which was carefully poured into a 90-mm Petri plate. The plates were incubated overnight at 25°C, and subsequently overlayed with 1% water agar (WA) containing 40 µg/ml hygromycin B. The plates (primary transformation plates) were again incubated at 25°C with transformants emerging 14 days later through the selective WA layer. Ten emerging colonies from the primary transformation plates were transferred to fresh selective PDA plates containing 40 µg/ml hygromycin B.

Epifluorescence microscopy. The *Pa. chlamydospora* hygromycin B resistant colonies were tested for GFP and DsRed-Express fluorescence using an epifluorescence Zeiss Axioscope (West Germany) microscope. Actively growing mycelium and conidia of transformants were used for microscopic observations. The wild type strain was used as negative control to ensure that no endogenous or auto fluorescence was present. The examined tissues were placed on a microscope slide, submerged in a water droplet, and covered with a glass cover slip. GFP fluorescence was viewed using a Endow GFP BP filter with excitation filter 470 nm, emission filter 525 nm and beam splitter of Q495lp (Chroma Technology Corp., Rockingham, VT, USA). DsRed-Express fluorescence was detected using a HQ:TRITC filter with

excitation filter of 545 nm, emission filter of 620 nm and beam splitter Q570lp (Chroma Technology Corp.). Images were captured with a Nikon digital camera DXM1200 and Automatic Camera Tamer (ACT-1) computer software. This was used without further manipulation of the objects being studied.

Selection of stable transformants. A subset of the transformants, five GFP and five DsRed-Express transformants, were tested for stable transformation. Prior to stability testing, each transformant colony was first single spored twice on PDA plates containing 40 µg/ml hygromycin B, in order to eliminate heterokaryons. GFP and DsRed-Express expression were tested microscopically after each successive generation. The stability of the transformants was tested by transferring a mycelial plug of each transformant for a further three consecutive generations onto non-selective media (PDA without hygromycin B) at 10 to 14 days intervals. The *Pa. chlamydospora* transformants were then transferred for a final generation onto selective PDA medium and tested for GFP and DsRed-Express expression.

Polymerase chain reaction (PCR) detection of reporter genes and hygromycin resistance genes. The five GFP and five DsRed-Express transformants that were stably transformed, as well as the wild type isolate PcWSA, were tested for the presence of the transgenes (*hph*, *DsRed-Express* and *GFP*) using PCR analyses. DNA used for the PCR analyses was obtained from mycelium of the isolates that were grown on non-selective media (PDA) for 14 days in the dark at 25°C. DNA was extracted using the Wizard® SV Genomic DNA Purification system (Promega Corporation, Madison, WI, USA) according to manufacturer's instructions.

Gene specific primer pairs were used to investigate the presence of the *GFP*, *DsRed-Express* and *hph* genes. *GFP* was detected using primer pair GFP1 and GFP2 (Lorang *et al.*, 2001). *DsRed-Express* was amplified using primer pair DsF (5' ATG GCC TCC TCC GAG GAC 3') and DsSeq (5' GTA CTG GAA CTG GGG GGA CAG 3'), which were designed based on the vector sequence of plasmid pDsRed-Express (Clontech Laboratories, Palo Alto, CA, USA). The *hph* gene was amplified with primer pair HygF (GGA TGC GAT CGC TGC GGC CG) and HygR (CTA TTC CTT TGC CCT CGG ACG), which were designed based on the Genbank accession AY142483. Each PCR amplification reaction consisted of a final volume of 40 µl containing 1 × Buffer (Bioline, Celtic Molecular Diagnostics, Wynberg, Cape Town,

South Africa), 15 mM $MgCl_2$, 0.2 µm each of the relevant primer pair, 0.2 mM dNTP, 1 mg/ml bovine serum albumib (BSA) Fraction V (Roche Diagnostics South Africa, Randburg, South Africa), 1 U *Taq* (Bioline), ddH_2O and 10 µl DNA. Amplification conditions consisted of an initial denaturation step at 94°C for 5 min, followed by 40 cycles each consisting of 30 s at 94°C, 30 s at 55°C (for GFP), 66°C (DsRed-Express) or 64°C (*hyg*), 90 s at 72°C, and a final extension step at 72°C for 7 min. Negative controls consisted of DNA of the wild type isolate (PcWSA) as well as a control receiving only sterile water and no template DNA. The positive controls consisted of plasmid DNA of pCT74 for *GFP* and *hph* gene amplifications, and plasmid pPgpd-DsRed for *DsRed-Express* amplifications.

***In vitro* biological characterisation of transformed and wild type isolates.**

The biological characteristics colony colour, conidial germination percentage, spore morphology and growth rate were studied in the two wild type *Pa. chlamydospora* isolates PcWSA and PcWNZ, as well as in five fluorescent transformants. The fluorescent transformants included two stably transformed GFP-labeled and two DsRed-Express labeled transformants derived from PcWSA, as well as a GFP-labeled *Pa. chlamydospora* transformant (pCT74-P7) from New Zealand (Bradshaw *et al.*, 2005).

Colony colour. The colony colour of each *Pa. chlamydospora* transformant and their respective wild type isolates were surface rated according to the colour charts of Rayner (1970) as previously described by Crous and Gams (2000). Colony colour was evaluated on isolates that were grown on 2% malt extract agar (MEA) (Biolab, Merck Bioscience, Modderfontein, South Africa). The colony colour was evaluated after 2 and 3 weeks of growth at 25°C in the dark.

Conidial germination. Spore suspensions of 5×10^5 spores/ml were prepared from 14-day-old cultures that were grown on PDA at 25°C in the dark. For each isolate, three PDA dishes were inoculated with 10 ml of spore suspension of which the spores were allowed to settle in a laminar flow for 1 hour before excess water were decanted. Inoculated plates were incubated at 25°C for 15 hours. Germination was evaluated on each Petri dish at 40 × magnification using a stereoscopic microscope. The germination percentage was calculated by counting the number of germinated spores, i.e. spores with germ tubes that were longer than the spore's

diameter, among 100 randomly chosen spores on each of the Petri dishes. The trial was repeated twice.

Spore morphology. Microscope slides with specimens were prepared by placing a small amount of mycelium containing spores from 14-day-old cultures into lactic acid on a microscope slide, and covering it with a glass cover slip. The length and width of 25 alpha conidia of each colony were measured, the averages were calculated and the minimum and maximum ranges were noted. The trial was repeated twice.

In vitro mycelium growth. The radial growth of isolates was determined at different temperatures. One mycelium plug (5-mm-diameter) of each isolate was taken from 14-day-old PDA cultures and placed on 90-mm 2% MEA plates and incubated for 8 days in the dark at four different temperatures ranging from 10-25°C in 5°C intervals. Each isolate was replicated three times at each temperature. Measurements were taken 24, 48, 72 and 96 hours after inoculation. The radial growth was calculated by making four radial measurements on each plate of which the average was calculated, and the original mycelium plug diameter (5 mm) was subtracted.

Statistical analyses. The conidial germination percentage, spore size and *in vitro* mycelium growth data was submitted to analysis of variance (ANOVA) using SAS version 8.1 (SAS Institute, Cary, North Carolina, USA). Student's t-LSD was calculated to compare means of significant effects at the 5% significance level.

Virulence characterisation. A trial was conducted to compare the virulence of five different *Pa. chlamydospora* isolates. These isolates included the wild type isolate PcWSA, and one GFP and one DsRed-Express labeled transformant derived from it, as well as a New Zealand GFP labeled transformant pCT74-P7 and its wild type isolate (PcWNZ). Isolates pCT74-P7 and PcWNZ were kindly provided by R.E. Bradshaw (National Centre for Advanced Bio-Protection Technologies, Institute of Molecular BioSciences, Palmerston North, New Zealand).

Plant material from *Vitis vinifera* cultivar Chenin blanc was used for the trial. Dormant six-node cuttings were collected in July 2005 at Delvera, Stellenbosch, South Africa, during the winter pruning season. The cuttings were dipped in sterile

water containing Sporekill (1 ml/liter) for 1 hour and then dried at room temperature, where after it was stored at 8°C until use. The cuttings received a hot water treatment (50°C for 30 min) just before being inserted into hydroponic systems (Fig. 1) that were placed on laboratory benches at 25°C in natural light. Cuttings were inoculated with the *Pa. chlamydospora* isolates by inserting a 5-mm-diameter mycelium plug from a 14-day-old PDA culture into a hole made with a sterilised 5 mm cork borer between the fifth and sixth node of the cuttings. Each inoculation site was covered with Parafilm. Sterile PDA plugs were used as controls. The cuttings were evaluated after 4 months for lesion development. The lesions were investigated by first removing the bark and measuring the lesions beneath the bark, followed by splitting the cutting longitudinally and measuring xylem discolouration inside the cutting. Isolations were made from the edges of xylem lesions onto PDA and PDA amended with hygromycin B, which were incubated for 10 to 14 days at 25°C. The percentage of re-isolated wild type isolates and their respective transformants were determined for each treatment and the results were compared to determine whether the transformants differed from their respective wild type isolates. The fluorescence of all the re-isolated transformants were investigated using epifluorescence microscopy.

The experiment was a randomised block design with six treatments randomly replicated within four blocks. Each treatment was replicated four times within each block. Data were subjected to analysis of variance using SAS version 8.1, and the Student's t-LSD was calculated to compare means of significant effects at the 5% significance level.

RESULTS

Screening for hygromycin B sensitivity. PcWSA was completely inhibited at a concentration of 40 µg/ml hygromycin B (Fig. 2). Therefore, this concentration was chosen as the hygromycin concentration used in the transformation experiments.

Transformation of *Pa. chlamydospora*. Colonies appeared approximately 14 days after incubation on the primary selective plates (PDA amended with 40 µg/ml hygromycin) containing protoplasts to which vector DNA was added. No colonies

appeared on primary selective plates to which no vector DNA was added, confirming that the hygromycin B concentration of 40 µg/ml was effective in suppressing the wild type protoplasts. The transformation method was repeated five times and yielded approximately one transformant per microgram vector DNA per 2×10^6 viable protoplasts each time.

All ten selected colonies from the primary transformation plates containing protoplasts transformed with GFP (pCT74) or DsRed-Express (pPgpd-DsRed and pHyg8) exhibited green fluorescence or red fluorescence, respectively. The ten transformants that were transferred from primary transformation plates to new selective plates, were all able to subsequently grow on the selective media, and were able to express the reporter genes when viewed using epifluorescence microscopy.

Selection of stable transformants. The stability of a subset of ten transformants including five GFP (PcG1, PcG5, PcG8, PcG9 and PcG10) and five DsRed-Express (PcR1, PcR2, PcR6, PcR9 and PcR10) transformants were evaluated. The evaluation showed that the transformants were all stable, since they retained their fluorescence after successive transfers onto non-selective media. After these transfers, the transformants were also able to grow on hygromycin media, showing that they retained expression of the *hph* gene.

Epifluorescence microscopy. There were no signs of auto-fluorescence when the wild type isolates growing on artificial media were viewed with epifluorescence microscopy. In transformants grown on artificial media, strong constitutive expression of GFP and DsRed-Express could be visualised in the cytoplasm of most of the individual hyphae and spores (Fig. 3 A-D), although the level of fluorescence varied between transformants.

In grapevine tissue a substantial amount of auto-fluorescence was observed using epifluorescence microscopy with the GFP as well as the DsRed-Express filter sets. This host tissue auto-fluorescence precluded the investigation and identification of transformant hyphae within inoculated grapevine tissue.

PCR detection of reporter genes and hygromycin resistance genes. PCR analyses revealed the presence of the *hph* gene in all the transformants, whereas no amplification was obtained from the wild type isolate (Fig. 4A, 5A). The *GFP* gene

was detected in all five GFP transformants (PcG1, PcG5, PcG8, PcG9 and PcG10) (Fig. 4B), and the *DsRed-Express* gene in all five red fluorescent transformants (PcR1, PcR2, PcR6, PcR9 and PcR10) (Fig. 5B). Amplification of the *GFP*, *DsRed-Express* and *hph* genes yielded the expected PCR amplification size products of 417 bp, 200 bp and 700 bp, respectively. The two reporter genes were not detected in the wild type isolates or in the water control (Fig. 4, 5).

***In vitro* biological characterisation of transformed and wild type isolates.**

Colony colour. Colony colour after 2 weeks of growth of three GFP transformants (PcG1, PcG10 and pCT74-P7) and two DsRed-Express transformants (PcR1 and PcR2) was similar to the wild type isolates (PcWSA and PcWNZ), ranging from Greenish Olivaceaous (23'''i) on the upper-side becoming paler towards the margin of the colony and Dark Herbage Green (27"k) on the reverse-side. However, evaluation of the colony colour after 3 weeks of growth showed that transformants PcG1 and PcR1 were different from the wild type isolate (PcWSA). Transformant PcG1 ranged from Dull Green (27"m) to Greenish Olivaceaous (23'''i) on the upper-side and Dull Green (27"m) on the reverse-side. Transformant PcR1 was sectorially white with a pink undertone on the upper-side as well as the reverse-side of the plate (Fig. 6).

Conidial germination. Analysis of variance of germination percentages indicated a significant difference between isolates (P = 0.0201; ANOVA table not shown). The mean germination percentages of transformants PcG1, PcR1 and PcR2 (89.33% to 90.00%) did not differ significantly from their derived wild type isolate (90.33%), whereas PcG10 (88.67%) differed significantly from these isolates (Table 1). The germination percentage of transformant pCT74-P7 (88.33%) did not differ significantly from its derived wild type isolate (87.67%).

Spore morphology. Analysis of variance of conidial length and width measurements indicated no significant difference between the *Pa. chlamydospora* transformants and wild type isolates (P = 0.9398 and 0.5545, respectively; ANOVA table not shown). Spore lengths averaged between 4.50 and 4.87 µm, with spore widths between 1.84 and 2.20 µm.

In vitro *mycelium growth.* Analysis of variance of the *in vitro* mycelium growth indicated a day × temperature interaction only ($P < 0.0001$), and no significant effect for isolates ($P > 0.05$; ANOVA table not shown). As the mycelial growth rate of transformants and wild types did not differ, the data were not analysed further.

Virulence characterisation. All the Chenin blanc cuttings that were inoculated with *Pa. chlamydospora* transformants and wild type isolates showed brown-black lesions under the bark tissue and black discolouration of the vascular tissue (lesions) (Fig. 7 A-C). Slight discolouration was also found on the control cuttings that were inoculated with a sterile agar plug, but this was clearly due to natural wound healing (Fig. 7D). Analysis of variance of data for lesion lengths beneath the bark and inside the vascular tissue showed a significant effect for treatment ($P = 0.0090$ and 0.0416, respectively). This was due to the fact that lesion lengths in inoculated cuttings differed significantly from the control (0 and 4.43 mm for bark and vascular lesions, respectively). In inoculated cuttings, bark and vascular lesion lengths did not differ significantly between transformants and wild type isolates, varying between 23.91 mm and 37.52 mm for vascular lesions and 21.87 mm and 28.38 mm for lesions beneath the bark (Table 2).

Phaeomoniella chlamydospora was re-isolated from all the inoculated cuttings. The *Pa. chlamydospora* transformants yielded a similar re-isolation percentage than their respective wild type isolates (data not shown). The isolated segments from the cuttings inoculated with the wild types (PcWSA and PcWNZ) grew on the PDA media but not on the selective media (PDA amended with hygromycin). Isolated segments from the cuttings inoculated with the transformants (PcG1, PcR1 and pCT74-P7) grew on the PDA as well as the selective media. No *Pa. chlamydospora* cultures were isolated from the control cuttings. The *Pa. chlamydospora* isolates obtained from cuttings inoculated with the reporter gene labeled isolates (PcG1, PcR1 and pCT74-P7) all retained their respective bright fluorescent phenotypes after being re-isolated from the cuttings. The isolations from the cuttings yielded various other fungi at very low frequencies including *Acremonium*, *Alternaria*, *Gliocladium*, *Penicillium* and *Trichoderma* spp.

DISCUSSION

The transformation procedure yielded stable transformants of *Pa. chlamydospora* (STE-U 5784) expressing the *GFP* and *DsRed-Express* reporter genes. The transformation frequency was low, yielding only one transformant per microgram plasmid DNA. Bradshaw *et al.* (2005) found similar low transformation frequencies when transforming a New Zealand isolate of *Pa. chlamydospora*, while this has also been reported for several other fungal genera (Hamada *et al.*, 1994; Bowen *et al.*, 1996). The reporter gene-labeled *Pa. chlamydospora* transformants showed constitutive expression of the reporter genes, with cytoplasmic fluorescence detected during all the different growth stages. Although all the reporter gene-labeled transformants showed fluorescence, the fluorescence levels varied between transformants, most likely due to the integration of different reporter gene copy numbers or due to positional effects. This is the first report on the stable transformation of *Pa. chlamydospora* expressing the red fluorescent protein DsRed-Express.

Characterisation of the biological and physiological characteristics of transformants is very important in order to confirm that mutations have not occurred prior to carrying out time-consuming ecological and interaction studies (Lo *et al.*, 1998). Characterisation of the colony colour, conidial size, germination percentage and growth rate of the *Pa. chlamydospora* transformants and wild type isolates showed that the transformants did not differ from their respective wild type isolates. The only exceptions were transformant PcG10 that had a significant lower germination percentage than the wild isolate, and transformants PcG1 and PcR1 that had different colony colours when compared to their wild type isolate (PcWSA). The differences in colony colour in PcR1 and PcG1 is most likely due to the presence of the DsRed-Express and GFP proteins respectively, which when expressed at high levels may add a light pink (Mikkelsen *et al.*, 2003) or green colour (A. McLeod, personal communication) to colonies respectively. Mikkelsen *et al.* (2003) observed that the white mycelium of some *Penicillium paxilli* transformants that contained DsRed-Express at a high level, turned pink on PDA plates. Similar observations have been made with *Fusarium oxysporum* transformants that express GFP and DsRed-Express at high levels (A. Mcleod, unpublished data).

The transformants were not altered in their virulence, since they caused similar lesion lengths beneath the bark, and inside the vascular tissue of cuttings than their respective wild type isolates. The *Pa. chlamydospora* transformants also yielded a similar re-isolation percentage than their respective wild type isolates, further confirming that the transformants are as virulent as the wild type isolates. Several other studies have also found that reporter genes did not alter the pathogenicity and virulence of transformants (Spellig *et al.*, 1996; Maor *et al.*, 1998, Nahalkova and Fatehi, 2003; Visser *et al.*, 2004). The virulence characterisation trial further confirmed the stability of the transformants, since all three reporter gene-labeled transformants retained their bright fluorescence phenotype after 4 months of host tissue colonisation.

The bright constitutively expressed red and green fluorescence observed in the well characterised transformants PcR1 and PcG1, respectively, will allow their discrimination from other non-fluorescing fungi within host tissue and soil. This will allow new approaches for observing and monitoring this slow-growing pathogen within grapevine tissue. The GFP and DsRed-Express *Pa. chlamydospora* transformants can be used in dual label studies with reporter gene-labeled biocontrol agents such as *Trichoderma harzianum* (Chapter 4) or other trunk disease pathogens including *Phomopsis viticola* (Chapter 3), for studying interactions among these fungi within host tissue. The reporter gene technology could further be used to determine whether soil is an active inoculum source, as well as how *Pa. chlamydospora* spreads from initial infections sites to the rest of the host plant. Altogether these aspects will answer important questions and enable the development of sufficient disease management strategies. As Morton (2000) stated "The story will not be complete until we follow the trail of black goo which has been hidden away from view, the one that moves from inside the wood tissue to the outside."

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Table 1. Mean germination percentages of *Phaeomoniella chlamydospora* reporter gene-labeled transformants (PcR1, PcR2, PcG1, PcG10, pCT74-P7) and wild type isolates (PcWSA and PcWNZ)

Isolates[x]	Mean germination percentage[y]
PcWSA	90.33 a
PcR1	89.33 ab
PcR2	90.00 a
PcG1	89.33 ab
PcG10	88.67 bc
PcWNZ	87.67 c
pCT74-P7	88.33 bc
LSD ($P = 0.05$)	1.283

[x] The GFP-labeled transformant PcG1 and DsRed-Express-labeled transformant PcR1 were derived from the wild type isolate PcWSA, whereas the GFP-labeled transformant pCT74-P7 was derived from the wild type isolate PcWNZ.

[y] Values in each column followed by the same letter do not differ significantly ($P < 0.05$).

Table 2. Mean lesion lengths on cv. Chenin blanc 4 months after inoculation with *Phaeomoniella chlamydospora* reporter gene-labeled transformants (PcR1, PcG1, pCT74-P7) and wild type isolates (PcWSA and PcWNZ)[x]

Treatment[y]	Lesion inside vascular tissue	Lesion beneath bark
Control	0.00 b	4.43 b
PcWSA	37.52 a	27.38 a
PcR1	23.91 a	21.87 a
PcG1	32.97 a	24.38 a
PcWNZ	35.92 a	26.94 a
pCT74-P7	33.97 a	28.38 a
LSD ($P > 0.05$)	**18.984**	**15.422**

[x] Mean lesion lengths were measured inside the vascular tissue of grapevine cuttings as well as beneath the bark. Values in each column followed by the same letter do not differ significantly ($P = 0.05$).

[y] The GFP-labeled transformant PcG1 and DsRed-Express-labeled transformant PcR1 were derived from the wild type isolate PcWSA, whereas the GFP-labeled transformant pCT74-P7 was derived from the wild type isolate PcWNZ.

Fig. 1. Hydrophonic system with grapevine cuttings, Chenin blanc, used for the *Phaeomoniella chlamydospora* virulence characterisation trial.

Fig. 2. *Phaeomoniella chlamydospora* growth on potato dextrose agar plates containing different concentrations of hygromycin B (0, 20, 40 and 80 µg/ml).

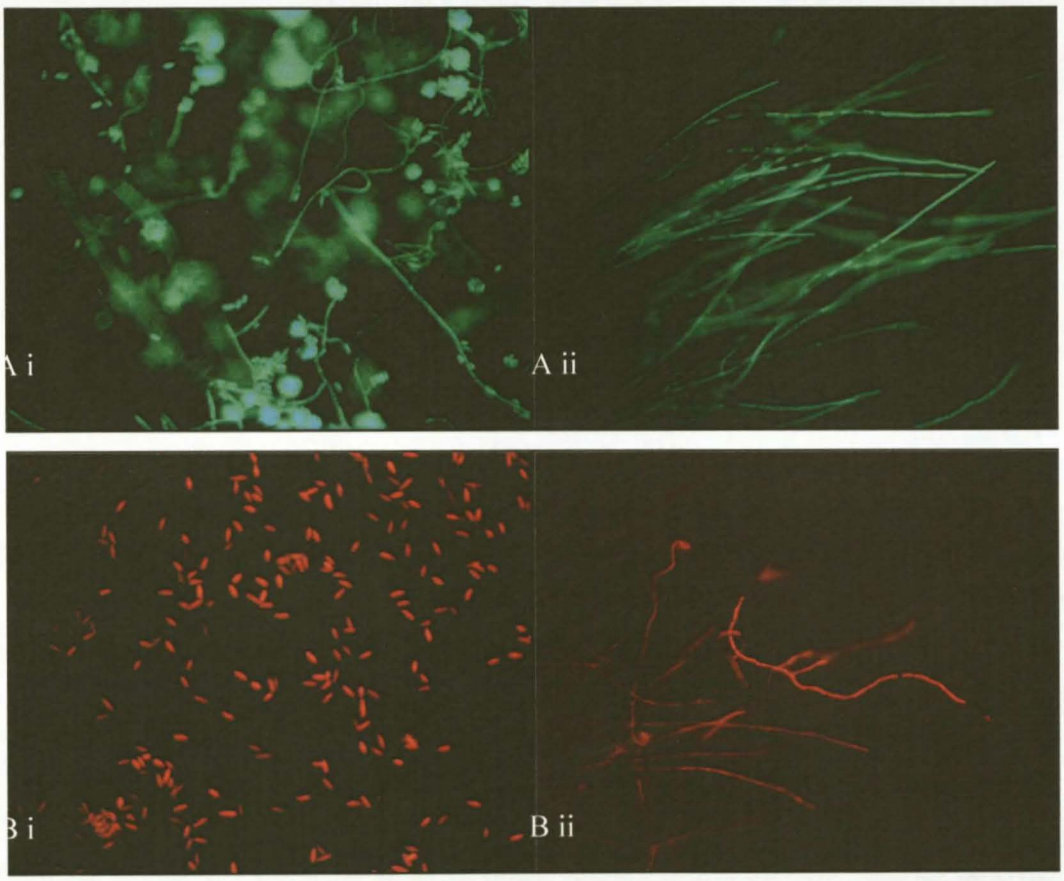

Fig. 3. *Phaeomoniella chlamydospora* expressing the (A) green fluorescence protein (GFP) and (B) the red fluorescent protein (DsRed-Express) in (i) conidia and (ii) mycelium.

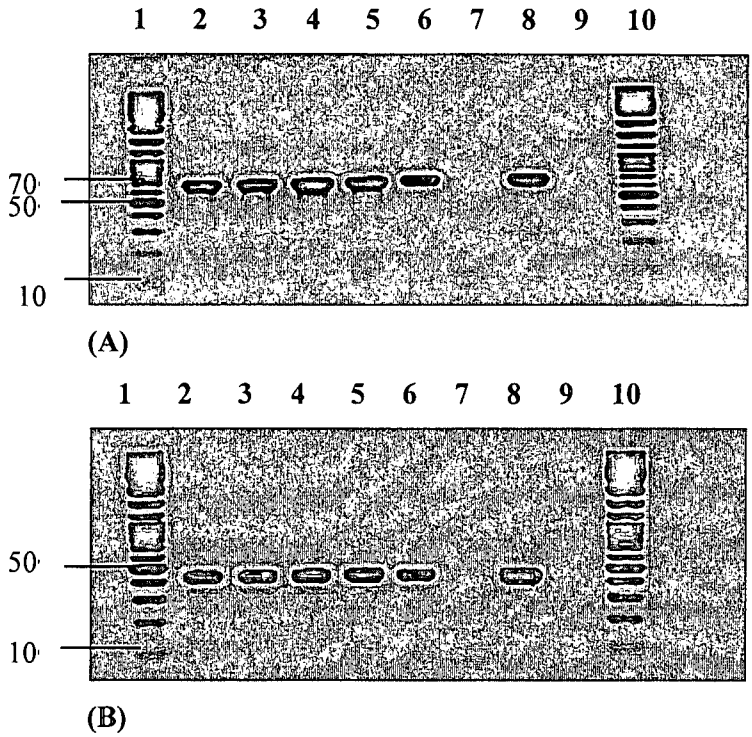

Fig. 4. Polymerase chain reaction (PCR) analyses of *Phaeomoniella chlamydospora* transformants confirming the presence of the (A) selectable marker gene hygromycin phosphotransferase (*hph*) and the (B) green fluorescent protein (GFP) gene. Transformants were derived from a South African *Pa. chlamydospora* isolate (PcWSA). PCR was conducted using primers specific to the (A) *hph* and (B) *GFP* genes, yielding a 700 bp or 417 bp PCR fragment respectively. Lane 1, molecular weight marker; lane 2-6, GFP transformants; lane 7, wild type isolates PcWSA; lane 8, plasmid vector pCT74 containing the *GFP* and *hph* gene; lane 9, water control; and lane 10, molecular weight marker.

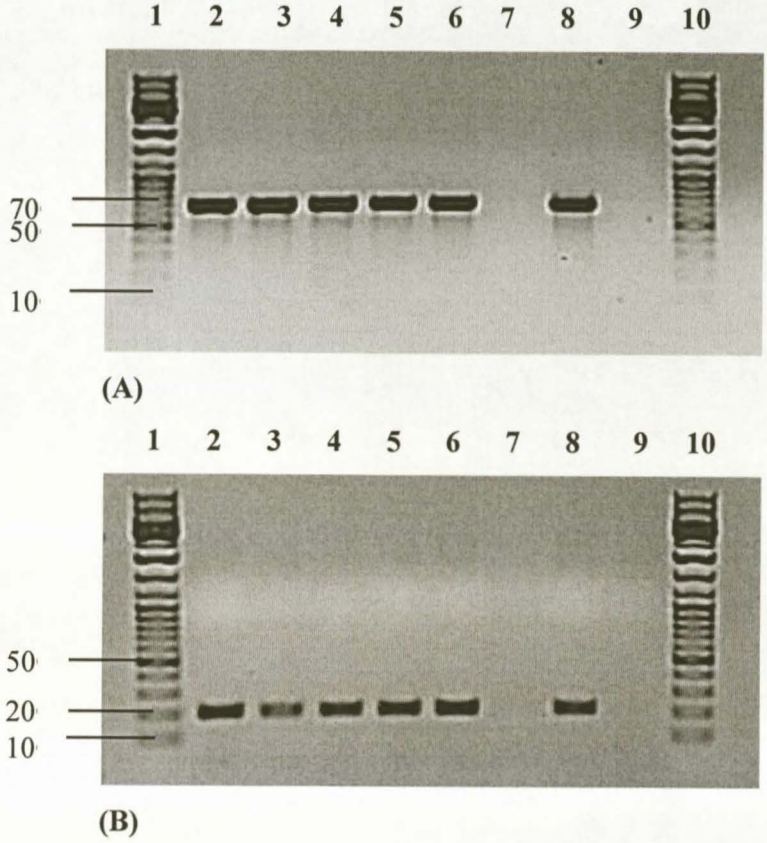

Fig. 5. Polymerase chain reaction (PCR) analyses of *Phaeomoniella chlamydospora* transformants confirming the presence of the (A) selectable marker gene hygromycin phosphotransferase (*hph*) and the (B) red fluorescent protein (DsRed-Express) gene. Transformants were derived from a South African *Pa. chlamydospora* isolate (PcWSA). PCR was conducted using primers specific to the (A) *hph* and (B) *DsRedExpress* genes, yielding a 700 bp or 200 bp PCR fragment respectively. Lane 1, molecular weight marker; lane 2-6, DsRed-Express transformants; lane 7, wild type isolates PcWSA; lane 8, plasmid vector pHyg8 containing the *hph* gene (A) or vector pPgpd-DsRed containing the *Dsred-Express* gene (B); lane 9, water control; and lane 10, molecular weight marker.

Fig. 6. Colony colour of *Phaeomoniella chlamydospora* wild type isolate PcWSA, GFP transformants PcG1 and PcG10, and DsRed-Express transformants PcR1 and PcR2 after 3 weeks of growth at 25°C in the dark on potato dextrose agar.

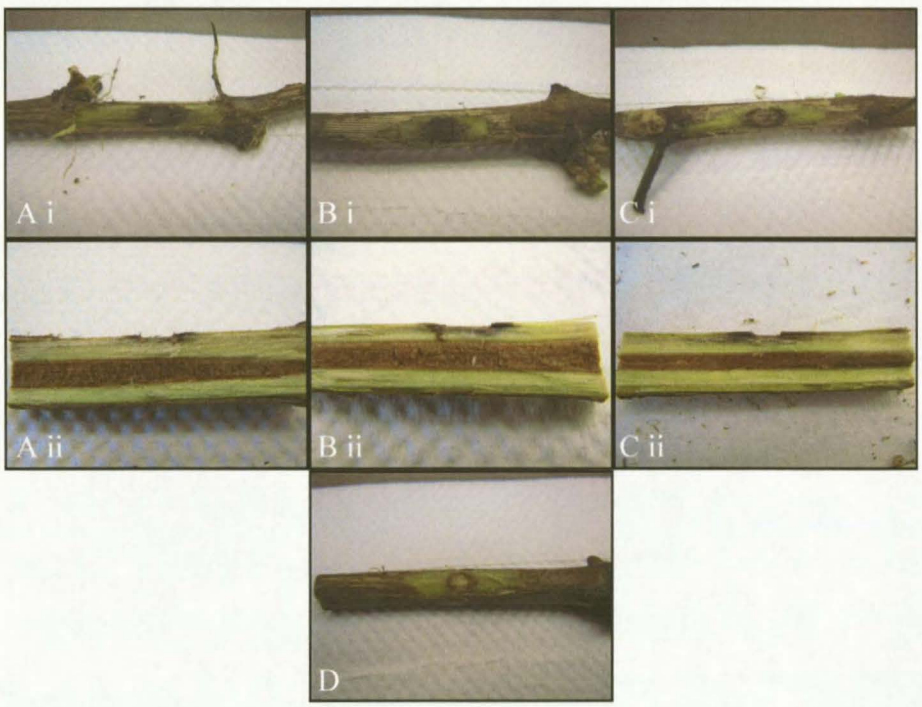

Fig. 7. Lesions caused by *Phaeomoniella chlamydospora* (A) wild type isolate PcWSA, (B) GFP transformant PcG1 and (C) DsRed Express transformant PcR1 on Chenin blanc grapevine cuttings (i) beneath the bark and (ii) inside the vascular tissue 4 months after inoculation. (D) The control cuttings that were inoculated with a sterile agar plug only showed discolouration due to natural wound healing.

3. STABLE TRANSFORMATION OF *PHOMOPSIS VITICOLA* WITH THE GREEN (GFP) - AND RED (DSRED-EXPRESS) FLUORESCENT PROTEIN GENES

ABSTRACT

Phomopsis viticola is well known as the pathogen that causes Phomopsis cane and leaf spot of grapevines. However, the pathogen is also implicated as one of the pathogens involved in the economically important grapevine trunk disease complex. The inoculum ecology, endophytic nature and disease cycle of *P. viticola*, especially with regard to trunk diseases, is still poorly understood. As a first step into elucidating some of these aspects, two *P. viticola* isolates (STE-U 6048 and STE-U 6049) were stably transformed with the green fluorescent protein (GFP) or the red fluorescent protein (DsRed-Express) reporter genes. All the GFP transformants exhibited low to intermediate levels of fluorescence in mycelia and conidia over a 6-week growth period, with no brightly fluorescing isolates being obtained. Similarly, all the DsRed-Express transformants also showed low to intermediate levels of fluorescence in the mycelia of 1- to 3-week-old cultures, whereas no fluorescence was observed in 4- to 6-week-old cultures. Contrarily, the conidia of DsRed-Express transformants showed very bright fluorescence in 3- to 4-week-old cultures. Selected GFP and DsRed-Express transformants retained their hygromycin resistance phenotype after sub-culturing onto non-selective media, as well as the *hph*, *GFP* and *DsRed-Express* genes as shown through polymerase chain reaction amplifications. The colony colour, spore size, germination percentage and growth rate at different temperatures of the two wild type *P. viticola* isolates and two stably transformed GFP and two DsRed-Express transformants of each isolate were characterised, and were found to be similar to their respective wild type isolates. Virulence characterisation showed that the transformants, one GFP and one DsRed-Express transformant derived from each wild type isolate, did not differ in their virulence from the wild type isolates on Chenin blanc grapevine cuttings. The GFP and DsRed-Express transformants that were re-isolated from the cuttings retained their fluorescence

phenotype, although DsRed-Express fluorescence was only visible 6 weeks after isolations were made.

INTRODUCTION

Phomopsis viticola (Sacc.) Sacc. is known as the causal agent of a disease known as Phomopsis cane and leaf spot (Mostert *et al.*, 2001). It was referred to as "excoriose" in Europe and as "dead arm" in American literature before the description of Eutypa dieback surfaced (Pearson and Goheen, 1994). Phomopsis cane and leaf spot occurs in most of the grape-growing areas reported in Africa, Asia, Australia, Europe and North America (Pearson and Goheen, 1994; Castillo-Pando *et al.*, 1997; Atia *et al.*, 2003). This disease causes major decline and reductions in yield of susceptible cultivars due to weakening of canes and poor fruit development or fruit loss (Pearson and Goheen, 1994).

Phomopsis cane and leaf spot is known to spread slowly, eventually causing slow die-back of vines (Emmett *et al.*, 1992; Atia *et al.*, 2003). Shoots tend to break off at the base and are stunted (Van Niekerk *et al.*, 2005). Dark brown, lens-shaped lesions appear early in the season, usually on the bottom two to three internodes of the grapevine shoot (Ferreira and Venter, 1996). Characteristically these lesions will turn brown, and as they widen the tissue cracks and become dry. Lesions can also occur on bunch stems and cause blackening and shrivelling of berries, resulting in reduced bunch set. Similar symptoms can appear on the petioles. Leaf symptoms include small black spots with yellow halos, which can give the leaf a wrinkled appearance. These spots may drop out and give the leaf a "shot hole" appearance (Pearson and Goheen, 1994; Melanson *et al.*, 2002).

Phomopsis viticola has not only been implicated as the casual agent of Phomopsis cane and leaf spot, but is also thought to play a role in the trunk disease complex. The pathogen has been frequently isolated from trunk disease symptoms within grapevine pruning wounds (Van Niekerk *et al.*, 2005; Bester, 2006). Furthermore, inoculation of detached grapevine shoots has shown that *P. viticola*

along with *Phomopsis amygdali*, are the most virulent *Phomopsis* spp. associated with grapevines (Van Niekerk *et al.*, 2005).

The life cycle of *P. viticola* is thought to be mainly asexual, since the sexual phase (perithecia) has only rarely been found in a few Australian vineyards (Scheper *et al.*, 1997; Mostert *et al.*, 2001). The pathogen generally overwinters as mycelium and pycnidia inside infected grapevine canes, spurs, pruning wounds and dormant buds (Pearson and Goheen, 1994). In the case of cane and leaf spot, *P. viticola* spores can penetrate uninjured young shoots or leaves directly (Pearson and Goheen, 1994). However, in the trunk disease complex it is not known whether *P. viticola* can directly infect fresh pruning wounds or whether it is a secondary wound invader. Furthermore, since *P. viticola* is often isolated from asymptomatic plants (Graniti *et al.*, 2000; Halleen *et al.*, 2003), it may also be present in vines in a latent form (Graniti *et al.*, 2000). It is still questioned whether pathogenic *P. viticola* colonising the grapevine is endophytic, a latent pathogen, a weak pathogen or a true vascular pathogen. Therefore, many aspects of the inoculum ecology, endophytic nature and pathogenicity of *P. viticola* are uncertain within the trunk disease complex. Studies aimed at investigating these aspects have been limited due to the fact that *P. viticola* can not be easily discriminated within the host tissue, and among other endophytes.

The labeling of fungi with fluorescent reporter genes is a very good tool for visualising and studying the biology and activity of fungi within complex environmental samples, including host tissue (Lorang *et al.*, 2001). Two fluorescent reporter genes that are often used for labeling of fungi include the green fluorescent protein (GFP) and red fluorescent protein (DsRed-Express) genes (Chapter 2). The unique and specific spectral properties of these two reporter genes also enable dual labeling studies, allowing discrimination of two different fungi within the same environment (Baird *et al.*, 2000; Mikkelsen *et al.*, 2003; Nahalkova and Fatehi, 2003).

The first objective of this study was to stably transform two isolates of *P. viticola* with plasmids harbouring the reporter genes *GFP* or *DsRed-Express*, using the hygromycin phosphotransferase (*hph*) gene as a selectable marker. Prior to this study, *P. viticola* has only been transformed with the *hph* and benomyl resistance (*Bml*^{r}) genes (De Guido *et al.*, 2003), but not with any reporter genes. The second aim of the study was to characterise the morphology and virulence of a subset of the

transformants that showed the highest level of fluorescence, in order to establish whether the transformation process has caused any mutations in the transformants.

MATERIAL AND METHODS

Fungal isolates. Two pathogenic *P. viticola* isolates (STE-U 6048 and STE-U 6049) were obtained from the Stellenbosch University culture collection (Plant Pathology Department, Stellenbosch University, South Africa). The isolates will hereafter be referred to as PW8 and PW9. Both isolates were obtained from vineyards in the Paarl Valley of the Western Cape province of South Africa. The wild type cultures as well as the transformed isolates were routinely grown on potato dextrose agar (PDA) (Difco Laboratories, MI, USA), and were stored in sterile distilled water as mycelial plugs, as well as in 15% glycerol at -80°C.

Screening for hygromycin B sensitivity. The specific hygromycin B concentration required to inhibit the wild type (non-transformed) *P. viticola* isolates PW8 and PW9 was determined as previously described for *Pa. chlamydospora* (Chapter 2).

Plasmid vectors. PW8 and PW9 were labeled with the *GFP* and *DsRed-Express* reporter genes, using the previously described plasmid vectors pCT74, pHyg8 and pPgpd-DsRed (Chapter 2). Hygromycin phosphotransferase (*hph*) was the selectable marker gene.

Transformation of *P. viticola*

Protoplast preparation. PW8 and PW9 were grown for 10 to 14 days on PDA media at 25°C in the dark to allow pycnidial production. Conidia oozing from the pycnidia were harvested into 100 ml of sterile distilled water to obtain a final concentration of $\pm 2 \times 10^6$ spores/ml. The spore suspension was divided into two 500 ml Erlenmeyer flasks, to which an equal volume of 2 × 2YEG (Chapter 2) was added. The spores were incubated for 15 hours at 28°C at 140 rpm to allow germination. Protoplasts were produced from the germinated spores as described for *Pa. chlamydospora* (Chapter 2), except that a different enzyme combination was used.

Evaluation of various enzyme combinations and concentrations (data not shown) showed that a combination of 30 mg/ml driselase (Sigma-Aldrich, Aston Manor, South Africa), 20 mg/ml lysing enzyme from *Trichoderma harzianum* (Sigma-Aldrich) and 15 mg/ml cellulase (Yakult Pharmaceuticals, LTD, Minato-KU, Tokyo, Japan) was best for protoplast production. Cell wall digestion of the germinated spores in the enzyme solution was conducted for 75 min at 28°C under mild shaking (50 rpm) to allow the release of protoplasts.

Transformation of protoplasts. The transformation of protoplasts was based on the method of Lu *et al.* (1994) as described in Chapter 2, except that the primary transformation plates, containing the protoplasts and regeneration media, were overlaid with 1% water agar (WA) containing 40 µg/ml hygromycin B. Transformants appeared after 8 to 10 days through the selective water agar. A subset of 40 putative transformant colonies (10 GFP and 10 DsRed-Express putative transformants of each wild type isolate) were transferred to fresh selective PDA plates containing 40 µg/ml hygromycin B. The fluorescence of these putative transformants was investigated after 1 week of growth.

Epifluorescence microscopy. The respective green and red fluorescence of GFP and DsRed-Express transformants were investigated using epifluorescence microscopy as previously described (Chapter 2). The fluorescence of the wild type isolates PW8 and PW9 was also investigated to determine if any level of auto-fluorescence was present within these isolates.

Selection of stable *P. viticola* transformants. A total of 12 (four DsRed-Express and eight GFP transformants) and nine (five DsRed-Express and four GFP transformants) transformants derived from isolates PW8 and PW9 respectively, showed some level of fluorescence after being transferred once to selective media. These fluorescent transformants were selected for stable transformation evaluation. These isolates were hyphal tipped four times onto PDA amended with hygromycin B in order to eliminate heterokaryotic and non-fluorescent cells. The stability of the transformants was tested by transferring a mycelial plug of each transformant for three consecutive generations on non-selective media (PDA without hygromycin). GFP and DsRed-Express expression was tested after each generation using epifluorescence microscopy. The *P. viticola* transformants were transferred for a final

generation on PDA amended with hygromycin B, allowing evaluation of the hygromycin resistance phenotype. Subsequently, the transformants were transferred back to non-selective medium, and their level of fluorescence in mycelium and conidia was evaluated over a period of 6 weeks on a weekly basis.

Polymerase chain reaction (PCR) detection of reporter genes and hygromycin resistance genes. Gene specific primers were used to investigate the presence of the *GFP*, *DsRed-Express* and hygromycin phosphotransferase (*hph*) genes in all the transformants that were selected for stable transformation evaluation. DNA was isolated from 10-day-old cultures that were grown on non-selective PDA, as described in Chapter 2. These cultures were stored for ± 4 months in water as mycelial plugs, prior to the DNA extractions. PCR amplifications for the different genes were conducted as described in Chapter 2, except that annealing temperatures were different, with *GFP* annealing at 55°C, *DsRed-Express* at 64°C, and *hph* at 66°C.

***In vitro* biological characterisation of transformed and wild type isolates.** Biological characteristics of the wild type *P. viticola* isolates PW8 and PW9 used in the transformation studies, as well as two GFP-labeled transformants of each isolate and two DsRed-Express-labeled transformants of each isolate were characterised further.

Colony colour, conidial germination, spore morphology and in vitro *mycelium growth at different temperatures.* The selected *P. viticola* transformants and wild type isolates were characterised in terms of colony colour, conidial germination, spore morphology, as well as *in vitro* mycelium growth at four different temperatures ranging from 10-25°C in 5°C intervals, as described in Chapter 2. The only modifications were that the cultures were incubated for 10 days before colony colour evaluation and mycelium radial growth evaluations, and for 14 days before conidial germination and spore morphology evaluations.

Virulence characterisation. The virulence of the *P. viticola* wild type isolates (PW8 and PW9) and one transformant of each isolate expressing GFP and DsRed-Express were conducted as previously described for *Pa. chlamydospora* (Chapter 2). Lesions were evaluated 4 months after inoculation by first removing the bark tissue from cuttings, and measuring the lesion length beneath the bark at each

inoculation site. Isolations were made from lesions on selective and non-selective media as previously described (Chapter 2). Hyphae that grew from the isolated woody tissue were hyphal tipped onto fresh selective and non-selective media. Cultures obtained from cuttings inoculated with the transformants were further evaluated by investigating their intensity of fluorescence on a weekly basis for 6 weeks using epifluorescence microscopy. Growth on hygromycin selective medium was also evaluated. Statistical analysis was done as previously described (Chapter 2).

RESULTS

Screening for hygromycin B sensitivity. The wild type isolates PW8 and PW9 were completely inhibited at a concentration of 40 µg/ml hygromycin B (Fig. 1). Therefore, this concentration was used in the transformation studies for selecting transformants.

Transformation of *P. viticola* isolates PW8 and PW9. Putative transformant colonies appeared approximately 8 to 10 days after incubation of the primary transformation plates. However, there were two different sized putative transformant colonies on these plates for both wild type isolates, i.e. fast-growing and slow-growing colonies (Fig. 2). In a preliminary trial it was found that only the fast-growing larger colonies grew when they were transferred to hygromycin B medium, whereas the slow-growing small colonies mostly showed no growth on the selective media. Therefore, only the fast-growing large colonies were transferred from the primary plates and were thus considered as putative transformants for calculating transformation efficiencies. The transformation method yielded approximately three and one transformants per microgram plasmid DNA for isolate PW8 and PW9, respectively. No growth was seen on the primary transformation plates containing only protoplasts with no vector DNA.

A total of 40 putative transformants, 10 GFP and 10 DsRed-Express transformants derived from each of the wild type isolates PW8 and PW9, were transferred to new hygromycin medium from the primary transformation plates. Eighty percent of the putative transformants derived from PW8 were able to

subsequently grow on selective media, whereas only 40% of putative transformants derived from PW9 were able to grow on selective media. Fluorescence evaluation of the putative transformants derived from PW8 showed that most (90%) of the transformants fluoresced, whereas all the transferred transformants derived from PW9 showed fluorescence. The level of GFP and DsRed-Express fluorescence in all the putative transformants consisted of very low to intermediate fluorescence levels after 1 week of growth on the selective medium. There were no brightly fluorescing transformants as seen when viewing transformants obtained from other fungi (Chapter 2 and 4). The low level of fluorescence in the *P. viticola* isolates were not considered as auto-fluorescence, since no fluorescence was present when examining the wild type isolates using epifluorescence.

Selection of stable *P. viticola* transformants derived from isolates PW8 and PW9. A subset of 21 of the putative 40 transformants derived from both wild type isolates which showed the highest level of fluorescence, were selected for stability testing. GFP transformants derived from isolates PW8 and PW9 will hereafter be designated as PG8.x and PG9.x, respectively. DsRed-Express transformants derived from PW8 and PW9 will hereafter be designated as PR8.x and PR9.x, respectively. The transformants that were tested for stability included the GFP transformants PG8.1, PG8.2, PG8.3, PG8.5, PG8.7, PG8.8, PG8.9, PG8.10, PG9.1, PG9.2, PG9.3 and PG9.4 as well as the DsRed-Express transformants PR8.1, PR8.2, PR8.3, PR8.7, PR9.1, PR9.2, PR9.3, PR9.4 and PR9.5. These transformants were all hyphal tipped three times to remove heterokaryons and non-fluorescent colonies. Stability evaluation showed that the transformants retained their hygromycin resistance and fluorescence phenotypes (Fig. 3), after being transferred for three generations onto non-selective media. The transformants also retained their resistance and fluorescence phenotype after ± 4 months of storage as mycelial plugs in sterile water.

Due to the low fluorescence seen in 1-week-old transformants, the level of fluorescence in transformant mycelia and conidia was further investigated over a 6-week growth period on a weekly basis. Fluorescence in conidia could only be evaluated once conidia started oozing from pycnidia in older cultures. Evaluation of the fluorescence in the mycelia of GFP transformants revealed low to intermediate

levels of fluorescence in the different transformants throughout the 6 week evaluation period. Transformants PG8.1, PG8.2, PG8.7, PG8.8, PG8.9, PG9.1, PG9.2 and PG9.4 exhibited intermediate levels of fluorescence, whereas the other remaining transformants only showed low fluorescence. The conidia of all GFP transformants exhibited a low level of fluorescence after 3 to 6 weeks of growth. The level of fluorescence in the mycelia of DsRed-Express transformants fluctuated over the 6-week evaluation period. The mycelia of all the DsRed-Express transformants only exhibited a low level of fluorescence when cultures were 1 week old. After 2 weeks of growth, most DsRed-Express transformants still had a low level of fluorescence in their mycelia (PR8.7, PR.9.4 and PR9.5), whereas a few transformants (PR8.1, PR8.2, PR8.3, PR9.1, PR9.2, PR9.3) showed a slight increase in fluorescence. After 3 weeks of growth, the fluorescence level in the mycelium of the aforementioned DsRed-Express transformants (PR8.1, PR8.2, PR8.3, PR9.1, PR9.2 and PR9.3) started decreasing again, whereas the other transformants (PR8.7, PR9.4, PR9.5) no longer fluoresced. Gradually, no fluorescence was observed in the mycelia of all transformants in the remaining 4- to 6-week growth period. Contrarily to the mycelia of DsRed-Express transformants, the conidia of these transformants fluoresced brightly after a 3- to 4-week growth period. At the end of the 6 week growth period, the conidia of most of the DsRed-Express transformants no longer fluoresced, except for two transformants (PR8.2 and PR8.3) that still showed medium fluorescence.

PCR detection of reporter genes and hygromycin resistance genes. PCR analyses revealed the presence of the *hph* gene in all of the transformants obtained from the wild type isolates PW8 and PW9 (Fig. 4, 5). The *GFP* gene was detected in all GFP transformants (PG8.1, PG8.2, PG8.3, PG8.5, PG8.8, PG8.9, PG8.10, PG9.1, PG9.2, PG9.3, PG9.4) (Fig. 4), and the *DsRed-Express* gene in all the red fluorescent transformants (PR8.1, PR8.2, PR8.3, PR8.7, PR9.1, PR9.2, PR9.3, PR9.4, PR9.5) (Fig. 5). Amplification of the *GFP*, *DsRed-Express* and *hph* genes yielded the expected PCR amplification size products of 417 bp, 200 bp and 700 bp, respectively. The two reporter genes were not detected in the wild type isolates or in the water control used in amplification reactions (Fig. 4, 5).

Biological characterisation of transformed and wild type isolates.

Colony colour. The colony colour of the selected transformants (PG8.1, PG8.2, PG9.1, PG9.3, PR8.1, PR8.2, PR9.2 and PR9.3) differed slightly from the wild type isolates (PW8 and PW9), but was similar to the natural variation seen in *P. viticola* colony colour when making sub-cultures from a single culture. The colour of the upper-side and reverse-side varied from buff (19"d) to shades of honey (19"b) and isabelline (17"i) according to Rayner (1970).

Conidial germination. Analysis of variance of germination percentages indicated no significant differences between the wild types and transformant isolates ($P = 0.3359$; ANOVA table not shown) with mean germination percentage ranging from 91 to 87% (Table 1).

Spore morphology. Analysis of variance of conidial length and width measurements indicated no significant differences between the *P. viticola* transformants and wild type isolates PW8 and PW9 ($P = 0.3042$ length and 0.5650 width; ANOVA table not shown). Spore lengths averaged between 10.13 and 9.07 µm, with spore widths between 3.39 and 3.04 µm.

In vitro mycelium growth. Analysis of variance of radial colony growth measurements of transformants (PG8.1, PG8.2, PG9.1, PG9.3, PR8.1, PR8.2, PR9.2 and PR9.3) and wild type isolates (PW8 and PW9) grown at a range of temperatures indicated a significant interaction between the day and temperature only ($P < 0.0001$; ANOVA table not shown). A significant interaction involving isolates was not found ($P > 0.05$), nor did the main effect of isolates prove to be significant ($P = 0.2626$). There were no significant differences in mycelial growth rate when the transformants were compared to each other and their wild types (results not shown).

Virulence characterisation. Cuttings of cultivar Chenin blanc inoculated with the *P. viticola* transformants (PG8.1, PG9.1, PR8.2 and PR9.3) and their respective wild type isolates (PW8 and PW9) caused a brown-black discolouration on the xylem vascular tissue beneath the bark (lesions) after 4 months of inoculation (Fig. 6). A slight discolouration was found on the vine cuttings inoculated with the sterile agar plug (control), but this was clearly due to natural wound healing. Analysis of variance of lesion lengths beneath the bark showed a significant effect for

treatment (P = 0.0084), which was attributed to the control differing significantly from the inoculated cuttings. Similar lesion lengths were measured for the *P. viticola* transformants and their respective wild type isolates (Table 2).

Isolation studies from the control cuttings that received only a sterile agar plug yielded no *P. viticola* growth. Isolations from cuttings inoculated with the transformant and wild type isolates onto non-selective media all yielded *P. viticola* growth. However, on hygromycin selective media, *P. viticola* growth was only observed on plates containing isolations made from cuttings inoculated with either of the four transformants. Epifluorescence microscopy of these cultures showed that the GFP transformants (PG8.1 and PG9.3) all exhibited a low level of fluorescence. However, DsRed-Express transformants did not exhibit any fluorescence 1 week after isolation, with fluorescence only being present after 6 weeks in transformant PR8.2 and no fluorescence in transformant PR9.3. The transformants PR8.2 and PR9.3 did however grow on the selective media. Isolations from the cuttings also yielded very low frequencies of various fungal cultures, including *Acremonium*, *Alternaria*, *Gliocladium*, *Penicillium* and *Trichoderma* spp.

DISCUSSION

A polyethylene glycol/calcium chloride transformation method was used to obtain stable transformants of two *P. viticola* isolates (PW8 and PW9) expressing the reporter genes *GFP* and *DsRed-Express*, using the *hph* gene as selectable marker. The transformants were shown to be stably transformed since they retained their hygromycin resistant phenotypes after three consecutive subcultures onto non-selective media, as well as after 4 months of storage. The presence of the transgenes after these time periods was also confirmed through PCR analyses.

The transformation frequencies of both *P. viticola* isolates were low, with approximately one and three transformants per microgram vector DNA for PW8 and PW9 respectively. The transformants were all selected using the *trpC* promoter of *Aspergillus nidulans* for driving expression of the selectable marker gene *hph* in GFP as well as DsRed-Express transformants. De Guido *et al.* (2003) obtained 5 to 10

transformants per microgram vector DNA when expression of the *hph* gene was driven by the *gpd* promoter of *A. nidulans*. However, when the *hph* gene was driven by the *OliC* promoter of *A. nidulans*, a much higher transformation efficiency of 107 to 330 transformants per microgram vector DNA was obtained (De Guido *et al.*, 2003). Therefore, higher transformation efficiencies might have been obtained in this study if the *hph* gene was driven by the *OliC* promoter, instead of the *trpC* promoter. Alternatively, it could be that the two isolates used in this study are not readily transformed. De Guido *et al.* (2003) found that the transformation rates varied from 79 to 330 putative transformants when investigating five *P. viticola* isolates representing four different vegetative compatibility groups (VCGs).

The transformation method yielded putative transformant colonies that consisted of either slow growing or fast growing colonies on the primary selective transformation plates. Transferring of these colonies to new selective media showed that most of the fast growing large colonies subsequently grew, whereas the small slow growing colonies mostly did not grow on selective medium. De Guido *et al.* (2003) also reported the presence of fast and slow growing colonies on primary transformation plates of *P. viticola* when using the *hph* gene as selectable marker. Slow growing putative transformant colonies are often found in fungal transformation experiments and are believed to be "abortive" transformants, resulting from vector DNA only being expressed in the cytoplasm and not being incorporated into nuclear DNA (Cullen *et al.*, 1987; Punt *et al.*, 1987; Timberlake and Marshall, 1989; Goosen *et al.*, 1991; Hamada *et al.*, 1994; Sánchez-Torres *et al.*, 1994; Riach and Kinghorn, 1996). However, De Guido *et al.* (2003) found that a variable number of their small slow growing colonies were able to grow on selective medium, and that these had a stable resistance phenotype. Most of these colonies had a reduced vigour when grown on non-selective medium, suggesting that the slow growing colonies included abortive transformants as well as transformants where the transgene was incorporated into genomic regions that affected colony growth (De Guido *et al.*, 2003).

The green fluorescence observed in the mycelia and conidia of GFP transformants derived from *P. viticola* isolates PW8 as well as PW9 was low to intermediate. There were no *P. viticola* transformants that fluoresced brightly, as often seen when viewing other fungi transformed with the *GFP* gene (Lorang *et al.*,

2001; Chapter 2; Chapter 4). The low GFP fluorescence seen in the *P. viticola* isolates could be due to instability of the mRNA or protein, a lack of function of the promoter, RNA silencing-related phenomena such as quelling and meiotic silencing by unpaired DNA, DNA methylation, or due to inefficient transcription of the *SGFP* gene, which has a plant optimised codon bias (Maor *et al.*, 1998; Lorang *et al.*, 2001; Fitzgerald *et al.*, 2003; Nakayashiki, 2005). The *SGFP* gene has been shown to function efficiently in a wide range of fungi, although a few exceptions exist. For example, Lugones *et al.* (1999) found that the efficient translation of the *SGFP* gene in *Schizophyllum commune* required the insertion of a *S. commune* intron after the *SGFP* stop codon for proper transcription. Another, more feasible reason for the low fluorescence levels are that the *ToxA* promoter, which has been shown functional in a wide range of fungi (Lorang *et al.*, 2001), might not function well in *P. viticola.* This is a feasible hypothesis since De Guido *et al.* (2003) found a large difference in the functionality of the *gpd* and *OliC* promoters in *P. viticola.* However, the exact reason for the low GFP fluorescence in *P. viticola* isolates remains unknown, since *P. viticola* has not been transformed with any reporter genes, along with the fact that *P. viticola* has rarely been studied at the genetic level (De Guido *et al.*, 2003).

The red fluorescence in the mycelium of all DsRed-Express *P. viticola* transformants derived from isolate PW8 as well as PW9 was low in 1-week-old cultures. However, after 2 and 3 weeks of growth a group of transformants (PR8.1, PR8.2, PR8.3, PR9.1, PR9.2 and PR9.3) showed higher fluorescence than the rest of the transformants. The difference in fluorescence levels between these transformants is most likely due to different *DsRed-Express* gene copy numbers being integrated into genomic DNA, or integration of *DsRed-Express* genes into different genomic regions, resulting in different levels of transcription (Sexton and Howlett, 2001). It can also not be ruled out that the expression of the gene in some transformants is under control of endogenous transcriptional regulatory elements. The fluorescence in the mycelium of all DsRed-Express transformants, contrarily to GFP transformants, disappeared after 4 weeks of growth. This is most likely due to the fact that the *gpd* promoter that drove expression of *DsRed-Express* is metabolically regulated, resulting in reduced transcriptional levels in older cultures where pycnidia formation has been initiated (Olivain *et al.*, 2006). The reason for the low fluorescence in the mycelia of all DsRed-Express transformants, similar to the GFP transformants, is most likely also

due to the poor functionality of the *gpd* promoter that drove expression of the *DsRed-Express* gene. The conidia of all DsRed-Express transformants fluoresced brightly after 3 weeks of growth. The bright fluorescence observed in young conidia of all the transformants could be due to the fact that the *gpd* promoter is metabolically regulated, and that the promoter drives high levels of transcription in conidia, although this has not been documented previously.

Evaluation of the biological characteristics and virulence of *P. viticola* wild type isolates and transformants showed that the isolates did not differ. Characterisation of the colony colour, conidial size, germination percentage and growth rate, did not reveal any significant differences amongst the studied isolates. The *in vitro* virulence characterisation trail proved that the transformation did not alter the virulence characteristic of the *P. viticola* isolates. This is essential for further studies on host-pathogen interactions. The *P. viticola* transformants retained their hygromycin resistance and fluorescence phenotype after being isolated from the inoculated cuttings, except for DsRed-Express transformant PR9.3 that no longer fluoresced and PR8.2 that only fluoresced after 6 weeks. The reason for the unusual fluorescence phenotypes of these transformants is unknown since PCR analysis was not conducted further to investigate the presence of the *DsRed-Express* gene.

Since *P. viticola* GFP and DsRed-Express isolates only exhibited low levels of fluorescence, these isolates would most likely be indistinguishable from other filamentous fungi in the woody host, having limited value in host-pathogen interaction studies. Nonetheless, the hygromycin resistance phenotype of the transformants obtained in this study can still be used to investigate the endophytic nature of inoculated transformants through isolations made onto hygromycin selective media. Future studies should evaluate different promoters, such as the *OliC* promoter, for driving the expression of the reporter genes in order to obtain brightly fluorescing transformants. Alternatively, very large numbers of transformants should be screened in order to identify brightly fluorescing transformants.

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Table 1. Mean germination percentages of *Phomopsis viticola* reporter gene-labeled transformants (PR8.1, PR8.2, PR9.2, PR9.3, PG8.1, PG8.2, PG9.1 and PG9.3) and wild type isolates (PW8 and PW9)

Isolates	Mean germination percentage[x]
PW8	89.33 ab
PW9	89.33 ab
PR8.1	88.33 ab
PR8.2	91.00 a
PR9.2	87.00 b
PR9.3	88.67 ab
PG8.1	91.33 ab
PG8.2	88.00 ab
PG9.1	89.67 ab
PG9.3	87.68 ab
LSD ($P = 0.05$)	3.417

[x] Values in each column followed by the same letter do not differ significantly ($P < 0.05$).

Table 2. Mean lesion lengths on cv. Chenin blanc 4 months after inoculation with *Phomopsis viticola* reporter gene-labeled transformants (PR8.2, PR9.3, PG8.1, PG9.3) and wild type isolates (PW8 and PW9)

Treatment	**Lesion length**[x]
Control	4.43 b
PW8	33.99a
PW9	42.76a
PR8.2	27.43a
PR9.3	45.72a
PG8.1	31.71a
PG9.3	30.67a
LSD ($P > 0.05$)	20.951

[x] Values in each column followed by the same letter do not differ significantly ($P = 0.05$).

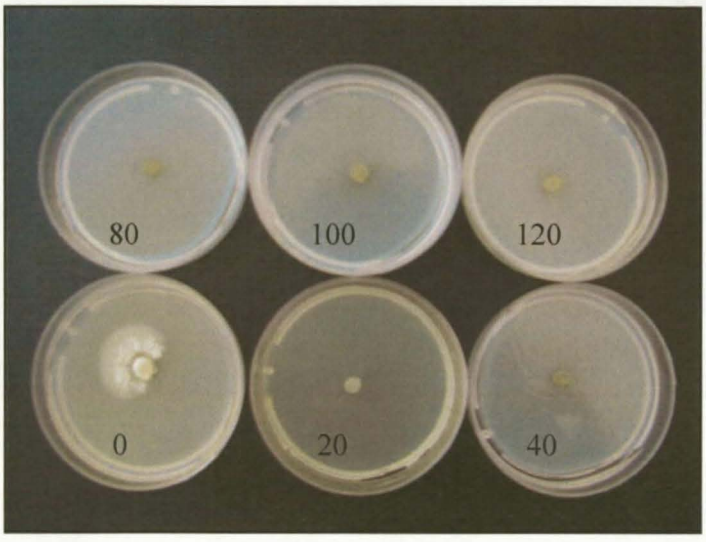


Fig. 1. *Phomopsis viticola* growth on potato dextrose agar plates containing different concentrations of hygromycin B (0, 20, 40, 80, 100 and 120 µg/ml), 5 days after inoculation.

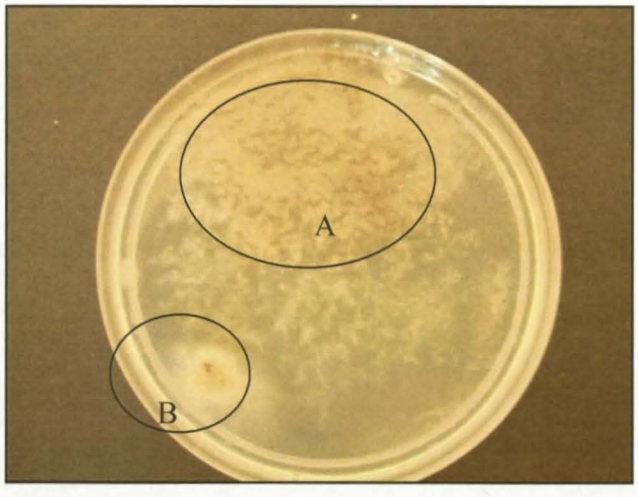


Fig. 2. Putative transformant colonies of *Phomopsis viticola* on primary transformation plates, which included slow growing (A) as well as large fast growing (B) putative transformants colonies.

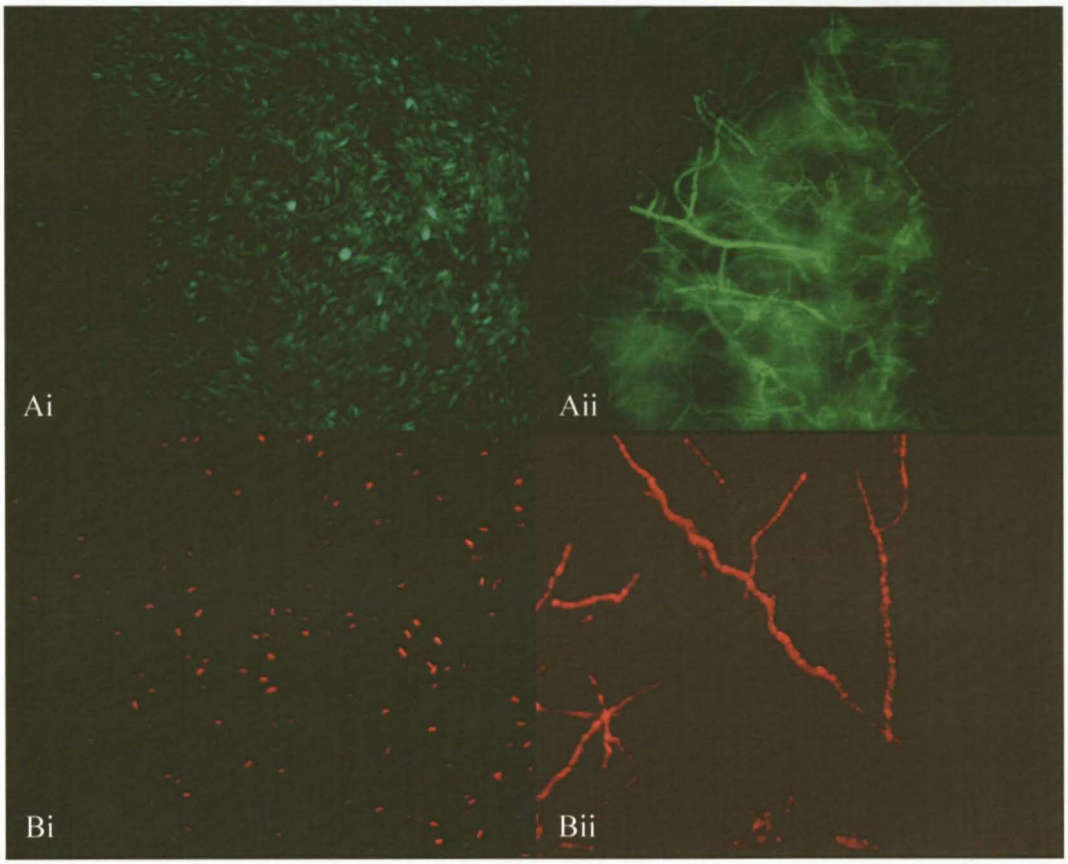
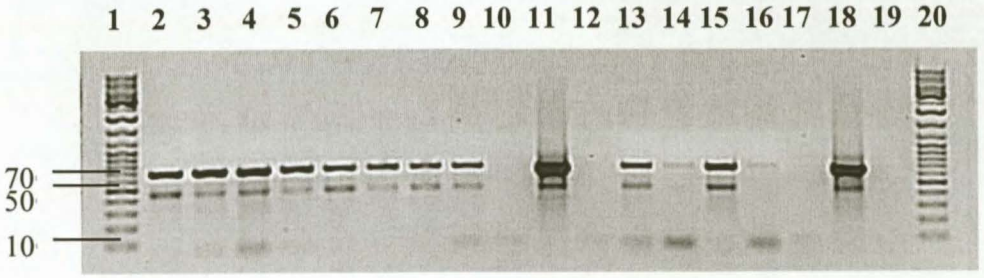
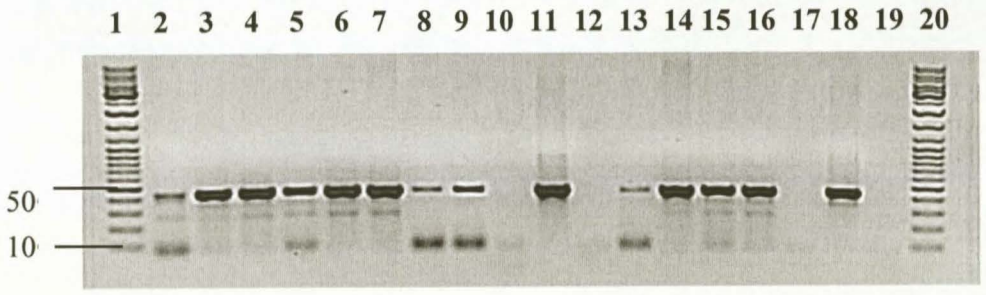


Fig. 3. *Phomopsis viticola* transformants expressing the (A) GFP and (B) Dsred-Express genes in (i) conidia and (ii) mycelium.



(A)



(B)

Fig. 4. Polymerase chain reaction (PCR) analyses of *Phomopsis viticola* transformants derived from two wild type isolates PW8 and PW9, confirming the presence of (A) the selectable marker gene hygromycin phosphotransferase (*hph*) and (B) the green fluorescent protein (GFP) gene. PCR was conducted using primers specific to the (A) *hph* and (B) *GFP* genes, yielding a 700 bp or 417 bp PCR fragment respectively. Lane 1, molecular weight marker; lane 2-9, GFP transformants (PG8.1, PG8.2, PG8.3, PG8.5, PG8.8, PG8.9, PG8.10) derived from wild type isolate PW8; lane 10, wild type isolates PW8; lane 11, plasmid vector pCT74 containing the *GFP* and *hph* gene; lane 12, water control; lane 13-16; GFP transformants (PG9.1, PG9.2, PG9.3, PG9.4) derived from wild type isolate PW9; lane 17, wild type isolate PW9; lane 18, plasmid vector pCT74 containing the *GFP* and *hph* gene; lane 19, water control; and lane 20, molecular weight marker.

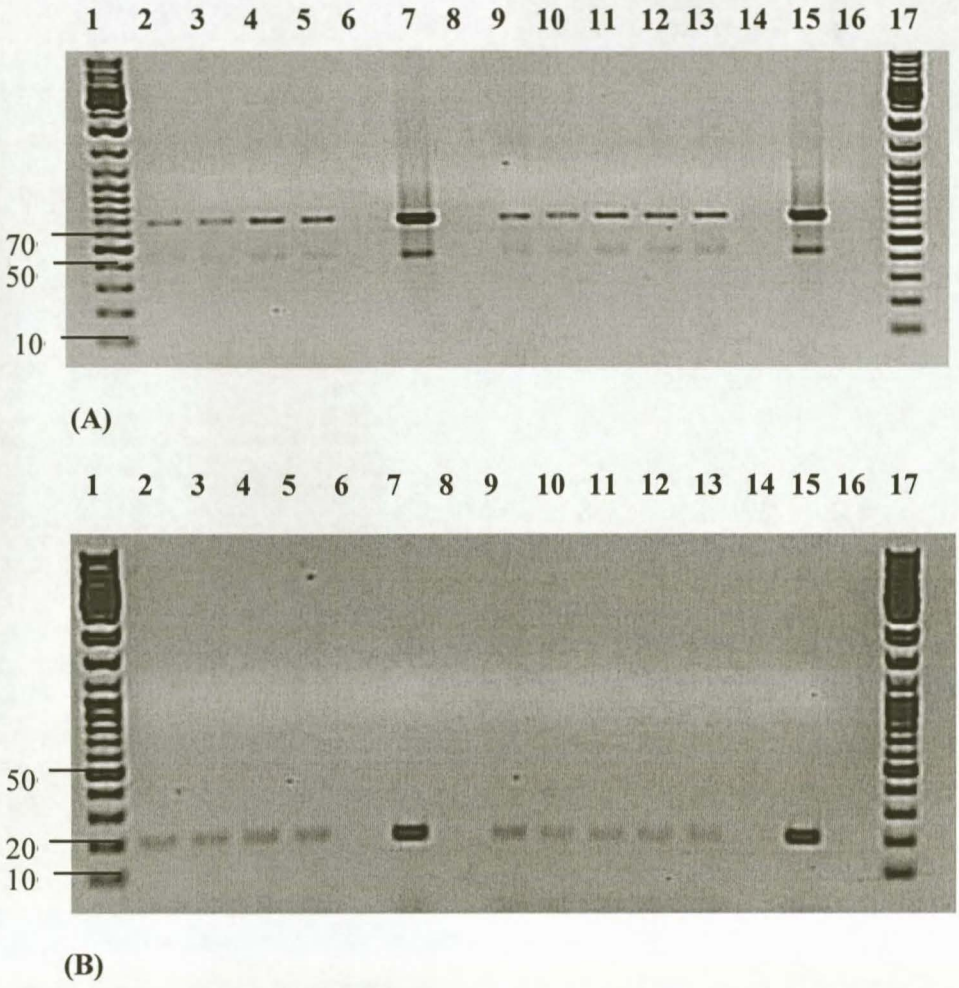


Fig. 5. Polymerase chain reaction (PCR) analyses of *Phomopsis viticola* transformants derived from two wild type isolates PW8 and PW9, confirming the presence of the (A) selectable marker gene hygromycin phosphotransferase (*hph*) and the (B) red fluorescent protein (DsRed-Express) gene. PCR was conducted using primers specific to the (A) *hph* and (B) *DsRed-Express* genes, yielding a 690 bp or 200 bp PCR fragment respectively. Lane 1, molecular weight marker; lane 2-5, DsRed-Express transformants (PR8.1, PR8.2, PR8.3, PR8.7) derived from wild type isolate PW8; lane 6, wild type isolates PW8; lane 7, plasmid vector pHyg8 containing the *hph* gene (A) or vector pPgpd-DsRed containing the *DsRed-Express* gene (B); lane 8, water control; lane 9-13; DsRed-Express transformants (PR9.1, PR9.2, PR9.3, PR9.4, PR9.5) derived from wild type isolate PW9; lane 14, wild type isolate PW9; lane 15, plasmid vector pHyg8 containing the *hph* (A) gene or vector pPgpd-DsRed containing the *DsRed-Express* gene (B); lane 16, water control; and lane 17, molecular weight marker.

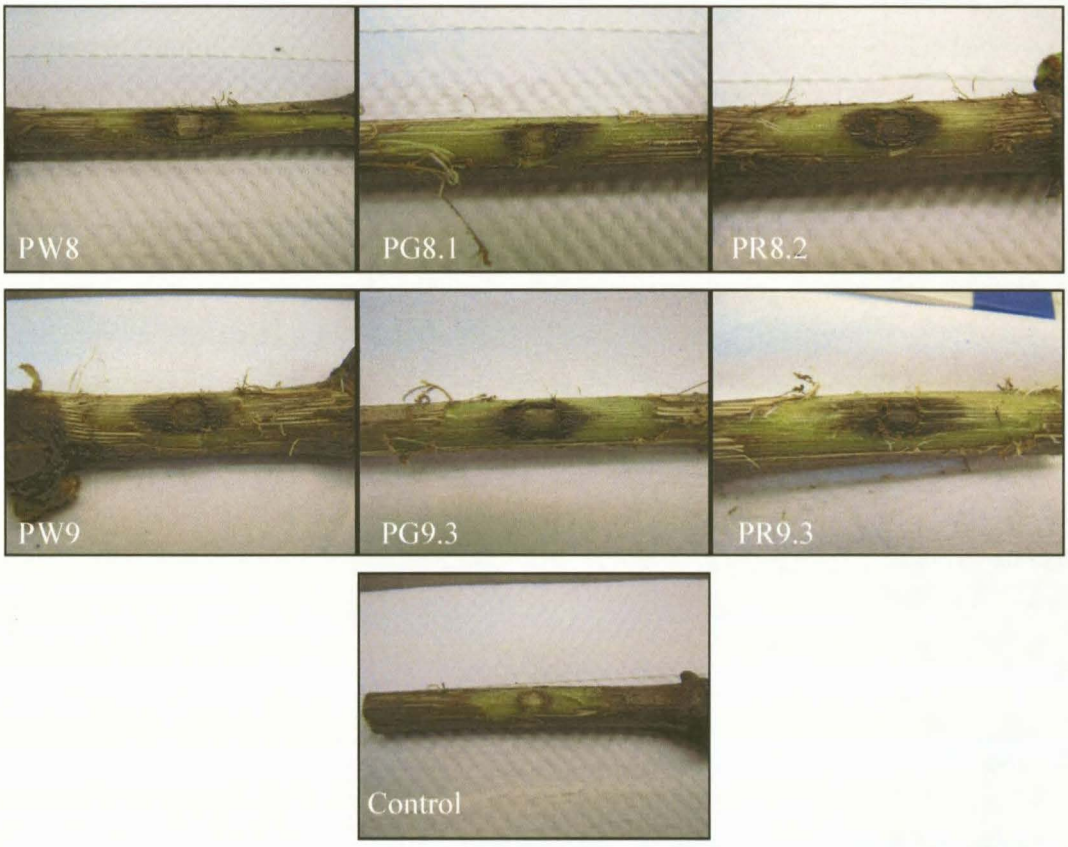


Fig. 7. Lesions beneath the bark caused by *Phomopsis viticola* wild type isolates (PW8 and PW9), GFP transformants (PG8.1 and PG9.3), and DsRed-Express transformants (PR8.2 and PR9.3) on Chenin blanc grapevine cuttings 4 months after inoculation. The control cuttings that were inoculated with a sterile agar plug only showed discolouration due to natural wound healing.

4. STABLE TRANSFORMATION OF *TRICHODERMA HARZIANUM* WITH THE GREEN (GFP) - AND RED (DSRED-EXPRESS) FLUORESCENT PROTEIN REPORTER GENES

ABSTRACT

Biological grapevine pruning wound protection agents such as *Trichoderma harzianum* have demonstrated the ability to colonise pruning wound sites, thereby providing a living barrier against trunk disease pathogen invasions. In order to facilitate studies of these host-pathogen-biocontrol interactions, the aim of this study was to transform *T. harzianum* strain T77 with the green fluorescent protein (GFP) or the red fluorescent protein (DsRed-Express) reporter genes, using a polyethylene glycol/calcium chloride transformation method. Transformation of *T. harzianum* strain T77 was difficult, since most transformants proved to be unstable, and did not retain the selectable marker gene (hygromycin phosphotransferase) or the reporter genes. Sub-culturing and purification of several putative transformants ultimately yielded only four stably transformed isolates, two expressing *GFP* (TG1 and TG2) and two expressing *DsRed-Express* (TR1 and TR2). Stable transformation of these isolates was proved through five successive transfers on non-selective media. The stable transformants were characterised further to determine whether the transformation process has altered their growth rate, morphology, conidial size and germination percentage, when compared to the wild type isolate. The transformants were not altered in any of these characteristics, with the exception of transformants TR1 and TG1. Compared to the wild type isolate, TR1 had a faster growth rate at low temperatures (10-15°C), a lower spore germination percentage as well as a lighter green colony colour. Transformant TG1 had a significant lower germination percentage than the wild type isolate. Transformants TR1 and TG1 were further characterised with regard to their ability to colonise grapevine cuttings of cultivars Chenin blanc and Merlot in a hydroponic system. Both *T. harzianum* transformants were able to colonise grapevine cuttings in a similar manner than the wild type isolate, and retained their fluorescent phenotype following isolation from the host.

All isolates including the wild type, showed a significantly higher colonisation rate on Merlot than on Chenin blanc.

INTRODUCTION

The development of efficient biocontrol agents has been at the forefront of agricultural development and research for years. Fungi belonging to the genus *Trichoderma* have dominated biocontrol studies over the past 70 years, providing biocontrol against a wide range of root, seed, foliar and storage rot diseases (Thrane *et al.*, 1995; Lo *et al.*, 1998; Harman, 2000; Howell, 2003; Hanson and Howell, 2004). Disease suppression and the protection exerted by *Trichoderma* spp. include antibiosis, mycoparasitism, competition for nutrients and space, triggering of plant defense reactions and inducing systemic resistance (Lo *et al.*, 1998; Harman, 2000; Steyaert *et al.*, 2003; Di Marco *et al.*, 2004; Harman *et al.*, 2004; Lu *et al.*, 2004; Carpenter *et al.*, 2005; Harman, 2006; Howell, 2006). These mechanisms will vary according to the specific biological control isolate used, the pathogen and the host involved in the interaction.

Trunk diseases have a detrimental impact in the grapevine industry due to decline and premature dieback of grapevines (Ferreira *et al.*, 1989; Mugnai *et al.*, 1999; Chiarappa, 2000; Halleen and Fourie, 2005). The main trunk disease pathogens that can cause a reduction in quantity and quality in yields include basidiomycetes (Fischer, 2006), species of Botryosphaeriaceae (including *Botryosphaeria* and aggregate genera, *Lasiodiplodia* and *Neofusicoccum*) (Van Niekerk *et al.*, 2004; Crous *et al.*, 2006; Van Niekerk *et al.*, 2006), *Eutypa lata* (Munkvold and Marois, 1995), *Phaeoacremonium* spp. (Mostert *et al.*, 2006a, b), *Phaeomoniella chlamydospora* (Mugnai *et al.*, 1999) and *Phomopsis viticola* (Van Niekerk *et al.*, 2005). These pathogens mainly enter the host through pruning wounds, which stay susceptible for several weeks after pruning (Munkvold and Marois, 1993; Munkvold and Marois, 1994; Halleen *et al.*, 2003; Van Niekerk *et al.*, 2006). This is problematic, since it is known that chemical treatments may not protect the pruning wounds for the specific period of susceptibility, due to residue breakdown (John *et al.*, 2004; John *et al.*, 2005). Therefore, it is crucial to develop sustained pruning wound

protection methods against trunk disease pathogens that can provide prolonged protection at commercially acceptable levels.

The application of *Trichoderma* spp. as a biological pruning wound protectant is a promising trunk disease management approach, since it may provide better long-term protection than fungicides (John *et al.*, 2004). Strains of *Trichoderma* have been shown to have *in vitro* activity against *Pa. chlamydospora* as well as *E. lata* (Hunt *et al.*, 2001; Di Marco *et al.*, 2004; John *et al.*, 2004). Harvey and Hunt (2006) showed in field studies that the timely and targeted application of *T. harzianum*, 5 hours after pruning, provided very good pruning wound protection and colonisation by the fungus. The biocontrol agent was also persistent in grapevine pruning wounds, and could be re-isolated 8 months after inoculation (Harvey and Hunt, 2006). Di Marco *et al.* (2004) also found in field studies that *Trichoderma* spray inoculated onto pruning wounds remained viable and persistent for up to 60 days, whereafter it gradually decreased. Their field studies further showed that *Trichoderma* was effective in preventing pruning wound infection by *Pa. chlamydospora* in pathogen-free planting material.

Although biological strategies are gaining more ground and some studies have been conducted using *Trichoderma* as wound protectant (Fourie *et al.*, 2001; Hunt, 2003), controversy still surrounds the recommendation of this fungus as protectant (John *et al.*, 2004). The mechanisms involved in biocontrol activity are not yet fully understood, nor to what extent colonisation takes place on different grapevine cultivars and whether it will persist. Histological studies on the activity of *Trichoderma* at the pruning wound sites can provide some key answers to these aspects. However, these studies have been hampered by the fact that the biocontrol agent can not be discriminated from other fungi within host tissue.

Manipulating biocontrol agents with reporter genes that convey a detectable phenotype, has proved to be a useful tool for detection and monitoring of introduced biocontrol agents in natural environments (Green and Jensen, 1995; Lo *et al.*, 1998). Two fluorescent reporter genes that are most often used for labeling fungi include the green fluorescent protein (GFP) gene and derivatives of the red fluorescent protein (DsRed) gene, which includes DsRed-Express (Chalfie *et al.*, 1994; Chalfie and Kain, 1998; Maor *et al.*, 1998; Lorang *et al.*, 2001; Hakkila *et al.*, 2002). These reporter

genes further have the advantage that their unique and different spectral properties allow dual labeling and visualisation studies of fungi (Baird *et al.*, 2000; Mikkelsen *et al.*, 2003; Nahalkova and Fatehi, 2003). Although various *Trichoderma* strains have been labeled with GFP (Bae and Knudsen, 2000; Orr and Knudsen, 2004), none of these isolates have been used to study pruning wound colonisation. The only reporter gene-labeled fungus that has been used to study fungi within aboveground wood tissue is the sapstain fungus, *Ophiostoma piceae*, and a potential biocontrol agent, *Ophiostoma piliferium*, which were transformed with GFP (Lee *et al.*, 2002).

Trichoderma harzianum strain T77 has been shown to have some efficacy against grapevine trunk disease pathogens (Halleen and Fourie, 2005). The aim of this study was to first produce mitotically stable transformants of *T. harzianum* strain T77 (ARC Nietvoorbij-Infruitec, Stellenbosch, South Africa) expressing the reporter genes *GFP* and *DsRed-Express*. The stable transformants that were obtained were subsequently characterised using a series of morphological, physiological and colonisation trials to determine whether the transformed isolates differed from the *T. harzianum* wild type (non-transformed strain).

MATERIAL AND METHODS

Growth and culturing of isolates. *Trichoderma harzianum* strain T77 used in the transformation experiments was kindly provided by ARC Infruitec-Nietvoorbij, Stellenbosch, South Africa. The isolate was maintained routinely on potato dextrose agar (PDA) (Difco Laboratories, MI, USA) at 25°C. The wild type culture as well as the transformed isolates were stored in sterile distilled water as mycelial plugs, as well as in glycerol at -80°C.

Screening *T. harzianum* strain T77 for hygromycin B sensitivity. The sensitivity of *T. harzianum* strain T77 to hygromycin B was determined as previously described (Chapter 2), except that 5-day-old cultures were used for inoculating plates and that the hygromycin B concentrations differed, consisting of concentrations of 40, 100, 150, 200, 250 and 300 µg/ml. The Petri dishes were rated for the presence or absence of hyphal growth after 5 days of incubation in the dark at 25°C.

Plasmid vectors. The plasmid vector pCT74 (Chapter 2) was used for labeling *T. harzianum* with GFP, whereas co-transformation with pHyg8 and pPgpd-Red (Chapter 2) was used for labeling the isolate with the red fluorescent protein (DsRed-Express). The hygromycin phosphotransferase (*hph*) gene was the selectable marker gene in vectors pCT74 and pHyg8.

Transformation of *T. harzianum*

Protoplast preparation. *Trichoderma harzianum* was grown on PDA plates for 5 days at 25°C in the dark. A spore suspension was prepared by flooding the plates with sterile distilled water, and filtering the suspension through Miracloth (Calbiochem, La Jalla, CA, USA). The spore suspension was adjusted to 4×10^6 spores/ml, where after an equal amount of double strength potato dextrose broth (PDB) (Difco Laboratories) was added and mixed. Approximately 100 ml of this solution was aliquoted into two 500 ml sterile Erlenmeyer flasks covered with cotton and foil. The spores were germinated overnight with incubation at 28°C and shaking at 150 rpm. The germinated spores, that formed a mycelial mat overnight, were harvested by filtration through Miracloth, and were subsequently washed with 0.8 M mannitol.

Trichoderma harzianum protoplasts were prepared from the germinated spores using a modified *Arabidopsis* mesophyll protoplast method (Sheen, 2002). Mycelia (approximately 0.5 g) were transferred into a sterile 50 ml Falcon tube containing 20 ml enzyme buffer (0.4 M mannitol, 20 mM KCl, 20 mM MES pH 5.7, 10 mM $CaCl_2$) and 40 mg/ml driselase (InterSpex Products, Inc., San Mateo, CA), 30 mg/ml lysing enzyme from *T. harzianum* (Sigma-Aldrich, Aston Manor, South Africa) and 15 mg/ml cellulase (Yakult Pharmaceuticals, LTD, Minato-KU, Tokyo, Japan). Several other enzyme combinations and concentrations were tested, which proved less effective (data not shown). Prior to dissolving enzymes in the buffer, the starch carrier in the driselase was first removed by mixing the driselase with the enzyme buffer and placing it on ice for 15 min, followed by centrifugation at a speed of 652 g for 5 min. The supernatant was poured off and added to the rest of the enzymes. Protoplasts were released from mycelia through incubation at 28°C under mild shaking (60 rpm), until a sufficient number of protoplasts were released as revealed through microscopic observations, after approximately 1 hour. Protoplasts were separated from the mycelial residues by filtration through a single layer of Miracloth

into a 50 ml Falcon tube. The protoplast solution was centrifuged for 4 min at 1258 g at 20°C in a swing bucket centrifuge (Eppendorf centrifuge 5810R) to pellet protoplasts. The supernatant was decanted and protoplasts were washed with 20 ml of W5 buffer [154 mM NaCl, 125 mM $CaCl_2$, 2 mM 2-morpholinoethanesulfonic (MES) pH 5.7 and 5 mM KCl]. The protoplast suspension was centrifuged again at 2000 rpm for 4 min at 20°C, the supernatant decanted and a few millilitres of W5 buffer was added to adjust the protoplast concentration to approximately 1×10^6 protoplasts/ml. Protoplasts were then incubated on ice for 20 min. The protoplast suspension was centrifuged again at 1258 g for 4 min at 20°C, the supernatant decanted and the protoplast pellet was resuspended in MMg solution (0.4 M Mannitol, 15 mM $MgCl_2$ and 200 mM MES pH 5.7) to a final concentration of 2×10^7/ml. The protoplast solution was then left at room temperature for 10 min, prior to transforming it with vector DNA.

Transformation of protoplasts. GFP transformation was conducted by adding 15 µl of vector pCT74 to a sterile 12 ml tube, whereas red fluorescent protein transformation was conducted using co-transformation with 10 µl pHyg8 and 20 µl pPgpd-DsRed. Subsequently, each tube received 100 µl of the protoplast solution that was mixed gently. A negative control, which consisted of protoplast receiving no vector DNA, was also included to ensure that the wild type nuclei were suppressed by the 300 µg/ml hygromycin concentration that was used for selecting transformants. After incubation for 15 min on ice, 2 ml of a sterile 40% polyethylene glycol 4000 (Fluka, Taufkirchen, Germany) solution (40% v/v PEG, 0.2 M mannitol, 0.1 M $CaCl_2$) was added in three aliquots of 500 µl, 500 µl and 1 ml, with gentle mixing between each step. After another 15 min incubation period on ice, 1 ml of 0.5 M mannitol/PDB solution was added, followed by the addition of another 8 ml. One hundred or 500 µl aliquots of the suspension were each mixed with 10 ml molten PDA (55°C) and poured into Petri dishes. Solidified plates were overlaid with 15 ml of a 1% water agar solution containing a final hygromycin B concentration of 300 µg/ml. These primary transformation plates were incubated for 5 days at 25°C in the dark. Putative transformants grew through the selective water agar layer on the primary transformation plates.

Epifluorescence microscopy. Putative *T. harzianum* hygromycin-resistant colonies that emerged on primary transformation plates after incubation of 4 to 5 days, as well as sub-cultured colonies that were single spored were tested for GFP and DsRed-Express fluorescence using epifluorescence microscopy as previously described (Chapter 2).

Selection of stable *T. harzianum* transformants. A subset of the putative transformants on the primary transformation plates that showed fluorescence were transferred to new PDA Petri plates containing 300 µg/ml hygromycin B. The plates were incubated in the dark for 1 to 2 days at 25°C. Subsequently, hyphal tip cultures were made from these cultures onto fresh hygromycin PDA plates. Each isolate was hyphal tipped four times and were tested regularly for GFP and DsRed-Express fluorescence using epifluorescence microscopy. Transformants that were hyphal tipped successfully were also single-spored twice on selective media. The stability of the transformants was tested by transferring a mycelial plug of each transformant onto non-selective media (PDA without hygromycin B) for five consecutive times at 5-day intervals. GFP and DsRed-Express fluorescence were again evaluated after this period using epifluorescence microscopy.

PCR detection of reporter genes and hygromycin resistance genes. Gene specific primers were used to investigate the presence of the *GFP*, *DsRed-Express* and *hph* genes in putative transformants that were hyphal-tipped and single-spored onto selective media, and subsequently grown on non-selective media for five transfers. The wild type isolate was included as a negative control. DNA isolations were made from mycelium of isolates that were grown on non-selective media (PDA) for 5 days in the dark at 25°C, using the Wizard® SV Genomic DNA Purification system (Promega Corporation, Madison, WI, USA) according to manufacturer's instructions. PCR amplification reactions and conditions were done as previously described (Chapter 2), except that different annealing temperatures were used, i.e. 55°C for *GFP*, 56°C for *DsRed-Express* and 66°C for the *hph* gene.

***In vitro* biological characterisation of transformed and wild type isolates.** Biological characteristics of the wild type isolate of *T. harzianum* that was transformed with the reporter genes, as well as two stably transformed GFP labeled and two DsRed-Express labeled transformants were compared with regard to colony

colour, conidial germination, spore morphology and mycelial growth rate at different temperatures.

Colony colour. The isolates were grown on PDA medium for 5 days in the dark at 25°C for evaluation. The colony colour of each *T. harzianum* isolate was evaluated using the colour chart of Rayner (1970).

Conidial germination. The isolates were plated onto PDA and incubated at 25°C in the dark for 5 days to promote sporulation. The germination percentage of a 1×10^6 spores/ml spore suspension was determined as previously described (Chapter 2), except that the germination percentage was determined after 8 hours of incubation. The trial was repeated twice.

Spore morphology. Microscope slides were prepared from 5-day-old cultures and the conidia length and width were measured as previously described (Chapter 2). The trial was repeated twice.

In vitro *mycelium growth.* The *T. harzianum* isolates were grown for 5 days on PDA, after which a colonised plug of each isolate was transferred onto 2% malt extract agar (Biolab, Merck Biosciences) and water agar. The plates were incubated for 8 days in the dark at four different temperatures raging from 10 to 25°C in 5°C intervals, with three repeats of each isolate per temperature. Radial colony diameters were measured twice perpendicularly 1, 3, 5 and 7 days after inoculation and the original mycelium plug diameter (5 mm) was subtracted.

Statistical analyses. The conidial germination percentage, spore size and *in vitro* mycelium growth data was submitted to analysis of variance (ANOVA) using SAS version 8.1 (SAS Institute, Cary, North Carolina, USA). Student's t-LSD was calculated to compare means of significant effect at the 5% significance level. *In vitro* mycelium growth data were also submitted to regression analysis.

***In vivo* colonisation ability of transformed and wild type isolates.** The *T. harzianum* wild type (TWT) and one transformed GFP and DsRed-Express isolate, that were also characterised morphologically, were used in host colonisation studies to determine whether the transformation procedure altered this characteristic relative to the wild type isolate.

Six-node cuttings of *Vitis vinifera* cultivars Chenin blanc and Merlot collected in July 2006 during the winter pruning season, were used in the colonisation studies. The cuttings were pruned ±2 cm above the sixth bud, and dipped in sterile water containing Sporekill (1 ml/liter) for 1 min followed by drying at room temperature. Subsequently, the cuttings were grown in a hydroponics system (Chapter 2) on laboratory benches at 28°C in natural light. Two days after planting, the cuttings received fresh pruning wounds, which were inoculated with 50 µl of a 1×10^6 *T. harzianum* spore suspension of each isolate. Control cuttings were treated with sterile water. Twelve weeks after inoculation the cuttings were pruned at the fifth bud. The harvested cuttings were cut into a 5-cm-long segment from the point of inoculation and split in half. One half of the cutting was used to make three isolations (each comprising two xylem sections of 1 × 0.5 mm), 5 mm apart, from just beneath the point of natural wound healing (Fig. 1) onto PDA. The other half of the cutting was used to make similar isolations onto PDA containing 300 µg/ml hygromycin B. The isolated segments were incubated for 4 to 5 days at 25°C. The percentage of re-isolated wild type and transformant isolates were recorded, as well as the ability of isolates to fluoresce.

The experiment was a randomised block design with four treatments randomly replicated within four blocks. Each treatment was replicated six times within each block. Data were subjected to analysis of variance and Student's t-tests as described above.

RESULTS

Screening for hygromycin B sensitivity. The wild type *T. harzianum* isolate still showed substantial growth at hygromycin concentrations of 40 to 150 µg/ml. Growth of the wild type isolate was completely inhibited at a concentration of 300 µg/ml hygromycin only (Fig. 2). This concentration was subsequently used for selection of transformants in transformation experiments.

Transformation of *T. harzianum* and selection of stable transformants. Colonies appeared after approximately 4 to 5 days incubation of the primary selective

plates, which received protoplasts transformed with vector DNA. No colonies appeared on primary selective plates containing protoplasts to which no vector DNA was added, confirming that the hygromycin concentration of 300 µg/ml was effective in suppressing the wild type protoplasts. A subset of the hygromycin-resistant colonies was transferred onto fresh selective media (PDA with 300 µg/ml hygromycin), of which 80% were able to subsequently grow on the selective media. The remaining 20% did not grow at all when transferred to new selective medium. Furthermore, when the colonies that did grow on new selective medium were inspected for fluorescence, only 5% showed some level of GFP or DsRed-Express fluorescence. Subsequent hyphal tipping of these putative fluorescent transformants, was mostly unsuccessful. Four independent transformation experiments yielded comparable results. Only seven fluorescent transformants (two GFP and five DsRed-Express) could be single-spored successfully on selective media after a series of hyphal tipping. Not all of these transformants retained their fluorescence phenotype after being transferred three consecutive times onto non-selective medium. Ultimately, only two GFP (TG1 and TG2) and two DsRed-Express (TR1 and TR2) transformants maintained their fluorescent phenotypes throughout the stability evaluation (Fig. 3).

PCR detection of reporter genes and hygromycin resistance genes. PCR analyses revealed the presence of the *hph* and *GFP* gene in the stable transformants TG1 and TG2, as well as the *hph* and *DsRed-Express* genes in the stable transformants TR1 and TR2 (Fig. 4). However, no PCR amplification was obtained from the three DsRed-Express transformants that lost their fluorescence phenotype when transferred onto non-selective media. No amplification was obtained from the wild type isolate (TWT) or the water control (Fig. 4).

***In vitro* biological characterisation of transformed and wild type isolates.**

Colony colour. Evaluation of the colony colour of the isolates revealed that there was a noticeable difference between the colony colour of TR1 and the wild type isolate (Fig. 5). Isolate TR1 had a Dark Herbage Green 27"k colour, whereas TWT, TG1, TG2 and TR2 isolates were all a dull green 27"m colour (Rayner, 1970).

Conidial germination. Analysis of variance of germination percentages indicated a significant difference between isolates (P = 0.0309; ANOVA table not

shown). Mean germination percentages ranged from 91.0% to 93.3%, with TR1 and TG1 (91.0 and 91.7%, respectively) having germination percentages significantly lower than TWT (93.3%) (Table 1).

Spore morphology. Analysis of variance of conidial length and width measurements indicated no significant difference between the *T. harzianum* transformants and wild type isolates ($P = 0.2352$ and 0.6956, respectively; ANOVA table not shown). Spore lengths averaged between 3.9 and 4.2 µm, with spore widths between 3.0 and 3.4 µm.

In vitro *mycelium growth.* Analysis of variance of radial colony growth measurements of transformants and wild type isolates grown at a range of temperatures indicated significant interactions between the day, temperature and isolate ($P < 0.0001$), as well as between growth medium, temperature and day ($P < 0.0001$; Table 2). As the latter interaction did not involve isolates, it was not investigated further. For the day × temperature × isolate interaction, a bivariate second order polynomial regression analysis of each isolate demonstrated that this interaction can be attributed to isolate TR1, which had a faster growth rate at low temperatures of 10°C and 15°C (Fig. 6). No significant differences were observed between the other isolates. Coefficients for the bivariate polynomial functions are shown in Table 3.

***In vivo* colonisation ability of transformed and wild type isolates.** Analysis of variance of isolation percentages from various positions in pruning wounds onto PDA medium showed a significant effect for *Trichoderma* strains ($P < 0.0001$; ANOVA table not shown). However, no significant differences were observed between mean isolation percentages for the wild type (51.4%), GFP (61.8%) and DsRed-Express (50.7%) isolates. The isolation percentages of *Trichoderma* inoculated treatments were all significantly higher than the water inoculated control treatment (1.91%). A significant effect was observed for cultivar ($P = 0.0179$). Merlot yielded a significantly higher mean isolation percentage (59.69%) than Chenin blanc (41.45%). A significant effect was not observed for isolation position ($P = 0.2498$), although a declining trend was observed with increased depth into the wound (51.3%, 41.9% and 38.3% for positions A, B and C, respectively).

Analysis of variance of isolation percentages on hygromycin amended PDA did, however, show a significant effect for isolation position ($P = 0.0132$; ANOVA table not shown). On the selective medium, a significant effect was also observed for *Trichoderma* strains ($P < 0.0001$). Only isolations from TG1 and TR1-inoculated cuttings yielded *Trichoderma* growth on the hygromycin amended PDA at similar percentages, while wild type inoculated and control cuttings did not yield any *Trichoderma* growth on the selective medium. All the re-isolated TG1 and TR1 isolates grew on selective as well as non-selective medium, and exhibited bright green and red fluorescence respectively when viewed using epifluorescence microscopy.

Isolations from cultivars Chenin blanc and Merlot cuttings yielded various fungi including *Acremonium* (2.16%), *Alternaria* (1.18%), Botryosphaeriaceae (0.43%), *Fusarium* (0.68%), *Gliocladium* (0.99%), *Penicillium* (6.36%) and *Rhizopus* (0.12%) species. Only two *T. harzianum* isolates were obtained from the water inoculated control cuttings.

DISCUSSION

Trichoderma harzianum strain T77 was stably transformed with the reporter genes *GFP* and *DsRed-Express* using a protoplast-based polyethylene glycol calcium chloride method and hygromycin as a selectable marker gene. The transformation procedure yielded many putative transformants on primary transformation plates. However, the majority of these transformants did not express the reporter genes or were unstable. Ultimately, only four stable reporter gene expressing isolates were obtained, two expressing *GFP* (TG1 and TG2) and two expressing *DsRed-Express* (TR1 and TR2). These four transformants retained their hygromycin resistance and fluorescent phenotypes after five successive transfers on non-selective media, as well as after being isolated from inoculated grapevine cuttings 12 weeks after inoculation.

Difficulty was experienced in obtaining transformants of *T. harzianum* strain T77 expressing the reporter genes. Many putative transformants on the primary transformation plates did not fluorescence, whereas those that did fluoresce did not grow when transferred to new selective media. Furthermore, three of the

transformants that could be single-spored on selective media, lost their fluorescence when grown on non-selective media. The loss of transgenes in these transformants was shown through PCR analyses, ruling out the involvement of RNA silencing-related phenomena such as quelling and meiotic silencing by unpaired DNA (Nakayashiki, 2005). Maor *et al.* (1998) found similar results with *T. harzianum* strain ATCC 32173, where hygromycin-resistant colonies did not exhibit fluorescence when transformed with the *GFP* gene fused to the *gpd* or *trpC* promoter of *Aspergillus nidulans*. In this study, the *GFP* gene fused to the *ToxA* promoter of *Pyrenophora tritici-repentis* (Lorang *et al.*, 2001), and the *DsRed-Express* gene fused to the *gpd* promoter of *A. nidulans* and was also not able to yield many fluorescent transformants. Maor *et al.* (1998) hypothesised that the mitotic stability of the DNA, stability of the mRNA or protein, or lack of function of the promoters could all possibly be the cause. However, a more feasible hypothesis provided by Maor *et al.* (1998), also considering results from this study, is that some *Trichoderma* isolates most likely spontaneously form heterokaryons (existence of two or more genetically different nuclei in the same cell) with great ease, which makes it very difficult to isolate single-spore hygromycin-resistant lines that will fluoresce (Maor *et al.*, 1998). Alternatively, it is possible that a particular genomic control system might be at work in some fungi that could interfere with the integration or the expression of foreign genes (Rolland *et al.*, 2003), in this case *GFP* and *DsRed-Express*.

The stably transformed isolates TG1, TG2, TR1 and TR2 were characterised to determine whether the transformation process altered their biology. The study showed that the transformants were not altered in their colony growth colour, conidial size, germination percentage, morphology and growth rate, with the exception of transformant TR1 and TG1. Transformant TR1 and TG1 were changed in their biological characteristics, emphasising the need to characterise transformants before conducting further laboratory or greenhouse trials. The phenotypic and biological changes in TR1 and TG1 may have been caused by the integration of some of the transgenes into an important gene-coding area or promoter region of the transformants.

The host colonisation studies showed that the *T. harzianum* wild type isolate and transformants TG1 and TR1 were able to colonise grapevine cuttings in a similar manner. Moreover, these isolates and the wild type *T. harzianum* isolate could be re-

isolated after 12 weeks of inoculation. The re-isolated transformants also retained their hygromycin resistance and fluorescent phenotypes. The presence of endogenous *Trichoderma* isolates was shown in the water inoculated control cuttings at a very low frequency, only two isolates. This could be due to the fact that *Trichoderma* isolates can be present endophytically in grapevines (Halleen *et al.*, 2005). However, it is also possible that the *Trichoderma* isolates in the water control originated from contaminating spores of *T. harzianum* T77.

Isolations from grapevine cuttings in this study revealed the presence of several endophytes, including some known pathogens. This is not unexpected, since it has been widely reported that known pathogens that were isolated in this study, occur within grapevine propagation material (Halleen *et al.*, 2003). *Trichoderma* strain T77 has a good competitive pruning wound colonising ability within grapevine cuttings, since it was able to colonise and establish in the wound site for 12 weeks, in the presence of natural occurring endophytes and putative pathogens within the grapevine cutting. The fresh pruning wounds that are rich in nutrients most likely enables *T. harzianum* to colonise the wounds, where it can compete with the pathogens or endophytes for nutrition, space and moisture (Anderson and Brodbeck, 1989).

An interesting observation from the inoculation studies was that *T. harzianum* strain T77 was able to colonise pruning wounds of Merlot significantly better than Chenin blanc. This could be due to the fact that the two cultivars differ in certain genes that influence the interaction with *T. harzianum* strain T77. Although the selection of specific beneficial microbial genotypes by specific plant genotypes is becoming an increasingly important theme in microbial soil ecology, the host genes involved in these interactions are still unknown (Smith *et al.*, 1999; Mazzola, 2004; Mazzola *et al.*, 2004; Picard and Bosco, 2006). Alternatively, this could be due to physiological and morphological differences between the two cultivars. Either way, this may hold an additional level of compilation for the broad scale commercial application of biocontrol agents, since a specific biocontrol agent genotype might not be effective in colonising and establishing on all the different host genotypes, i.e. cultivars, which are grown commercially.

The well characterised fluorescently labeled isolate TG1 and TR1 will unfortunately have limited use in future studies, since they have physiological and morphological characteristics that differ from the wild type isolate. Therefore, the other *Trichoderma* transformants TR2 and TG2 should be characterised further in future host colonisation studies to determine if they differ from the wild type isolate in this aspect. If these transformants are similar to the wild type isolate in their host colonisation ability, they can be used in future laboratory studies to investigate the biocontrol agent's colonisation and survival on different cultivars, as well as mechanism of pathogen control. Although laboratory studies can not always be extrapolated to the field, they are better suited for initial investigations since a controlled environment approach offers the ability to study the effect of single factors that can not be elucidated in a complex field situation.

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Table 1. Mean germination percentages of *Trichoderma harzianum* strain T77 (TWT) as well as GFP-labeled (TG1 and TG2) and DsRed-Express-labeled (TR1 and TR2) transformants derived from *T. harzianum* T77

Isolates	Mean germination percentage[x]
TWT	93.33 a
TG1	91.67 bc
TG2	92.33 ab
TR1	91.00 c
TR2	92.33 ab
LSD ($P = 0.05$)	1.171

[x] Values in each column followed by the same letter do not differ significantly ($P < 0.05$).

Table 2. Analysis of variance for the mean radial colony growth measurements at 1, 3, 5 and 7 days after inoculation of *Trichoderma harzianum* strain T77 and reporter gene-labeled transformants (TG1, TG2, TR1 and TR2) which were grown on 2% malt extract agar and water agar medium at a range of temperatures (10-25°C)

Source	DF	Sum of Squares	Mean Square	F Value	P
Block (B)	1	652.1	652.1	11.56	0.0273
Isolate (I)	4	308.5	77.1	1.37	0.3846
Error a *	**4**	**225.6**	**56.4**		
Temperature (T)	3	84494.0	28164.7	173.06	<0.0001
Growth medium (M)	1	681.5	681.5	4.19	0.0483
M × T	3	322.9	107.6	0.66	0.5814
I × T	12	4715.1	392.9	2.41	0.0211
I × M	4	646.9	161.7	0.99	0.4239
I × M × T	12	578.1	48.2	0.30	0.9860
Error b*	**35**	**5696.0**	**162.7**		
Day (D)	3	153170.4	51056.8	1403.48	<0.0001
I × D	12	959.9	80.0	2.20	0.0197
T × D	8	67490.1	8436.3	231.90	<0.0001
M × D	3	4322.8	1440.9	39.61	<0.0001
M × T × D	5	2393.6	478.7	13.16	<0.0001
I × T × D	27	4722.6	174.9	4.81	<0.0001
I × M × D	12	459.5	38.3	1.05	0.4115
I × M × T ×D	16	522.3	32.6	0.90	0.5746
Error c*	**77**	**2801.2**	**36.4**		
Sample Error	**483**	**2782.9**	**5.8**		
Corrected Total	725	337946.0			

***Error a:** Isolate × Block
***Error b:** Block (Isolate × Growth medium × Temperature)
***Error c:** Block (Isolate × Growth medium × Temperature × Day).

Table 3. Coefficients (± standard error) of *Trichoderma harzianum* strain T77 (TWT), GFP-labeled (TG1 and TG2) and DsRed-Express-labeled (TR1 and TR2) transformants, which were used for the bivariate second order polynomial function ($z = a+bx+cy+dx^2+ey^2+fxy$) of mean radial colony growth measurements at 1, 3, 5 and 7 days after inoculation on 2% malt extract agar and water agar medium and grown at a range of temperatures (10-25°C)

Functions	TG1	TG2	TR1	TR2	TWT
Intercept (a)	-33.8 (10.42)	0.9 (9.41)	-66.8 (11.23)	-12.6 (8.79)	-3.2 (7.83)
Temperature (b)	4.0 (1.11)	0.2 (1.00)	7.3 (1.22)	1.7 (0.94)	0.8 (0.83)
Temp2 (c)	-0.1 (0.03)	-0.03 (0.03)	-0.2 (0.03)	-0.1 (0.03)	-0.04 (0.02)
Day (d)	-5.4 (2.43)	-11.1 (2.18)	8.7 (2.49)	-7.0 (2.05)	-10.3 (1.77)
Day2 (e)	-0.5 (0.21)	-0.2 (0.18)	-0.8 (0.23)	-0.2 (0.18)	-0.1 (0.15)
Temp × Day (f)	1.6 (0.09)	1.5 (0.09)	0.3 (0.08)	1.1 (0.07)	1.3 (0.07)
R-Square	0.933	0.990	0.959	0.969	0.991

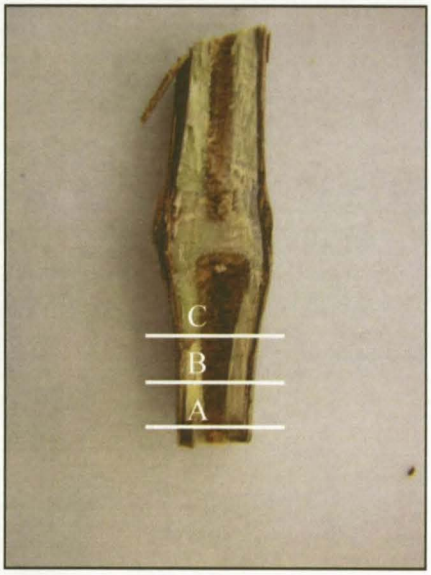


Fig. 1. Three positions (A, B and C) in a longitudinally split grapevine cutting from which isolations were made in the *Trichoderma harzianum* colonisation studies.

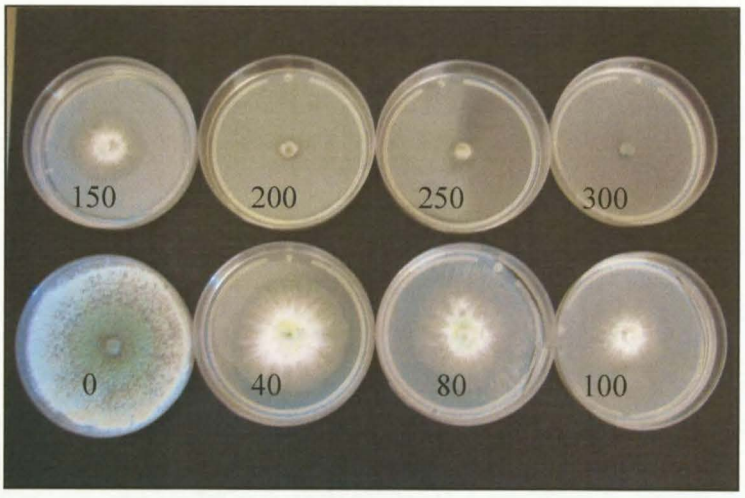


Fig. 2. Growth of *Trichoderma harzianum* strain T77 on PDA Petri plates containing different concentrations of hygromycin B (0, 40, 80, 100, 150, 200, 250 and 300 µg/ml).

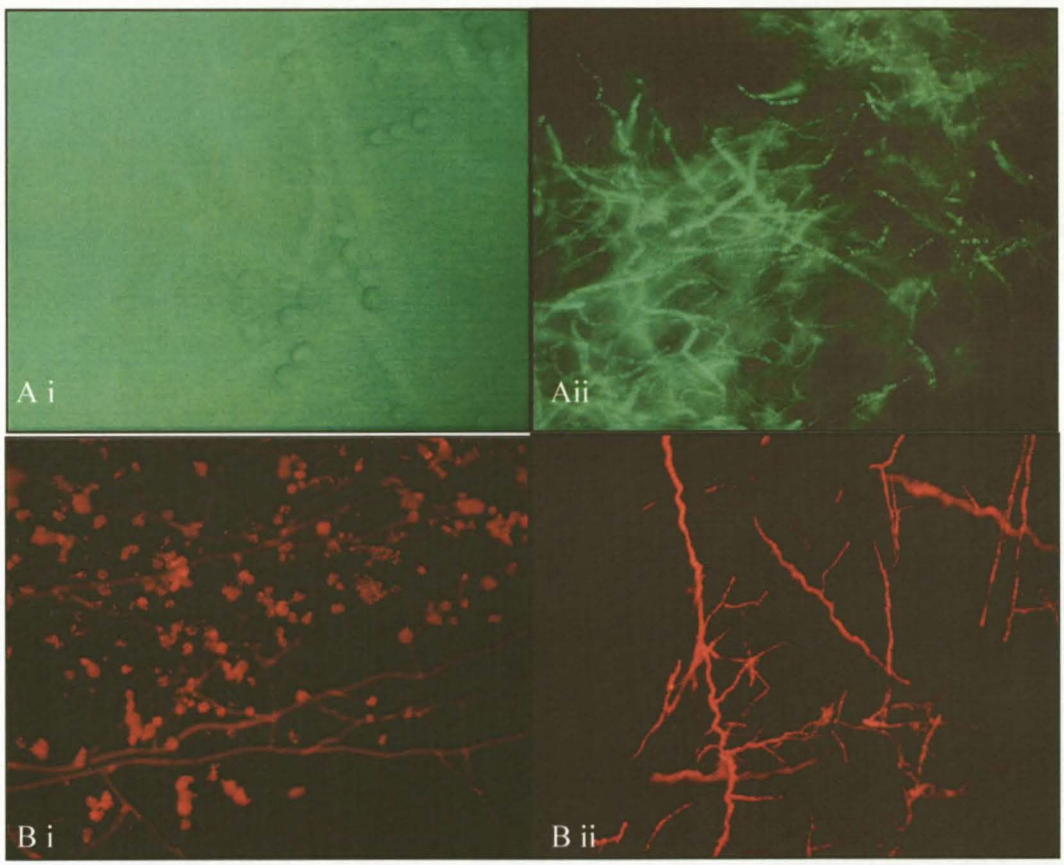
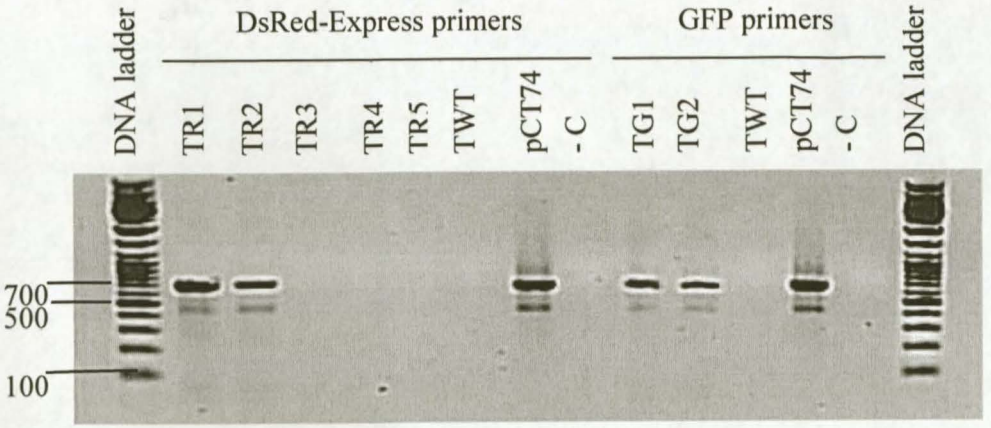
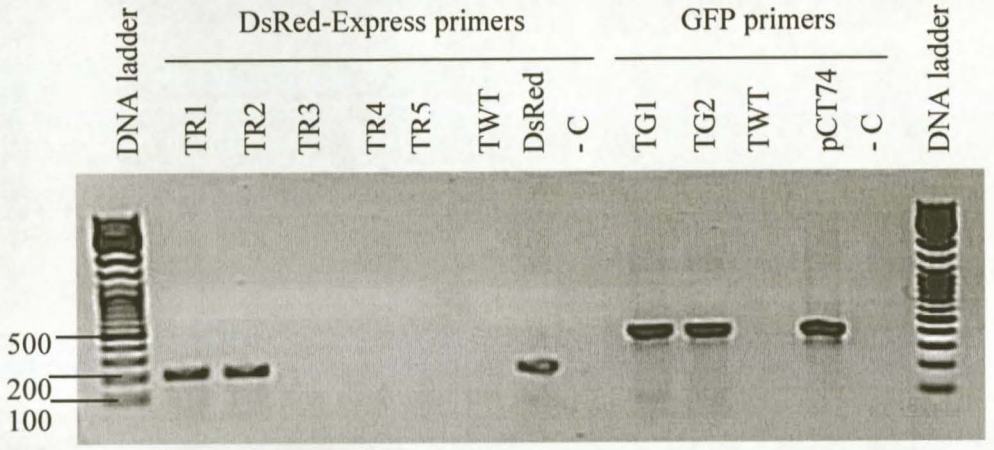


Fig. 3. *Trichoderma harzianum* transformants expressing the (A) *GFP* and (B) *Dsred-Express* genes in (i) conidia and (ii) mycelium.



(A)



(B)

Fig. 4. Polymerase chain reaction (PCR) analyses of *Trichoderma harzianum* transformants (TR1, TR2, TR3, TR4, TR5, TG1 and TG2), confirming the presence or absence of the (A) selectable marker gene hygromycin phosphotransferase (*hph*), and (B) reporter genes *DsRed-Express* and *GFP*. Transformants were derived from *T. harzianum* strain T77 (TWT), which was included as a negative control along with a water control. PCR was conducted using primers specific for *hph*, *DsRed-Express* and *GFP* genes yielding a 700, 200 or 417 bp PCR fragment respectively. Lane 1, molecular weight marker; lane 2-6, DsRed-Express transformants; lane 10-11, GFP transformants; lane 7 and 12, wild type isolate (TWT); lane 9 and 14, water control; lane 8A and lane 13, plasmid vector pCT74 containing the *hph* and *GFP* genes; lane 8 B, plasmid vector pPgpd-DsRed containing the *DsRed-Express* gene; lane 15, molecular weight marker.

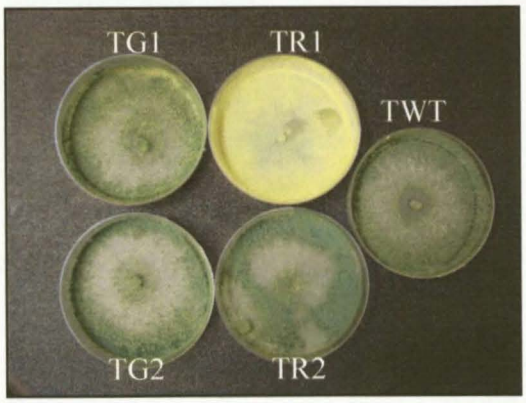


Fig. 5. Colony colour of *Trichoderma harzianum* wild type isolate strain T77 (TWT), DsRed-Express transformants TR1 and TR2, and GFP transformants TG1 and TG2 after 5 days of growth at 25°C on potato dextrose agar.

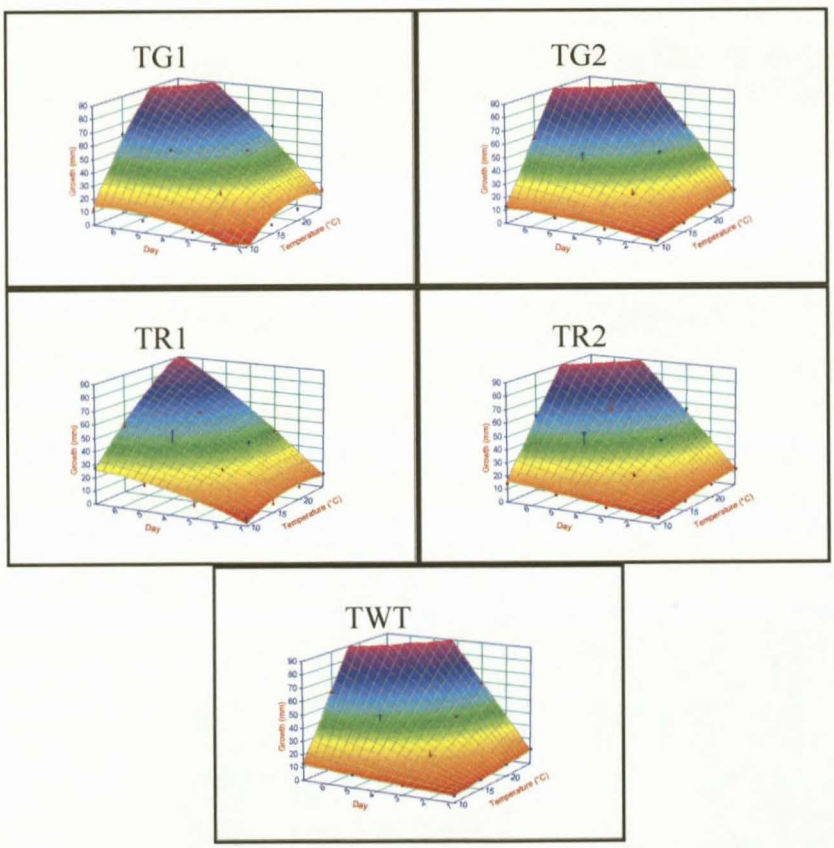


Fig. 6. Bivariate second order polynomial functions of mean radial growth (measured 1 to 7 days after inoculation) of *Trichoderma harzianum* wild type strain T77 (TWT) and reporter gene-labeled transformants (TG1, TG2, TR1 and TR2) which were grown on 2% malt extract agar and water agar medium at a range of temperatures (10-25°C).