Characterization and expression of an endopolygalacturonase gene from a lupin anthracnose fungus identified as *Colletotrichum lupini* var. *setosum*

By

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Declaration

I, the undersigned, hereby declare that the work contained in this dissertation is my own original work and that I have not previously in its entirety or in part submitted it at any University for a degree.

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Table of contents

Summary

Endopolygalacturonases (PGs) are the first cell wall degrading enzymes that are produced when pathogenic fungi encounter the host cell wall (Albersheim and Anderson, 1971). The role that these enzymes play in pathogenicity has been investigated for numerous pathogenic fungi. Although the results are not conclusive, there is evidence for some fungi that these enzymes are significant for their pathogenecity. Furthermore, plants contain polygalacturonase inhibiting proteins (PGIPs) in their cell walls, which are able to inhibit PGs (De Lorenzo *et al*, 2001; 2002).

Colletotrichum SHK2148 is a pathogenic fungus causing anthracnose of lupin plants in South Africa. The identity of the fungus has been described as *Colletotrichum tortuosum* (Koch, 1996). However, this was based on morphological evidence only. Thus, the classification of the South African lupin- associated *Colletotrichum* isolates was re-assessed by comparing *Colletotrichum* SHK2148 on a morphological and molecular level to the recently described *Colletotrichum lupini* species (Nirenberg *et al*, 2002) as well as previously described *Colletotrichum acutatum* lupin anthracnose isolates (Talhinas *et al*, 2002). Based on the culture morphology, ITS and β-tubulin sequence data, it was concluded that *Colletotrichum* SHK2148 groups with *C. lupini*, more specifically, *C. lupini* var. *setosum*.

The fungus, renamed *Colletotrichum lupini* SHK2148, was evaluated for its PG activity in pectin media (pH 5) over a 12 day growth period by using an agarose diffusion assay. The specific PG activity reached its highest level after three days, whereafter it decreased. Previous studies performed at the ARC, revealed that the fungus produced PG activity and this crude activity was inhibited by a PGIP produced in apple. A study was launched to isolate and characterise the gene(s) responsible for PG production. PG gene sequences from *Colletotrichum gloeosporioides* f.sp. *malvae* and *Colletotrichum lindemuthianum* were compared and conserved regions were identified from which primers were designed to amplify a fragment of a PG gene from *C. lupini* SHK2148. Inverse PCR was used to resolve the 5' and 3' sequences of the PG gene whereafter a complete copy of the gene was isolated from the genome of the

fungus and characterised. The isolated gene was approximately 1Kb, contained a single intron of 59 bp and was very similar to the PG gene from *C. gloeosporioides* f.sp. *malvae* (*cmpgII*) as well as one of the PG genes (*clpg2*) from *C. lindemuthianum*. Southern blot analyses revealed that the gene was present as a single copy in the genome of the fungus. The *in vitro* expression of the PG gene from *C. lupini* SHK2148, grown in pectin media (pH 5), was investigated via northern blot analyses as well as RT-PCR, which revealed that the gene was expressed in the same time period that the highest PG activity was observed. A full cDNA copy of the PG gene was isolated using mRNA harvested from mycelia that was grown for 4 days on pectin. The cDNA copy confirmed the predicted intron position of the previously isolated genomic PG gene.

Due to the unavailability of a full cDNA copy of the *C. lupini* SHK2148 PG gene at the time when expression studies were initiated, a complete cDNA copy was constructed by swapping an internal cDNA PG fragment with its counterpart in the complete genomic PG gene copy. The resulting cDNA PG copy was used as a template from which PG constructs were prepared for expression in *Pichia pastoris*. Constructs containing the PG gene with its native signal peptide, the PG gene with the α-MF signal peptide factor as well as hybrid constructs where the N terminal part of the mature PG proteins of *Fusarium moniliforme* and *C. lupini* SHK 2148 were exchanged, were transformed into *P. pastoris*. No PG activity was observed with an agarose diffusion assay for any of the *Pichia* clones. SDS-PAGE analyses were used to evaluate total protein isolations from the *P. pastoris* clones. The supernatant and cells of the clones were subjected to western blot analyses using antibodies directed against *Aspergillus niger* PG as well as *F. moniliforme* PG. The only positive hybridisation signal was observed between the *A. niger* antibody and a protein in supernatant extracts of the *P. pastoris* clones. However, the size of the hybridising band was very large. This could be due to glycosylation of the *C. lupini* SHK 2148 PG in *P. pastoris*, although the size increase is unusually large. The results indicated that it is unlikely that the *C. lupini* SHK 2148 PG was expressed in *P. pastoris* transformed with any of these constructs.

Opsomming

Endopolygalakturonase ensieme is van die eerste selwand degraderende ensieme wat plant patogeniese swamme produseer sodra hulle in kontak kom met plantselwande (Albersheim and Anderson, 1971). Die patogeniese rol van dié ensieme is al menigmaal ondersoek en alhoewel dit nog nie absoluut duidelik is nie, is daar wel gevalle waar die ensieme krities was vir die virulensie van sekere swamme (hoofstuk 1). Alhoewel, in plantselwande word poligalakturonase-inhiberende proteïne aangetref wat daartoe instaat is om endopolygalakturonases van swampatogene te inhibeer (De Lorenzo *et al*, 2001; 2002).

Colletotrichum SHK2148 is ‘n patogeniese swam wat antraknose veroorsaak op lupiene plante in Suid Afrika. Die swamme is vroeër geklassifiseer as *Colletotrichum tortuosum* (Koch, 1996), maar die klassifikasie studie was slegs gebasseer op morfologiese kenmerke. Dus is die identiteit van die *Colletotrichum* SHK 2148 swam, wat antraknose van lupiene veroorsaak, geherevalueer deur die swamme op ‘n molekulêre en morfologiese vlak te vergelyk met die onlangs geïdentifiseerde *C. lupini* isolate (Nirenberg *et al*, 2002) as ook vorige geïdentifiseerde *C. acutatum* isolate (Talhinas *et al*, 2002). Volgens die morfologiese, ITS en β-tubulin resultate, lyk dit asof die *Colletotrichum* SHK 2148 isolaat baie naby verwant is aan die *C. lupini* spesie of meer spesifiek een van die spesie se variante, *C. lupini* var. *setosum*.

Die PG aktiwiteit sowel as die proteïen produksie van die *Colletotrichum lupini* SHK 2148 swam, wat vir ‘n 12 dag periode gegroei is in ‘n pektien medium (pH 5), is onderskeidelik bepaal met ‘n agarose diffusie en Bradford toets. Die hoogste PG aktiwiteit was waargeneem nadat die swam 3 dae in die pektien medium gegroei het, die aktiwiteit het daarna afgeneem. Vorige studies wat plaasgevind het by die LNR, het daarop gedui dat die *Colletotrichum* swamme, wat antraknose van lupiene veroorsaak, PG aktiwiteit besit en dat ‘n ongesuiwerde PG ekstrak van *Colletotrichum* se aktiviteit geïnhibeer word deur PGIP wat uit appels geïsoleer is. Dus is ‘n studie onderneem om die geen/gene te identifiseer wat verantwoordelik was vir die PG aktiwiteit. PG gene van *C. gloeosporioides* f.sp. *malvae* (*cmpgII*) en

C. lindemuthianum (*clpg2)* is met mekaar vergelyk om gekonserveerde gebiede te identifiseer vir die ontwerp van voorlopers wat aangewend is om ‘n interne fragment van die PG geen te isoleer van die genoom van *Colletotrichum lupini* SHK 2148. Om die ontbrekende dele van die PG geen te bepaal is ‘n inverse polimerase ketting reaksie (PKR) gebruik. Gevolglik is die volledige PG geen van die genoom van *Colletotrichum lupini* SHK 2148 geamplifiseer en gekarakteriseer. Die geen was ongeveer 1 Kb groot, onderbreek deur ‘n 59 bp intron byna identies aan die *cmpgII* geen van *C. gloeosporioides* f.sp. *malvae* asook die *clpg2* geen *C. lindemuthianum*. Volgens die “Southern” klad, word die geen aangetref as ‘n enkel kopie in die genoom van die swam. Die geen se *in vitro* uitdrukking is bepaal, in pektien medium (pH5), met behulp van ‘n “Northern” klad en omgekeerde transkripsie polimerase ketting reaksie. Die geen word uitgedruk terselfdetyd as wat die hoogste PG aktiwiteit waargeneem is. Die geen word nie uitgedruk wanneer die swam vir 5 of 12 dae in die pektien medium gegroei het nie. ‘n Volledige cDNS (kopie dieoksieribonukleïensuur) kopie van die PG geen is geïsoleer van die boodskapper ribonukleïensuur (mRNS) van die swam wat vir 4 dae in die pektien medium (pH 5) gegroei het. Die cDNS kopie het die posisie van die voorspelde intron in die genomiese PG geen kopie bevestig.

Aangesien nog geen cDNS PG geen kopie beskikbaar was tydens die gis uitdrukkings studies nie, is ‘n cDNS kopie gekonstrueer deur ‘n interne cDNS PG fragment te ruil met die ooreenstemmende fragment in die genomiese PG kopie. Dié gekonstrueerde cDNS PG geen is as templaat gebruik waarvan PG konstrukte saamgestel is vir die uitdrukking studies in *P. pastoris*. Onder andere is ‘n PG geen met sy eie seinpeptied, ‘n PG geen met die α-MF (α-mating factor) seinpeptied sowel as hibried PG gene, waarvan die N-terminale van die volledige PG proteïene van *F. moniliforme* en *Colletotrichum lupini* SHK 2148 met mekaar omgeruil is, uitgedruk in *P. pastoris*. Geen PG aktiwiteit is waargeneem met die agarose difussie toets vir enige van die konstrukte se klone nie. ‘n SDS-PAGE toets is gedoen om die proteïen isolasies van die klone te evalueer. Die supernatant sowel as die selle van die klone is gebruik in ‘n “Western” klad analise met die teenliggame vir die PG gene van *A. niger* PG en *F. moniliforme*. Die enigste positiewe sein wat waargeneem is, was met die teenliggaam van die *A. niger* PG en die supernatant van die klone. Die sein se bandjie

het baie groot vertoon. Glikosilering van die PG proteïen mag moontlik die grootte van die proteïen verhoog, maar waarskynlik nie tot so ‘n groot mate nie. Die resultate toon waarskynlik dat geen van die PG konstrukte in *P. pastoris* uitgedruk is nie.

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List of abbreviations

α-MF	alpha mating factor
AA	amino acids
ADA	agarose diffusion assay
AFLP	amplified fragment length polymerisation
AOX1	alcohol oxidase gene 1
ARC	Agricultural Research Council
BCIP	5-bromo-4-chloro-3-indolyl-phosphate
bp	base pair
BSA	bovine serum albumin
cDNA	complementary DNA
CWDE	cell wall degrading enzymes
CreA protein	DNA binding protein involved in carbon catabolite repression
DIG	digoxygenin
dATPs	deoxy adenosine triphosphate
dH_2O	distilled water
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
EDTA	ethylenediamine tetraacetic acid
EndoPGs	endopolygalacturonases
EtBr	etidium bromide
EtOH	ethanol
ExoPGs	exopolygalacturonases
FA	formaldehyde
GAP	glyceraldehyde 3-phosphate dehydrogenase
GFP	green fluorescent protein
IPTG	isopropyl-β-D-thiogalactopyranoside
ITS	internal transcribed spacer region
kB	kilo base pair
kV	kilo volt
LB	Luria Bertani
LMW	low molecular weight
LRR	leucine-rich repeat
LSLB	low salt LB
MCS	multiple cloning site
$MgCl_2$	magnesium chloride
mM	millimolar
M-MLV	Moloney Murine Leukemia Virus
mRNA	messenger ribonucleic acid
NaCl	Sodium chloride
NaOH	Sodium hydroxide
NBT	nitroblue tetrazolium chloride
ng	nanogram
OD	optical density
OGAs	oligogalacturonides
PAGE	polyacrylamide gel electrophoresis

PCR	polymerase chain reaction
PDA	potato dextrose agar
PGA	polygalacturonic acid
PGs	polygalacturonases
PGIPs	polygalacturonase Inhibiting Proteins
RAPD	random amplified polymorphic DNA
RNA	ribonucleic acid
RNAse	ribonuclease
ROS	reactive oxygen species
Rpm	revolutions per minute
RT-PCR	reverse transcription polymerase chain reaction
SDS	sodium dodecyl sulphate
Sec.	seconds
SNA	saltwater nutrient agar
SOE PCR	splicing by overlap extension by PCR
SSC	sodium chloride/sodium citrate
TAE	Tris-acetate ethylenediamine tetraacetic acid
TCA	trichloroacetic acid
TE	Tris ethylenediamine tetraactic acid
TNE	Tris-sodium chloride EDTA
TT	transcription termination
U	unit
UV	ultraviolet
µg	microgram
µl	microlitre
µM	micromolar
VCG	vegetative compatibility grouping
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactoside
YPD	Yeast Extract Peptone Dextrose
BMMY	Buffered methanol complex medium
HCl	Hydrochloric acid

Index of figures

Index of tables

Aim of study

Lupins have been cultivated for at least 2000 years and of the more than 300 existing species only five are cultivated worldwide today. The plants are grown as both forage and grain legume in the USSR, Poland, Germany, South Africa and the Mediterranean, while Australia cultivates lupin primarily as an export product for European and Asian feed markets. Lupins are mainly used as feedstock for poultry and livestock, however, a human food market, which includes lupin flour, lupin pasta and hulls for dietary fiber, is also being developed in the USA (www.ext.nodak.edu/extpubs/alt-ag/lupin.htm). The annual turnover from the lupin industry in South Africa is approximately 60 million rands.

Lupin *spp.* however are highly susceptible to anthracnose. This disease is considered to be the most devastating disease of lupin industries, causing crop losses that range from 50% in narrow leafed lupins (*Lupinus angustifolius*) to 100% in white lupins (*Lupinus albus*) (Oelofse *et al*, 2003). The identity of the anthracnose causing *Colletotrichum* isolates is still a much-debated issue. Thus, one of the aims in this study was to resolve the current taxonomic placing of the South African lupin anthracnose isolates.

Plant pathogenic fungi produce several extracellular enzymes that facilitate the invasion and colonization of the pathogens. Endopolygalacturonases are only one of these enzymes that are produced by the pathogen when it is cultivated on isolated plant cell walls as well as during the infection process (Albersheim and Anderson, 1971; Cooper *et al*, 1981). The literature review (Chapter 2) discusses the importance of these enzymes as role players in pathogenicity and elicitors of the plant defense response. However, endo-PGs are inhibited by polygalacturonase inhibiting proteins (PGIP), which are leucine-rich repeat proteins associated with plant cell wall (Caprari *et al*, 1993; Rha *et al*, 2001; Di Pietro *et al*, 1996). By inhibiting fungal PGs, PGIPs directly interfere with host cell wall degradation and provide a first line of defense against a fungal attack. Thus, PGIP genes provide a potential tool that can be used to increase the resistance of lupin plants through genetic modification.

Apple PGIP1, expressed in transgenic tobacco plants at the ARC-Roodeplaat, was able to inhibit PG activity from the *Colletotrichum* SHK pathogens (Oelofse *et al*, 2003). Thus the aim of the study was to isolate the gene(s) responsible for the PG activity and characterize them in terms of their copy number, similarity to other fungal PG genes and their *in vitro* expression on pectin. The subsequent aim was to express and purify the protein in a yeast system and then test the inhibition effectiveness of the purified apple PGIP1 against the purified *Colletotrichum* PG.

In this research project, Chapter 1 provides a literature review, which includes information on the characterization of the casual agent of lupin anthracnose, the important fungal endoPG gene families, the role that some of these endoPGs play in pathogenicity, the ability of PGs to elicit a plant defense response and the structural domains in both PGs and PGIPs and their inhibition interaction with each other are discussed. In chapter 2 the taxonomic identity of the South African lupin anthracnose isolates are re-evaluated and compared with the recent classifications of lupin anthracnose causing *Colletotrichum* isolates. The rest of the project, Chapter 3 and Chapter 4, includes molecular studies. Chapter 3 describes the *in vitro* PG activity of the fungus, the isolation and characterization of a single PG gene from *C. lupini* as well as the *in vitro* transcription of this gene. Chapter 4 discusses the construction and expression of a cDNA copy of the PG gene in *Pichia pastoris*. Chapter 5 contains the concluding discussion of the results obtained in this project.

Chapter 1

Literature Review

1.Introduction

Lupin anthracnose is considered to be one of the most devastating diseases of lupin industries world wide, causing crop losses of up to a hundred percent. The disease has been reported worldwide, including Europe (France, UK, Russia and the Ukraine), Africa, Oceania and Western Australia (Nirenburg *et al*, 2002). In South Africa the disease was first reported in the Free State in 1993-1994, and later in the Western Cape, one of the main lupin producing areas of South Africa (Koch, 1996). This seed-borne disease is typically characterized by wilting of growth tips, followed by curling and twisting of stems, as disease severity increases. Diamond shaped brown lesions, displaying an orange centre of conidial masses, are visible on the branches. The disease can also affect flowers and pods causing circular lesions (Koch, 1996).

Several taxonomists and researchers have attempted to resolve the true identity of the fungus causing lupin anthracnose using either conventional classification methods or by including molecular techniques. Lupin-associated *Colletotrichum* isolates have previously been identified as *Colletotrichum gloeosporioides* (Yang *et al,* 1998) and *Colletotrichum acutatum* (Talhinas *et al*, 2002). Some authors proposed that the causal agent of lupin anthracnose displays enough characteristics to be located, as a new species, into the taxonomic hierarchy (Koch, 1996; Nirenburg *et al*, 2002). Nirenburg *et al*. (2002) proposed a species that constitutes two variants *Colletotrichum lupini* var. *lupini* and *Colletotrichum lupini* var. *setosum.* However, a final taxonomic identification of the fungus has not yet been established, thus for the purpose of this study the pathogen will be referred to as *Colletotrichum lupini.*

Plants are constantly under attack by bacterial, fungal and insect pathogens and have evolved numerous strategies for resisting these attacks. The plant cell wall is the first resistance barrier that phytopathogenic fungi must overcome during an attack on the plant. A main component of the plant cell wall and the matrix of the middle lamella is composed of pectin (Ridley *et al,* 2001). The middle lamella is primarily made up of polysaccharides of 1,4-linked α-D-Galactosyluronic acid residues (Gal*p*A). Three major polysaccharide domains; homogalacturonan and rhamnogalacturonans I and II

were previously recognized. In each of these domains the Gal*p*A residues are arranged in a certain fashion and show some modifications, such as methylation, and acetylation (Ridley *et al*, 2001).

Fungal pathogens can enter their host through stomata, wounds or by degrading the cell wall. They gain entry into the plant host by relying on an array of cell wall degrading enzymes (CWDE), which facilitate the degradation of complex structural compounds in plant cell walls (Collmer and Keen, 1986; Esquerré-Tugayé *et al*, 2000). These enzymes include polygalacturonases (PGs), pectin and pectate lyases and pectin esterases (Markovič *et al*, 2001). Lyases cleave pectic acid and pectin by removing a water molecule. Pectin esterases act specifically on the methyl ester of polygalacturonic acid, while polygalacturonases hydrolyze the α 1,4 linkages of galacturonic acid residues within the homogalacturonan domain. Endopolygalacturonases are the first CWDE that are produced when the pathogen encounters the plant cell wall (Albersheim and Anderson, 1971; Cooper *et al*, 1981). Several roles have been implicated for PGs: they provide the fungus with an energy source, they indirectly facilitate other cell wall degrading enzymes to further degrade host cell wall polymers and are indirectly involved in the elicitation of host defence responses by releasing oligogalacturonic acid residues from the host cell wall that are involved in the induction of several defence responses (Collmer and Keen, 1986, reviewed by Oeser *et al*, 2002).

PGs often encounter inhibitors in the vicinity of the plant cell wall as they invade the host. Polygalacturonase inhibiting proteins (PGIPs) are leucine rich repeat proteins that are associated with plant cell walls and have the ability to inhibit fungal PGs. Not all PGIPs can inhibit fungal PGs with the same effectiveness. For example, PGIPs from the same plant possess different inhibition capabilities towards PGs from fungal pathogens and PGIPs from different plant sources display differential inhibition potential to a fungal PG (De Lorenzo *et al*, 2001, 2002).

PGs and PGIPs are thought to play an important role in the host pathogen interaction (Collmer and Keen, 1986). Therefore, numerous genes have been isolated and characterized for fungal PGs and plant PGIPs. Several domains have been identified in both PGIPs and PGs that are involved in the binding interaction between these two

proteins. These domains can be genetically manipulated by altering single or multiple residues to enhance, weaken or broaden the binding affinity of PGIP proteins for PGs (De Lorenzo *et al*, 2001, 2002; Federici *et al,* 2001).

However, even though the role of CWDEs in pathogenicity has been investigated for various pathogens, results thus far are not conclusive. It appears that pectinolytic enzymes such as the PGs, are implicated more strongly than other CWDEs as definite pathogenicity factors (reviewed in section 4). Therefore, much more needs to be learned about the role of these interesting proteins in the host pathogen interaction.

2. The taxonomic riddle of the lupin anthracnose fungi

The classification of the casual agent of lupin anthracnose remains a debatable issue. Two approaches have been followed in the past to determine the taxonomic location of the pathogen. The first approach follows the traditional way of classification by using morphological and cultural characteristics such as conidial shape and size, colony morphology, pigmentation, growth rate and appresoria shape. The second approach includes molecular techniques such as RFLP, RAPD and PCR to facilitate the classification of organisms into genera, species, subspecies etc. Some taxonomists used either one of these approaches while others combined the two in their search for the true identity of the lupin anthracnose-associated pathogen.

The first description of the lupin anthracnose fungus was by Bondar in 1912 (Nirenberg *et al*, 2002). This isolate from white lupin was named *Gloeosporium lupinus.* A lupin anthracnose fungus from the USA was later described as *Glomerella lupinicola* Daernes (Nirenberg *et al*, 2002). Neither one of these descriptions were published, nevertheless they were included in the "Host index of the fungi of North America" as *Gloeosporium lupinicola* Dearness. Plant pathologists however never used these names. Descriptions that were more popular included *Colletotrichum gloeosporioides*, *Glomerella cingulata* (the name of the suspected teleomorph), *C. trifolii* from alfalfa and *C. fragariae* from strawberries and *C. gloeosporioides* f.sp *aeschynomenes* that was also reported to be pathogenic on blue lupins (Nirenberg *et al*, 2002).

More recently, several groups of researchers made an asserted effort to classify the casual agent of lupin anthracnose. A summary of their classification views is presented in table 1.1. A more detailed discussion of their views of the taxonomic placing of lupin anthracnose isolates will now be discussed.

Table 1.1 Summary of classification views of lupin anthracnose associated *Colletotrichum* isolates.

Causal agent	Characteristics used	Reference
C. tortuosum	Morphological characteristics	Koch (1996)
C. gloeosporioides	RAPDs, VCGs, morphological and cultural characteristics	Yang *et al* (1998)
C. acutatum	Molecular (ITS, His, β-tubulin, AFLP) morphological and cultural data	Talhinas *et al* (2002)
C. lupini	Morphological, physiological and molecular (ITS) data	Nirenberg *et al* (2002)

2.1 *Colletotrichum gloeosporioides* according to Yang *et al* (1998)

Most of the previous reports, including the original description, classified the pathogen as *Colletotrichum gloeosporioides*. Yang *et al* (1998) supported this taxonomic placing by using VCG grouping, morphological and cultural characteristics as well as RAPD analysis to investigate *Colletotrichum* isolates of lupin from several countries including *C. gloeosporioides* and *C. acutatum* reference isolates. Based on the VCG and RAPD data, the isolates were classified into three vegetative compatibility groups; VCG-1 contained three isolates from France and one isolate from Canada; and VCG-2 included the majority of isolates from several countries including the *Colletotrichum* SHK 788 and *Colletotrichum* SHK 1033 from South Africa, while VCG-3 only included the isolates from Portugal. The VCG-3 isolates were classified as *C. acutatum*, since their RAPD profile indicated that they grouped closely with some of the *C. acutatum* reference isolates with which they also shared several similar conidial, cultural and morphology properties. The VCG-1 and VCG-2

groups each had a unique RAPD profile, which had a low similarity coefficient with all the other *C. acutatum* and *C. gloeosporioides* isolates, nevertheless they were classified as *C. gloeosporioides* based on their conidial shape and colony properties that were similar to the reference *C. gloeosporioides* isolates. Even though the majority of the VCG-1 and VCG-2 isolates had cylindrical conidia like those of *C. gloeosporioides*, there was a slight percentage of the VCG-1 and VCG-2 isolates that had clavate conidia. Yang *et al.* (1998) suggested that a separate *formae specialis* in the *C. gloeosporioides* species should accommodate these isolates.

Yang *et al.* (1998) also evaluated benomyl sensitivity and diagnostic PCR with ITS species specific primers for classifying the lupin anthracnose isolates. Benomyl sensitivity has been reported as a useful tool to discriminate between *C. acutatum* and *C. gloeosporioides*. *C. acutatum* isolates are relevantly tolerant to benomyl while *C. gloeosporioides* isolates are highly sensitive (Adaskaveg *et al,* 1997). According to the PCR results and benomyl tests, the lupin anthracnose isolates were more closely related to *C. acutatum* than *C. gloeosporioides*. These results did not support the morphological, RAPD and VCG data and were disregarded. Thus, the lupin isolates belonging to VCG-1 and VCG-2 were classified as *C. gloeosporioides* while VCG-3 isolates were classified as *C. acutatum*.

2.2 *Colletotrichum acutatum* according to Talhinas *et al* (2002)

The previous study of this group used ITS sequence data to classify isolates associated with lupin anthracnose (Sreenvasaprasad *et al*, 1994). Their results were however questioned by Yang *et al* (1998), who argued that some variation in their study were compromised by the small number of isolates investigated and that the important fusiform conidial shape of *C. acutatum* that should not be ignored when placing isolates into this species.

In a second study Talhinas *et al* (2002) obtained various isolates from different countries, including isolates from lupin and other hosts as well as several reference isolates. Information gathered from conidial shape, colony morphology and benomyl sensitivity grouped the isolates closer to *C. acutatum.* ITS data confirmed this grouping and were supported from further evidence obtained from sequence data of

his4 and *tub2* genes. AP-PCR and AFLP data could discriminate groups within the species but were not very useful to resolve species level relatedness (Talhinas *et al*, 2002).

Talhinas *et al* (2002) identified two main groups that were supported by spore shape, colony morphology, growth rate and molecular data. The first slower growing group, containing the majority of lupin isolates, consisted of two subgroups of which one contained mostly acute spores and the other spores with one round and one acute end. The second faster growing group consisted of isolates from citrus and the *C. gloeosporioides* isolates that mainly produced round-ended spores. Based on colony pigmentation, two groups were distinguished for the lupin isolates, the first group had a characteristic pinkish, low density colony with significant spore masses while the second group had grey-olive, felty, medium density colonies with fewer spore masses (Talhinas *et al*, 2002).

According to the molecular data the isolates were classified into two distinct groups that correlated with the broad groups outlined by the morphological data. The one main group consisted of four subgroups. Subgroup A1, which correlates to COL2/VCG-2 group of Yang *et al* (1998), contained all the lupin-associated isolates including *Colletotrichum* SHK 788 from South Africa. The other subgroups were less significant, subgroup A2 corresponds to *C. acutatum* groups pathogenic to strawberries, subgroup A3 includes *C. acutatum* reference isolates from ornamentals and subgroup A4 includes *C. acutatum* groups of European strawberries.

The study of Talhinas *et al* (2002) was contradictory to the classification of Yang *et al*. (1998). Since both groups provided contradictory scientific evidence, the classification of the causal agent was still unresolved.

2.3 Nirenberg *et al* (2002) suggests two *forma speciali*

Nirenberg *et al*. (2002) compared more than 100 *Colletotrichum* isolates from diseased plants with other *Colletotrichum* species on lupin as well as other hosts, using morphological, physiological, and molecular data. The isolates represented

Germany, other European countries as well as South and North America. However, no *Colletotrichum* isolates from South Africa were used in their study.

On the molecular front RAPD analysis and phylogenetic analysis, using the 18S rDNA, ITS1, 5.8 rDNA and ITS2 sequence data were performed on the isolates. The morphological and physiological characteristics, such as colony morphology and colour as well as conidial shape and size, were carefully assessed using more than one growth medium and growth condition. The variation of the latter apparently influenced the production of conidiomata, appressoria, colour of mycelium and shape and size of the conidia. Nevertheless, based on the molecular and morphological data that was gathered, the *Colletotrichum* isolates from lupin could be classified into two groups *C. lupini* var. *lupini* and *C. lupini* var. *setosum.* These two variants were distinctly different from *C. acutatum*, *C. gloeosporioides*, *C. trifolii* and *C. fragaria* isolates. The latter was the only species similar to both *C. lupini* varieties based only on morphology. The RAPD patterns supported the two variant groupings, however the differences in both the RAPD and ITS data were small. ITS sequence data revealed that the two variants were differentiated by one base pair (Nirenberg *et al*, 2002).

The studies of Nirenberg *et al* (2002) and Talhinas *et al* (20002) are well supported by both molecular and morphological evidence, and both used a variety of isolates from different countries, these isolates however differ for each study, which limits the comparison between the studies.

2.4 Koch suggests a new species

Apart from Nirenberg *et al* (2002), Koch (1996) studied isolates associated with lupin anthracnose from France and South Africa and also proposed that these isolates should be grouped in a separate species. The study focussed on morphological and cultural characteristics and did not include molecular data. The isolates were distinguished from *C. gloeosporioides* and *C. acutatum* (as well as *C. musae*). Based on the apparent unique properties of this isolates, a new name *C. tortuosum* was proposed (Koch, 1996).

Koch (1996) described the conidia as oblong to cylindrical and tapered towards a truncated base. Nirenburg *et al* (2002) described conidia, from *Colletotrichum sp.*, that are produced on aerial mycelia as having different sizes and shapes and therefore included conidiomatal conidia in their species differentiation studies. Both variants identified by Nirenburg *et al* (2002) produced conidiomatal conidia that were pointed at one end and rounded at the other. The conidia produced on hyphae were subglobose to cylindrical with rounded ends (*C. lupini* var. *lupni*) or mostly cylindrical with rounded ends (*C. lupini* var. *setosum*).

Although these authors used different growth media and conditions, which influence morphology and culture growth, it could be informative to investigate these proposed new species of Koch (1996) and Nirenburg *et al* (2002) on both a molecular and conventional taxonomic level and determine how they compare with each other. This might provide insightful data for the debate around the taxonomy of the lupin anthracnose *Colletotrichum* isolates, especially regarding the South African isolates.

3. Polygalacturonase encoding genes

PG enzymes have been described for several bacterial, fungal, plant as well as insect systems. The PGs in these systems fulfil different roles; in plants they act mostly in the development of the plant, while in bacteria, fungi and insects they form part of the attack system of these organisms.

3.1 Polygalacturonase encoding genes in plants

In plants there are large gene families that encodes numerous PGs, which have diverged sequences and are expressed in a wide range of tissues and developmental stages (Ridley *et al*, 2001; Dal Degan *et al*, 2001). For example, there are 52 PG genes in *Arabidopsis thaliana*, the largest known family of polysaccharide hydrolases (The A*rabidopsis* Genome Initiative (2002). All plant PGs are secreted proteins and are associated with fruit ripening, cell separation processes such as leaf and flower abscission, pod and anther dehiscence, pollen grain maturation, pathogen defense,

plant host interaction and processes of cell expansion, growth and xylogenesis (Atkinson *et al*, 2002). Previously, plant PGs were classified into three clades (A, B & C), however, a recent study of Torki *et al* (2000) revealed two additional classes of PGs (D & E) as well as a PG from a gymnosperm, *Cryptomeria japonica* (Torki et al, 2000). Genes of a certain clade have similar structures such as intron conservations and apparently perform specific functions in the plant. Class A PGs are found in fruit and/or abscission zones, contain no propeptide sequence and displays an endo-mode of action. Class B PGs are found in dehiscence zones, also display an endo-activity mode, but differ from the other classes by having a long N-terminal domain following the signal peptide (Torki et al, 2000). This domain is speculated to be involved in the secretion of the protein (Dal Degan *et al*, 2001). Class C PGs lack a prosequence, have an exo-mode of action and were found in pollen (Dal Degan *et al*, 2001). The expression pattern for class D enzymes is not yet established, whereas the class E enzymes are present in young seedlings and roots (Torki *et al*, 2002). Phylogenetic analysis revealed that the two new additional classes D and E are more related to class B (Torki *et al*, 2000).

Plant PGs differ from fungal PGs by having a lower activity than fungal PGs (Federici *et al*, 2001). It has been reported that banana and tomato PG were a 100 fold less active than the PG from *Fusarium moniliforme* (reviewed by Federici *et al*, 2001). This might be due to the different purposes that plant PG fulfil compared to fungal PGs; fungal PGs need to degrade significant amounts of complex wall components, while plant PGs play a role in the development of the plant (Dal Degan *et al*, 2001; Torki *et al*, 2000; Collmer and Keen, 1986; Esquerré-Tugayé *et al*, 2000)

3.2 Fungal polygalacturonase encoding genes

Similar to plants, fungal PGs are encoded by members of multigene families, however these families are relatively small compared to those found in plants. It is not clear why certain fungi contain only a few representatives (Centis *et al,* 1996, 1997; Li *et al*, 2002) while others are capable of producing numerous PGs (Bussink *et* al, 1992; Ten Have *et al*, 2002). It was proposed that the members present in a particular

pathogen are dependant on the host range of the pathogen (Esquerré-Tugayé *et al*, 2000). For instance *Botrytis cinerea* has a wide host range and produces a whole set of PGs (Cabanne *et al* 2002, Johnston *et al*, 1992; Ten Have, 2000), while only two PGs have been described for *C. lindemuthianum,* a pathogen that only infects *Pheaseolus vulgaris* (Centis *et al*, 1996, 1997) and one PG has been described for *C. gloeosporioides* f.sp. *malvae* which infects round-leafed mallow (Li *et al*, 2002). Currently, six PG genes from *B. cinerea* have been described; each one of these PGs has also been detected in other *Botrytis* species investigated - *Botrytis aclada, Botrytis gladiorum, Botrytis paeoniae* and *Botrytis squamosa* (Ten Have, 2000). While *B. cinerea* has a broad host range, some of the latter species can only infect a single host and yet contained homologous genes to the broad PG family of the *B. cinerea* (Wubben *et al*, 1999). Thus, the size of a PG gene family is not necessarily an indication of the host range of that pathogen.

PG genes from several fungal gene families, large or small, were isolated via numerous recombinant DNA techniques for characterization and expression purposes. Nucleotide or amino acid sequence data were used to isolate the genes with several PCR based methods. Sequence data obtained for the genes were applied in phylogenetic studies (Markovič *et al,* 2001, Torki *et al*, 2000), gene expression studies (Centis *et al,* 1999), studies regarding domains involved in PG activity or PGIP recognition (Federici *et al*, 2001) and for gene disruption studies to evaluate the role of PGs in pathogenicity (Gognies *et al*, 2002*;* Nakamura *et al*, 2001; Oeser *et al*, 2002; Shieh *et al*, 1997; Tenberge *et al,* 2002; Ten Have *et al*, 1998).

Most of the fungal PG genes characterised to date are typical of other protein coding fungal genes with regard to regions upstream from the translation start codon, and regions downstream from the translational stop codon. For instance the transcriptional start site is often found in the form of a PyAAG motif. Usually a CT-rich sequence precedes the transcription initiation site. This CT rich region is characteristic of genes lacking TATA and CAAT boxes and might facilitate the initiation of transcription (Gurr *et al*, 1987). The TATA box is found at approximately 30 bp upstream of the transcription initiation site and in numerous fungal genes it takes the form of a consensus TATAAA sequence. Other genes have a TA rich region that resembles the TATA box, however these TA rich sequences can

be situated further upstream than the conventional 30 bp position of the TATA box. The CAAT box, when present, is situated 60 to 120 bp upstream (Gurr *et al*, 1987). Furthermore, as other fungal genes, fungal PGs also contain a consensus sequence that is involved in polyadenylation of the 3' end of the mRNA gene copy. This consensus region, when present, is represented by the sequence AAUAAA or a more abbreviated AUAA form (Gurr *et al*, 1987). The translation of fungal PGs is also usually initiated at the first ATG start codon, like other fungal genes. The Kozak consensus sequence CCACC**ATG**GC, present around the ATG, usually has a purine at the –3 position and this is usually an adenine (Gurr *et al*, 1987). Fungal PG genes also resemble other fungal genes by usually containing small introns, less than 100 bp, which usually have three conserved regions; two conserved splice boundaries and an internal consensus lariat sequence PyGCTAACN. Two characteristic nucleotide pairs often border the introns, GT at the 5' end and AG at the 3' end. There are however some exception to this border rule. Not only is the conservation of the border and lariat sequences observed between fungal PGs, but also the remaining intron sequence and position were conserved between some similar fungal genes (Gurr *et al*, 1987).

Two types of PGs can be distinguished, endopolygalacturonases (endoPGs) and exopolygalacturonases (exoPGs). The main difference between the enzymes is their mode of action on the pectin cell wall; endoPGs catalyse the random hydrolytic cleavage of the galacturonic acid residues, while exoPGs are responsible for the removal of single galacturonic acid residues from the non-reducing end of galacturonan (Marcovič *et al*, 2001). Furthermore, exoPGs are apparently not subjected to inhibition by PGIPs, while endoPGs are (reviewed by De Lorenzo *et al*, 2001). Table 1.2 displays some properties of different PG genes and their protein products.

Table 1.2 Characteristics of important PGs isolated from fungal sources.

Fugal species	PG genes isolated	Introns (bp)	Size (kDA)	Amino acids of protein	Reference
Aspergillus flavus	*pecA*, *pecB*	58, 81 66, 65, 54	37.6	363 383	Whitehead *et al* (1995)
Aspergillus niger	*pgaI* *pgaII* *pgaA* *pgaB* *pgaC* *pgaD* *pgaE*	 62, 52 54 70, 74 75,56, 53 50, 50, 59	 36.2 50.7 35.58	 353 370 362 383 495 378	Bussink *et al* (1990, 1992); Pařenicová *et al* (2000)
Botrytis cinerea	*BcPG1*(one of six proteins identified)	intron-less	36		Ten Have *et al (1998, 2002)*
Colletotrichum lindemuthianum	*Clpg1* *ClpgII*	70 53		363	Centis *et al* (1996, 1997)
Fusarium moniliforme	*FmPg*	54,54,50,52		359	Caprari *et al* (1993)
Colletotrichum gloeosporioides f.sp. *malvae*	CmpgII	64		363	Li *et al* (2002)

3.3 Isolation and characterization of:

3.3.1 The endoPG genes from *Colletotrichum lindemuthianum*

Colletotrichum lindemuthianum causes anthracnose on bean plants and contains a smaller PG gene family than B. *cinerea*. Two PG genes have been cloned and characterised for this pathogen (Centis *et al*, 1996 and 1997). *Clpg1* was cloned and sequenced by using primers that was derived from the amino acid sequence. These primers amplified a segment of the PG gene from the genome of *C. lindemuthianum* that was used as a probe to screen a partial genomic library of the fungus. A positive clone was sequenced and revealed an ORF, interrupted by a single intron of 70bp, which represented a PG gene from *C. lindemuthianum*. The *Clpg1* gene encoded a protein of approximately 363 AA, with a signal peptide of 26 AA and one potential glycosylation site. The sequenced revealed regulatory sequences such as an upstream binding site for a regulatory proteinA as well as a downstream potential translation termination site. The *Clpg1* contained domains that are conserved in fungal PGs. Compared to other fungal PGs; it had the highest sequence homology to the *Sclerotinia sclerotium* endopolygalacturonase gene (*SCEEPG*) gene from *S. sclerotium* (Centis et *al*, 1996).

From the southern blot analysis, it appeared as if the *Clpg1* gene was present as a single copy in the genome, however previous analysis with a endoPG probe from *C. carbonum* showed that two other polygalacturonase genes might be present in the genome (Centis *et al*, 1996).

A second PG gene (*clpg2*) was isolated by using information of *clpg1* to isolate *clpg2* from a subgenomic library of *C. lindemuthianum* race ß. A PCR fragment designed from *clpg1* was used to probe the subgenomic library. The c*lpg2* gene was interrupted by a single intron of 53 bp and encoded a protein of approximately 365 AA. The two PGs had very similar biochemical properties such as molecular weight and isoelectric points. The *clpg1* gene showed a 61% AA identity to *clpg2*. The major differences between these two PG genes were their *in vitro* and *in vivo* expression pattern. During *in vitro* growth on pectin, both genes are expressed while only *clpg1* seems to be present after a longer culture period. RT-PCR was used to evaluate the *in vivo* expression pattern of these genes. *Clpg1* was expressed during the saprophytic and necrotrophic phase while *clpg2* was only expressed during the initial stages of infection (Centis *et al*, 1997). This difference in expression of the two PG genes were also confirmed by fusing the *gfp* gene (green fluorescent protein) to the regulating sequences of *clpg2* and monitoring the expression of the *gfp* by fluorescence microscopy during different stages of infection (Dumas et al, 1999).

3.3.2 The endoPG gene from *Colletotrichum gloeosporioides* f.sp *malvae*

C. gloeosporioides f.sp *Malvae* causes anthracnose on *Malva pusilla* (round-leaved mallow) (Li *et al*, 2002). A PG gene was isolated from this fungus by using degenerate primers, designed from highly conserved residues in PGs, to obtain an internal fragment of the gene (Li *et al*, 2002). The remaining gene sequence was obtained by genome walking. The resulting *Cmpg2* gene consisted of 1840 bp, contained a single intron of 64 bp, which had the typical conserved sequences. The predicted protein sequence consisted of 363 AA, with a potential signal peptide of 21 AA. The gene had a high sequence similarity to *clpg2* from *C. lindemuthianum*. Southern blot analysis revealed that the gene was present as a single copy in the genome. The *in vitro* expression of the *Cmpg2* showed that gene expression varied according to carbon source and pH level. Using RT-PCR, transcripts were only

detected using mallow cell wall extract (MCWE) as the carbon source at a pH of 3.5. No expression were detected when pectin or glucose were used as a carbon source or with MCWE at a pH of 7.5. *In vivo* expression showed that the gene was expressed in both the biotrophic and necrotrophic phase, with the highest expression levels in the early stages of the latter (Li *et al*, 2002).

4. Polygalacturonases as role players in pathogenicity

Polygalacturonases are present in several organisms such as insects, plants, fungi and bacteria (Markovič *et al*, 2001). In these organisms they fulfil different roles of which some are defined and other remain to be resolved. The role of these CWDEs in pathogenicity remains uncertain. For bacteria it seems that pectic enzymes are essential for pathogenicity (Colmer and Keen, 1986), while this also seem to hold true for fungal pathogens (Reviewed by D' Ovidio *et al*, 2004).

In bacteria, fungi and insects, PGs are usually part of the arsenal of weaponry used by these pathogens to infect their hosts. Microbial pathogens produce an array of enzymes to penetrate their host cell walls. EndoPGs are some of the first enzymes secreted when the pathogen has contacted the host surface and thus might be a crucial role-player in the virulence of these pathogens (Albersheim and Anderson, 1971; Cooper *et al*, 1981; Colmer and Keen, 1986). The PG enzymes furthermore facilitate the penetration of the pathogen and increase the substrate availability for other pectinases. They also seem to play an indirect role in the activation of the plant defence responses via pectin subunits that are released during the degradation of the plant cell wall (Ridley *et al*, 2001). These act as signal molecules for the plant defence system (Ridley *et al*, 2001; Esquerré-Tugayé *et al*, 2002; D' Ovidio *et al*, 2004).

Nakamura *et al* (2001) investigated, on a molecular level, the potential role that PGs might play in the pathogenicity of *Geotrichum candidum* on citrus fruits. PGs of two isolates, a pathogenic race and a non-pathogenic race, were cloned and their expression levels were compared. The two races were morphologically indistinguishable, however the PG activity of the pathogenic isolate seemed to be

higher on inoculated lemon peel and in culture filtrates, than that of the non-pathogenic isolate and the activity correlated with the pathogenicity of the pathogenic isolate. Cloning and characterization of the genes revealed that both genes contained all the amino acids conserved in PGs and they shared a similarity level of 68% (Nakamura *et al* 2001). Since the PG of the pathogenic isolate is responsible for the development of sour rot symptoms (Barash *et al*, 1984) and transcripts for the PG gene of only the pathogenic isolate were obtained from inoculated lemon peel broth, pectin and glucose (Nakamura *et al*, 2001), it strongly supports the hypothesis that the PG gene is a potential virulence factor for the *G. candidum* pathogen.

More concrete evidence for pathogenicity roles of PGs can be provided from targeted gene disruption studies. However, due to the complexity of CWDE sets, the plant cell wall composition and the host defence system, it is clear that a single, double or even triplicate mutations of these potential virulence genes do not always lead to a reduction of virulence in the relevant pathogen. However in some cases targeted gene disruption studies have revealed the importance of PG genes as pathogenicity factors (D' Ovidio *et al*, 2004).

Six PG genes have been reported for *Botrytis cinerea*. One of these genes, *Bcpg1*, was eliminated and the resulting mutant showed a reduced virulence on tomato and apple host tissue (Ten Have *et al*, 1998). Although no difference were observed in the primary stages of infection, secondary infection such as the radial growth from the lesions were significantly reduced for the mutants. It seems then that *Bcpg*1 doesn't play a crucial role in the penetration of the fungus, however without the PG activity, the fungus did not reach its full pathogenic potential on the host tissues. This is either due to a limited ability to degrade the complex pectin or the lack of enough pectin monomers to induce other polygalacturonases (Ten Have *et al*, 1998).

Deletion of the *pecA* gene in *A. flavus* reduced lesion development in cotton balls, whereas the expression of this gene in *A. flavus* strain, that lacks PG activity, increased lesion size (Shieh *et al*, 1997).

PG genes, present in some *Alternaria* spp. on citrus fruits, are required for full virulence of the pathogens (Isshiki *et al*, 2002). The endoPG genes of two pathogens

of citrus, *Alternaria citri* and *Alternaria alternata* were mutated and the effect on pathogenicity was evaluated. The two pathogens caused different disease symptoms; *Alternaria citri* caused alternaria black rot, while *Alternaria alternata* caused alternaria citrus brown spot. The effect of the mutation on the disease symptoms differed significantly. *Alternaria citri* were drastically impaired in its ability to colonise and cause symptoms, while the pathogenicity of *Alternaria alternata* were basically uninfected. The PG genes of these two pathogens had the same biochemical properties and were highly similar (99.6%) (Isshiki *et al*, 2002). This illustrates that PG genes, although very similar play different pathogenic roles for different pathogens.

The role of PGs in the virulence of a biotrophic fungus has also been assessed by Oeser *et al* (2002). *Claviceps purpurea* is an organ-specific pathogen of rye. It constitutively produces two PG genes, *cppg1* and *cppg2*. Mutants lacking both *cppg1* and *cppg2* were generated. These mutants had no defect in their vegetative properties, they did however seem to lose their pathogenicity by not being able to produce sclerotia and honeydew. One of the mutants were transformed with a construct containing copies of these PG genes and its virulence was restored to that of the wild type, thus proving that the PG genes were essential for pathogenicity of this fungus (Oeser *et al*, 2002). Targeted disruptions of other potential virulence genes such as cellulase, xylanase and catalase genes did not show such adverse effects on this organism's pathogenicity (reviewed by Oeser *et al*, 2002).

Cochliobolus carbonum, a pathogen causing Nortern leaf spot of corn, secretes numerous CWDE such as the pectin degrading enzymes endopolygalacturonases, exopolygalacturonases and pectin methylesterases (reviewed by Scott-craig *et al* (1998). Previously, the *PGN1* gene, which encoded the endopolygalacturonase, was investigated for its role in pathogenicity. The results obtained revealed that the endopolygalacturonase did not play a crucial role in pathogenicity, since the fungus were still pathogenic, grew well on pectin and PG activity was only reduced by 60 % (Scott-craig *et al*, 1990). However, the pathogenicity role of the other pectinases of the fungus remained questionable. An exopolygalacturonase mutant of *C. carbonarum* was constructed and as in the case of the endopolygalacturonase mutant, did not show reduced virulence (Scott-craig *et al*, 1998). Remarkably not

even a double mutant of the endo and exoPG genes affected the pathogenicity or the growth (on pectin) of the fungus although the total PG activity was reduced to less than 1% of that of the wild type (Scott-craig *et al*, 1998). The authors gave several explanations for their results including the possibility of additional pectinases that has not been identified yet (Scott-craig *et al*, 1998).

Altogether, the data suggest that PGs are definite candidates for pathogenicity factors. However, more studies regarding this issue should be conducted to determine if PGs are true pathogenicity factors and what parameters, such as substrate composition, host inhibitors and structural domains in both PGIPs and PGs, influence their pathogenicity.

5. Polygalacturonases as defence response elicitors

EndoPGs hydrolyses the linkages between galacturonic acid residues of the homogalacturonan region of pectin, releasing oligogalacturonic acid (OGA) fragments (Esquerré-Tugayé *et al*, 2000; Ridley *et al*, 2001). These OGAs are composed of two to thirty α - 1, 4 – D- galactopyranoslyuronic acid residues which are not only used by the pathogen as a carbon source but also seem to play a very important role as elicitors of the plant defence response (Ridley *et al*, 2001; D' Ovidio *et al*, 2004; Esquerré-Tugayé *et al*, 2000).

The overall effect of OGAs is the activation of several defence related genes and responses such as the oxidative burst, the hypersensitive response (HR) and systemic acquired resistance (SAR) (Ridley *et al*, 2001; D' Ovidio *et al*, 2004).

The response to OGAs differs from plant to plant, chemical composition of the OGA and pathogen involved (Boudart *et al*, 1998). Two near-isogenic lines of *Phaseolus vulgaris* of which one was susceptible and one was resistant to *C. lindemuthianum* were exposed to pure endoPG purified from this fungus. The resulting OGAs that were released from the respective lines were different in their chemical composition and elicitor activity. The elicitor effect in both lines was higher in response to the

OGAs from the more resistant line (Boudart *et al*, 1998). The different responses observed between the resistant and isogenic line can also be due to other factors; the methylation profile of pectin might differ from cell walls in resistant and susceptible lines, leading to different cleaving patterns and thus different OGAs (Bonnin *et al*, 2002). The level of PGIPs might be higher in the more resistant line than in the susceptible line (Lafitte *et al*, 1993). The role of the PGIP is to hamper the activity of PGs and thus prevent the hydrolysis of oligogalacturonides to inactive monomers.

Thus, the chemical composition and structure of OGAs play a key role in their biological activity; it seems that OGAs with a degree of polymerisation ranging from 10 – 16 has the highest biological activity, although there have been reports of OGAs with lengths of 2 – 30 that also induced a biological response (Reviewed by Ridley *et al*, 2001). Due to the apparent variety in composition of the OGAs it is reasonable to expect that the receptors for these molecules might also be very different in both affinity and structure, which might explain why some plants are more resistant than others (Ridley *et al*, 2001).

Another question that arises is if the elicitation effect is solely due to the OGAs or do the PGs themselves have an influence on the defence response triggering in plants? Poinssot *et al* (2003) reported that a purified PG from *Botrytis cinerea* induced defence related responses that were different from those observed in reaction to OGAs, it seemed as if the OGAs induced the defence responses (calcium influx, reactive oxygen species production, mitogen activated kinases activation, defence gene transcript accumulation and phytoalexin production) to a lesser extend than the PG enzyme. The OGAs released by the PG might however be different than the OGAs used (Poinssot *et al*, 2003). A lower defence response was also observed with another PG from *A. niger* that had the same enzymatic properties as the PG from *B. cinerea* (Poinssot *et al*, 2003). This can also be due to the possibility that the *A. niger* PG might release different OGAs with a different elicitation effect. They furthermore proposed that the PG enzyme from *B. cinerea* contained motifs that are recognised by the host cell, which can trigger the defence response.

Thus, PGs have a direct (the PGs themselves) and indirect (via OGAs) potential to induce a defence response in a plant of which the outcome varies according to the

hydrolysing ability and PG activity of the pathogen, the composition of the OGA and the plant host itself.

6. Conclusion

Lupin anthracnose is an important disease worldwide. Therefore concerted efforts have been made at classifying the lupin anthracnose pathogen. These efforts have not yet lead to a conclusive classification of the pathogen, since certain studies only included morphological data and used only a few isolates (Koch, 1996). Other studies included isolates from all over the world and used morphological and molecular information, however the molecular data contradicted the morphological data (Yang *et al*, 1998). In some studies the molecular and morphological data from the study supported the classification, however, isolates used from over the world differed between the studies, which makes it difficult to compare them (Talhinas *et al*, 2002; Nirenberg *et al*, 2002).

Polygalacturonases are not only secreted by lupin anthracnose pathogens, but are also present in plants, insects, fungi and bacteria in which they fulfil different roles. In several fungal pathogens investigations have shown a role for PGs as potential pathogenicity factors. Several examples showed that mutated PG genes from different fungal PGs affected the pathogenicity of the fungus. Therefore, the putative role of PGs as pathogenicity factors in the lupin anthracnose host pathogen interaction needs to be investigated further.

The review also discussed another important role of these enzymes; the role that they play as elicitors of the plant host defence response, illustrating that the overall effect of the plant defence system depends on the host, pathogen, presence of inhibitors and the chemical and structural composition of cell wall fragments that are released during an attack.

Residues present in PGs and PGIPs are crucial for enzyme activity and complex formation. These residues can seemingly adapt under positive selection to confer a recognition/discrimination between them. The knowledge on the interaction between

PGs and PGIPs has only been gained through thorough characterization of these genes and their products. Therefore, concerted efforts must be made to clone and characterize these genes from different host pathogen interactions including that between lupin anthracnose fungus and lupin plants.

Chapter 2

Taxonomic identification of *Colletotrichum* isolates associated with anthracnose in South Africa

Abstract

The South African lupin anthracnose-associated isolates have previously been identified as *Colletotrichum tortuosum* (Koch, 1996), *Colletotrichum gloeosporioides* (Yang *et* al, 1998) and *Colletotrichum acutatum* (Talhinas *et al*, 2002) while a recent study proposed that lupin anthracnose-associated isolates should be classified as a new species with two variants *Colletotrichum lupini* var. *setosum* and *Colletotrichum lupini* var. *lupini* (Nirenberg *et al*, 2002). Thus, a study was conducted to re-evaluate the classification of the South African lupin anthracnose isolates. The study focussed on the colony morphology as well as the shapes and sizes of the spores of the cultures incubated under different growth conditions. ITS and β- tubulin sequence data of the South African isolates, *Colletotrichum* SHK2148, SHK1033 and SHK 788, were compared with the two *C. lupini* type cultures as well as previously described *C. gloeosporioides* and *C. acutatum* sequence data. The colony morphology of *Colletotrichum* SHK2148 and *C. lupini* var. *setosum* were very similar under all growth conditions while that of *C. lupini* var. *lupini* differed considerably. There was no significant difference between the shapes and sizes of the conidia for all three isolates incubated under different growth conditions. Phylogenetic analysis of both the ITS and β- tubulin sequence data supported the basic groupings from previous classifications and indicated that *Colletotrichum* SHK2148 grouped with *C. lupini* var. *setosum*.

2.1 Introduction

Anthracnose is considered to be the most devastating disease to lupin industries worldwide, with crop losses as high as a hundred percent. The disease has been reported in several countries including South Africa (Koch, 1996).

The classification of the causal agent of lupin anthracnose is an on-going debate. Yang *et al* (1998) identified the lupin anthracnose isolates as *Colletotrichum gloeosporioides* using VCG groupings, RAPDs, morphological and cultural data. They classified the isolates in three VCG groups of which VCG-1 and VCG-2 (which contained the South African isolates SHK 788 and SHK 1033) grouped more closely to *Colletotrichum gloeosporioides*, while VCG-3 grouped with *C. acutatum.* Each group had a unique RAPD profile distinct from each other. The RAPD profiles of VCG-1 and VCG-2 shared a higher similarity coefficient, which was lower with that of the *C. gloeosporioides* and *C. acutatum* isolates. They strongly emphasised the spore shape difference between *C. gloeosporioides* and *C. acutatum*, and indicated that the spore shape of VCG-1 and VCG-2 resembled that of *C. gloeosporioides*. The molecular data obtained contradicted their morphological findings; *C. gloeosporioides* and *C. acutatum* specific primers were used to screen the isolates, which gave a positive signal with the *C. acutatum* specific primers but not with the *C. gloeosporioides* specific primers (Yang *et al*, 1998). Nevertheless, the lupin anthracnose isolates were classified as *C. gloeosporioides*.

Talhinas *et al* (2002) grouped lupin anthracnose-associated isolates as *Colletotrichum acutatum* based on molecular (ITS, β- tub and AFLPs), morphological and cultural properties. Morphological data indicated that *C. gloeosporioides* isolates are faster growing and contained spores with mainly round ends at both sides, while the C. *acutatum* isolates are slower growing and had spores with either two acute ends or one of the ends rounded. The ITS analysis revealed two main groups, which was supported by the β- tubulin sequence analysis, species specific PCR screening and morphological data (Talhinas *et al*, 2002). The one main group (B) contained the *C. gloeosporioides* reference isolates, while the other main group (A) contained all the lupin isolates, isolates from other hosts as well as *C. acutatum* isolates and was

divided into four smaller groups. Of these smaller groups, A1 contained all the lupin isolates, including the South African *Colletotrichum* SHK 788 and *Colletotrichum* SHK 1033 isolates, as well as one isolate from cinnamon and according to Talhinas *et al* (2002) corresponded to the VCG-2 group of Yang *et al* (1998) (Talhinas et al, 2002). Their results indicated that the lupin isolates grouped closer to C. *acutatum* (Talhinas *et al*, 2002).

Nirenberg *et al* (2002) used morphological, cultural and molecular (ITS and RAPDs) information and suggested that lupin anthracnose *Colletotrichum* isolates could be distinguished from both *C. gloeosporioides* and *C. acutatum* and should be classified as a new species, *Colletotrihum lupini*, with two variants; *C. lupini* var. *lupini* and *C. lupini* var. *setosum*. The research focussed on conidiomatal conidial shapes and sizes as well as colony morphology of several isolates incubated under different growth conditions. Significant morphological differences between the two variants included the total absence of setae in *C. lupini* var. *lupini*, while setae were detected in *C. lupini* var. *setosum* under black light conditions (Nirenburg *et al*, 2002). Molecular analysis indicated that the two isolates were distinguished by a single base pair in their ITS-2 region. Furthermore, slightly different RAPD profiles were recognised for the two variants, however they were more similar to each other than to other *Colletotrichum* species (Nirenburg *et al*, 2002).

Koch characterised the lupin anthracnose isolates from South Africa and France as a new species, *Colletotrichum tortuosum* (Koch, 1996). The study however focussed mainly on morphological characteristics such as spore shape and colony morphology. It was only later that studies indicated that the South African isolates grouped closer to *C. gloeosporioides* (Yang *et al*, 1998) or *C. acutatum* (Talhinas *et al*, 2002). No study has been conducted up to now to compare the South African lupin anthracnose isolates with the two variants of *C. lupini* species. Thus, in this study a South African isolate *Colletotrichum* SHK 2148 was compared, on a morphological and molecular level, with the two type cultures of *C. lupini* to determine how they relate to the newly described species of Nirenberg *et al* (2002) and to re-evaluate the phylogenetic relationship of anthracnose-associated *Colletotrichum* isolates.

2.2 Materials and Methods

2.2.1 Fungal isolates

Three *Colletotrichum* islolates, SHK 788, SHK 1033 and SHK 2148, that were isolated from lupins with anthracnose symptoms were obtained from Dr. S. H. Koch (ARC-PPRI, Roodeplaat, Pretoria, South Africa). SHK 788 was collected during 1994 in Bethlem, Free State (personal communication Dr. S. H. Koch), SHK 1033 was collected in Elsenburg, Stellenbosch during 1995 while SHK 2148 was collected in Langgewens, Malmesbury during 1999 (Koch *et al*, 2002). The isolates were maintained on potato dextrose agar (PDA) (Biolabs, Merck Laboratory Supplies, Gauteng, South Africa) plates containing 50 mg/ml streptomycin (Sigma, Missouri, USA) and 50 mg/ml chloramphenicol (Sigma). Two type cultures *C. lupini* var. *lupini* (CBS 109225) and *C. lupini* var. *setosum* (CBS 109221) described by Nirenberg *et al* (2002) were obtained from CBS (Centraalbureau voor Schimmelcultures). For the purpose of this study, they were designated as C51 for *C. lupini* var. *lupini* and C52 for *C. lupini* var. *setosum*.

2.2.2 Growth conditions

The isolates were grown on different media and light conditions using the conditions described by Nirenberg *et al* (2002) as a guideline to set up the experimental layout (Table 2.1). The colony and spore morphology were investigated after a period of approximately 14 days of growth. The colour chart of Rayner *et al* (1970) was used to describe the colours displayed by the cultures under growth condition D.

Table 2.1 Growth conditions outlined for the morphological comparison of *Colletotrichum* SHK 2148 with *Colletotrichum lupini* var. *setosum* and *Colletotrichum lupini* var. *lupini*.

Exp. Number	Media	Light conditions	Temp
A	PDA	Darkness	20 ° C
B	SNA with strips of filter paper added to the surface	Darkness	20 ° C
C	SNA with strips of filter paper added to the surface	Continuous light conditions*	20 ° C
D	PDA	Natural day night rhythm	20 –22 ° C

* Spore formation under continuous light conditions were investigated, since a UV facility was not available to investigate spore formation under near-UV light conditions.

After 14 days, the culture morphology as well as the spore size and shape for the different isolates were observed for all the growth conditions. Measurements of approximately 20 spores for each isolate under each condition were recorded and average sizes were determined.

2.2.3 Microscopic analysis of conidia

The size and shape of the conidia for each isolate under all growth conditions were recorded using a light microscope (Carls Zeiss, West Germany) and Auxiovision software. Culture suspensions were made in water or naphtophenol.

2.2.4 ITS and β- tubulin PCR amplification

Genomic DNA was isolated according to the method of Raeder and Broda (1985). DNA samples were evaluated on a 1% TAE agarose gel containing EtBr.

Glass *et al* (1995) reported several primer sets that are useful for phylogenetic analysis of filamentous ascomycetes. Primer pairs ITS1and ITS4 (Table 2.2) were used to amplify a section of the ITS1, ITS2 and the 5.8 S rDNA region from isolates *Colletotrichum* SHK 2148, *C. lupini* var. *setosum* and *C. lupini* var. *lupini* (Fig. 2.1). Primer sets βt2a, βt2b, βt1a and βt1b (Table 2.2) were used to amplify fragments of

the β-tub1 and β-tub2 region from isolates *Colletotrichum* SHK 2148, *C. lupini* var. *setosum* and *C. lupini* var. *lupini* (Fig. 2.1).

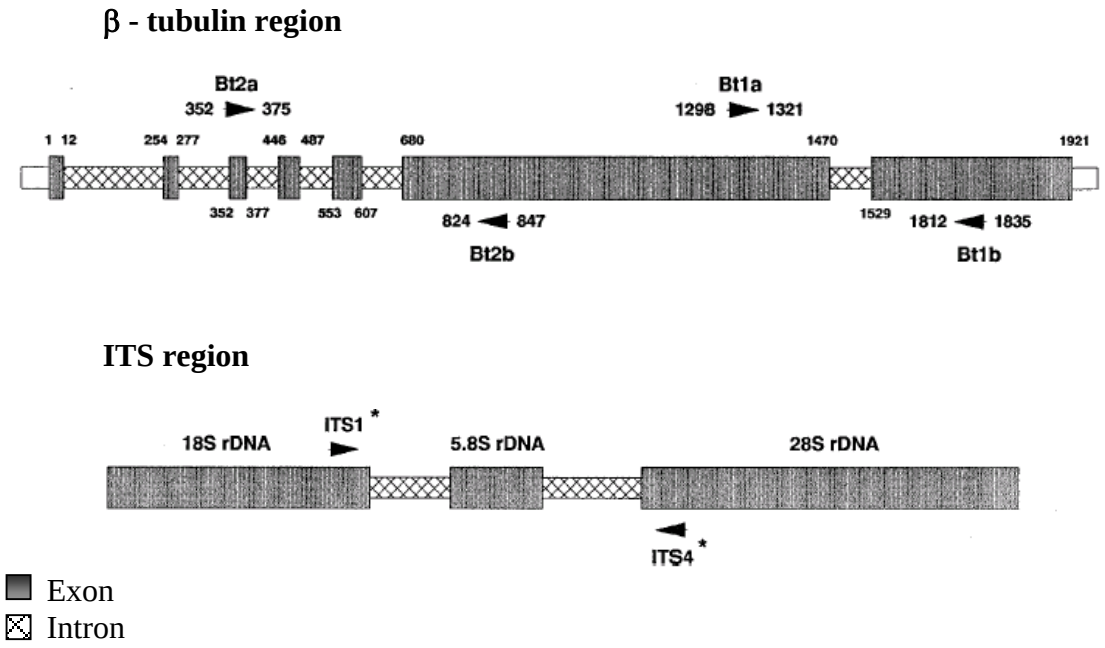


Fig. 2.1 Schematic representation of the ITS and β-tubulin regions as well as the primers used to amplify sections of these regions (Glass *et al*, 1995).

Table 2.2 Information of primers used in this study to amplify the β-tubulin and ITS regions of the isolates.

Target region	Sequence	Primer
ITS	5' TCCGTAGGTGAACCTGCGC 3'	ITS 1
	5' TCCTCCGCTTATTGATATGC 3'	ITS 4
β- Tubulin		
Region 2	5' TTCCCCCGTCTCCACTTCTTCATG 3'	βt1a
	5' GACGAGATCGTTCATGTTGAACTC 3'	βt1b
Region 1	5' GGTAACCAAATCGGTGCTGCTTTC 3'	βt2a
	5' ACCCTCAGTGTAGTGACCCTTGGC 3'	βt2b

The ITS PCR reaction was set up using 30ng genomic DNA from each isolate, 0.25µl Biotaq (1.25U/µl), 1.5mM $MgCl_2$, 1× PCR reaction buffer, dNTPs (200µM of each) and 0.1µM of each of the relevant primers. The ITS PCR cycle, performed in a GeneAmp 2700 thermal cycler (ABI Advanced Biotechnological Institute, Perkin-Elmer Corporation, Foster City, USA), was based on the cycle used by van Wyk *et al* (2004) and included an initial denaturation step at 96 °C for 2 min, followed by a ten-cycle step, which included another denaturation step at 94 °C for 20 sec., an annealing step at 55 °C for 45 sec. and an elongation step at 72 °C for 45 sec. This was followed with another cycle, consisting of a denaturation step at 94 °C for 30 sec.; an annealing step at 55 °C for 40 sec. and an elongation step at 72 °C for 45 sec., which was repeated thirty times. The final elongation step was carried out at 72 °C for 7 minutes. The β-tubulin PCR reactions consisted of an initial denaturation step of 95°C for 5 min, a 33 cycle repeat of a denaturation step at 94 °C for 20 sec., an annealing step where β-tub region 1 primers were annealed at 60°C and β-tub region 2 primers were annealed at 58 °C respectively for 45 sec., and an elongation step at 72 °C for 45 sec. The cycle was completed with a final elongation step at 72°C for 5 minutes.

The PCR reactions were evaluated on a 1% (w/v) TAE agarose gel with EtBr. The fragments were excised and purified using the Qiagen PCR purification kit (Qiagen, Germany). The purified products were sequenced using the ITS and β- Tubulin primers (10µM each) with the PCR BigDye Terminator v 3.1 cycle sequencing kit (Applied Biosystems). All sequence reactions were performed using 4µl Big Dye® v 3.1, 1µl primer (10µM) and 50 –100ng DNA template in a final volume of 10µl. The PCR sequencing cycles consisted of a denaturation step at 96 °C for 1 min., followed by a 25 cycle repeat of three steps; 96°C for 10 sec., and annealing step carried out at 50 °C for 5 sec and an elongation step at 60°C for 4 min. The sequencing reactions were purified in 0.5 ml eppendorf tubes in which 2µl NaOAc (3M, pH 4.6) and 50µl ice cold Ethanol (96%) was added to the sequencing mixture. The mixture was centrifuged at maximum speed for 30 minutes; whereafter the pellet was washed twice with 250µl Ethanol (70%) and air-dried. The sequencing reactions

were performed in an ABI prism 3000 sequencer (ABI Advanced Biotechnological Institute, Perkin-Elmer Corporation, Foster City, USA).

2.2.5 Phylogenetic analysis

ITS analysis

ITS sequence data of *Colletotrichum* SHK 2148, SHK 1033 and SHK 788 as well as Genbank sequences, obtained for 25 additional taxa, including an outgroup (Table 2.3), were analysed.

β- Tubulin analysis

β- Tubulin sequences were experimentally obtained for *Colletotrichum* SHK 2148, SHK 1033, SHK 788 and the type cultures *C. lupini* var. *setosum* and *C. lupini* var. *lupini*. The sequence data for these isolates were compared to that of 16 other taxa as well as outgroup (Table 2.4).

Phylogenetic analysis was performed in PAUP* (Swofford, 1998), version 4.0 b10. Phylogenetic trees were constructed using the Kimura-2P model and a neighbour joining (NJ) algorithm (Saitou and Nei, 1987). The parsimony analyses of the β-Tubulin sequences was conducted by using the heuristic search option and a strict consensus tree was constructed. The validity of the trees obtained from all the analyses performed, was tested by bootstrap analysis of a 1000 random re-samplings and a 60 % threshold.

Table 2.3 Taxa included for the phylogenetic analysis of the ITS region (The reference isolates, C51 and C51, obtained from CBS are indicated with an asterisk)

Accession number	Isolate code	Previously identified as	Host
AF081292[A]		*C. acutatum*	*Olea*
AF090853[A]		*C. acutatum*	*Fragaria*
AJ300558[A]	C2897	*C. acutatum*	*Fragaria*
AF090855[A]		*C. gloeosporioides*	Citrus
AJ311391[A]	HY09	*C. acutatum*	*Lupinus albus*
AJ300559[A]	HO19	*C. gloeosporioides*	Citrus
AJ313178[A]	CR 45	*C. gloeosporioides*	Citrus
AJ300560[A]	CR 21	*C. gloeosporioides*	Citrus
AJ300563[A]	CR 46	*C. acutatum*	*Vitis vinifera*
AJ300557[A]	JG 05	*C. acutatum*	*Ceanothus* sp.
AJ300561[A]	CMG12	*C. acutatum*	*Cinnamonium zeylanicum*
AJ300562[A]	TN47	*C. acutatum*	*Eriobotrya japonica*
AJ301964[B]	BBA71292	*C. acutatum*	*Lupinus albus*
AJ301981[B]	BBA71370	*C. acutatum*	*Cyclamen*
AJ301982[B]	BBA71371	*C. acutatum*	*Cyclamen*
AJ301916[B]	BBA70344	*C. lupini* var *setosum*	*Lupinus*
AJ301918[B]	BBA70346	*C. lupini* var *setosum*	*Lupinus*
AJ301923[B]	BBA70352	*C. lupini* var *setosum*	*Lupinus albus*
AJ301927[B]	BBA70073	*C. lupini* var *setosum*	*Lupinus polyphyllus*
AJ301928[B]	BBA70317	*C. lupini* var *setosum*	*Lupinus albus*
AJ301930[B]	BBA63879	*C. lupini* var *lupini*	*Lupinus mutabilis*
AJ301933[B]	BBA70358	*C. lupini* var *setosum*	*Lupinus albus*
AJ301934[B]	BBA68334	*C. lupini* var *setosum*	*Lupinus*
AJ301935[B]	BBA70385	*C. lupini* var *setosum*	*Lupinus angustifolius*
AJ301948[B]	BBA70884	*C. lupini* var *lupini*	*Lupinus albus*
AJ301959[B]	BBA71249	*C. lupini* var *lupini*	*Lupinus albus*
AJ301968[B]	BBA71310	*C. lupini* var *setosum*	*Lupinus luteus*
AJ301975[B]	BBA71330	*C. lupini*	*Urtica dioica*
AJ301984[B]	BBA71527	*C. coccodes*	*Lupinus polyphyllus*
AJ301985[B]	BBA71528	*C.* cf. *truncatum*	*Lupinus polyphyllus*
M13906[A]		*Neurospora crassa*	

[A] Talhinas *et al* (2002)
[B] Nirenberg *et al* (2002)

Table 2.4 Taxa [A] included for the phylogenetic analyses of the β- Tubulin region

Accession number		Isolate code	Previously identified as	Host
β-Tub 1	**β- Tub2**			
AJ314718	AJ314717	C2897	*C. acutatum*	*Fragaria*
AJ409290	AJ409289	CFA12	*C. falcatum*	*Saccharum officinarum*
AJ409292	AJ409291	315	*C. gloeosporioides*	*Fragaria*
AJ314720	AJ314719	96A4	*C. acutatum*	*Lupinus albus*
AJ409298	AJ409297	CR02	*C. acutatum*	*Lupinus albus*
AJ314716	AJ314715	CR20	*C. acutatum*	*Fragaria*
AJ314714	AJ314713	CR21	*C. gloeosporioides*	Citrus sp.
AJ292249	AJ292248	CR45	*C. gloeosporioides*	Citrus sp.
AJ311668	AJ292252	CR46	*C. acutatum*	*Vitis vinifera*
AJ314722	AJ314721	HO01	*C. acutatum*	*Lupinus albus*
AJ292241	AJ292242	HO19	*C. gloeosporioides*	Citrus sp.
AJ409302	AJ409301	JG05	*C. acutatum*	*Ceanothus* sp.
AJ409300	AJ409299	JR03	*C. acutatum*	*Lupinus albus*
AJ300709	AJ300708	JR15	*C. acutatum*	*Lupinus albus*
AJ314712	AJ314711	PT29	*C. acutatum*	*Lupinus albus*
AJ292250	AJ292251	PT30	*C. acutatum*	*Lupinus albus*
M13630			*Neurospora crassa*	

[A] Talhinas *et al* (2002)

2.3 Results

2.3.1 Colony morphology

The cultures were grown in four conditions (Table 2.1). From the colony morphologies observed for these different growth conditions, it seems as if *Colletotrichum* SHK 2148 is more similar to *C. lupini* var. *setosum* than to *C. lupini* var. *lupini* (Fig. 2.2 – Fig. 2.7). On SNA this similarity is not so evident (Fig. 2.3 and Fig. 2.4), although growth of *Colletotrichum* SHK 2148 (Fig. 2.3 d and Fig. 2.4 g) and *C. lupini* var. *setosum* (Fig. 2.3 e and Fig. 2.4 h) appears to be more vigorous on the filter paper than the growth observed for *C. lupini* var. *lupini* (Fig. 2.3 f and Fig. 2.4 i). When the isolates are grown on PDA under a natural day night rhythm (Fig. 2.5, Fig. 2.6 and Fig. 2.7) one can clearly distinguish *Colletotrichum* SHK 2148 and *C. lupini* var. *setosum* from *C. lupini* var. *lupini* (Fig. 2.5 n, o and Fig. 2.6 r); *Colletotrichum* SHK 2148 (Fig. 2.5 j, k and Fig. 2.6 p) and *C. lupini* var. *setosum* (Fig. 2.5 l, m and Fig. 2.6 q) are faster growing under normal day night rhythm lab conditions (condition D). These two isolates have a white margin with a darker centre that displays light pale olivaceous grey patches (Rayner *et al*, 1970; sheet 8, VI, 12o,d). As the culture grows older (approximately three weeks), some areas in the centre and the margin are turning a light salmon colour (Rayner *et al*, 1970; sheet 1, II, 41) (Fig. 2.5 k and m). When viewed from the bottom of the plates, the cultures can be described as a light saffron colour (Rayner *et al*, 1970; sheet 1, I, 10) interrupted with small black spots (Fig. 2.7 s and t). *C. lupini* var. *lupini* are slower growing, displays patches of light smoke grey (Rayner *et al*, 1970; sheet 8, V, 105) and straw yellow (Rayner *et al*, 1970; sheet 2, II, 46) (Fig. 2.5 n and Fig. 2.6 r), no salmon colour is observed after three weeks (Fig. 2.5 o). From the bottom of the plate the yellow colour is much brighter and could be described as pure yellow (Rayner *et al*, 1970; sheet 2, I, 14) (Fig. 2.7 u). This yellow colour is also displayed by *C. lupini* var. *lupini* when the isolates are grown on PDA in total darkness (Fig. 2.2 c). The brightness of the colour however is not so evident under these conditions and the isolates may look very similar to each other, although *C. lupini* var. *lupini* is much lighter than *Colletotrichum* SHK 2148 (Fig. 2.2 a) and *C. lupini* var. *setosum* (Fig. 2.2 b). The main morphological characteristics observed are summarized in Table 2.9.

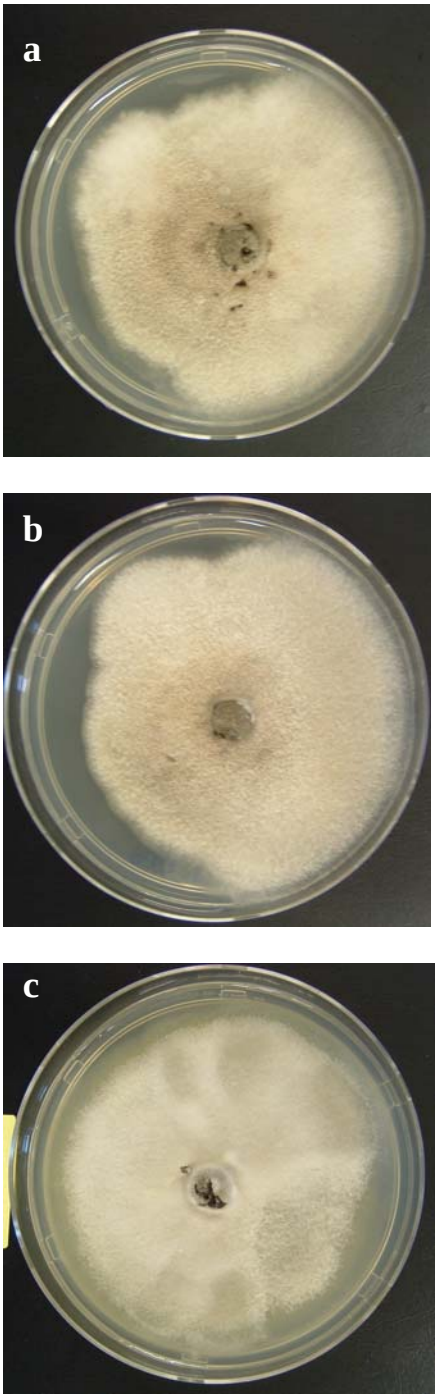


Fig. 2.2 Culture morphology displayed when *Colletotrichum* SHK 2148 (a) and *Colletotrichum lupini* var. *setosum* (b) and *Colletotrichum lupini* var. *lupini* (c) were grown on PDA in total darkness at 20 °C (top view of plates).

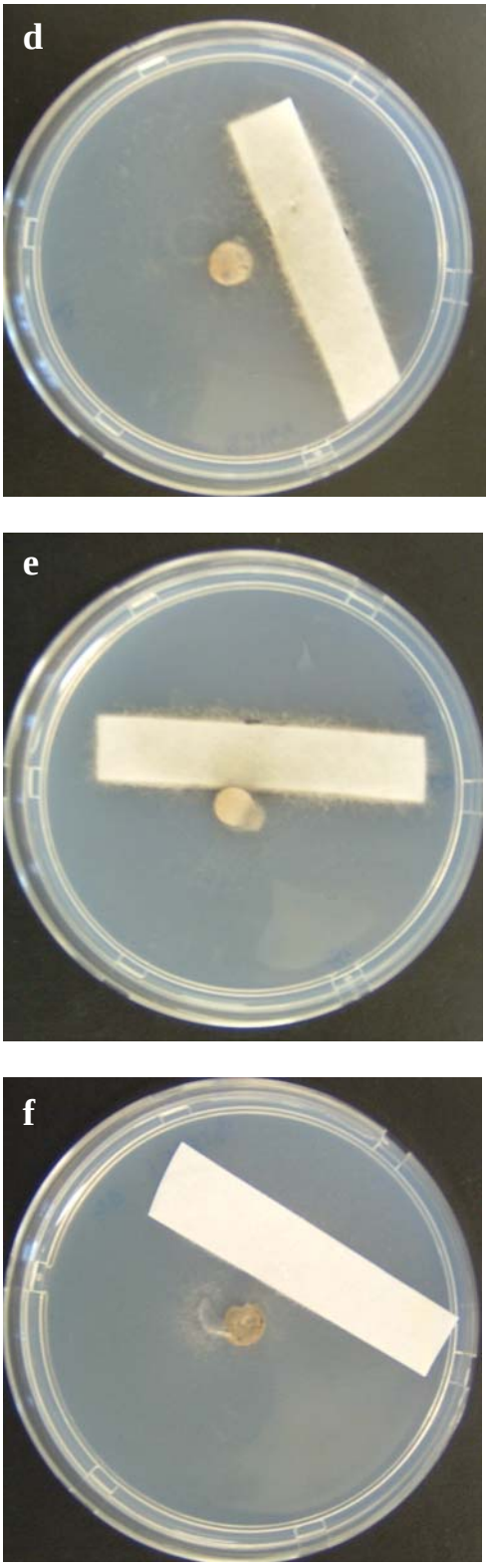


Fig. 2.3 Culture morphology displayed when *Colletotrichum* SHK 2148 (d) and *Colletotrichum lupini* var. *setosum* (e) and *Colletotrichum lupini* var. *lupini* (f) were grown on SNA, with strips of filter paper, in total darkness at 20 °C (top view of plates).

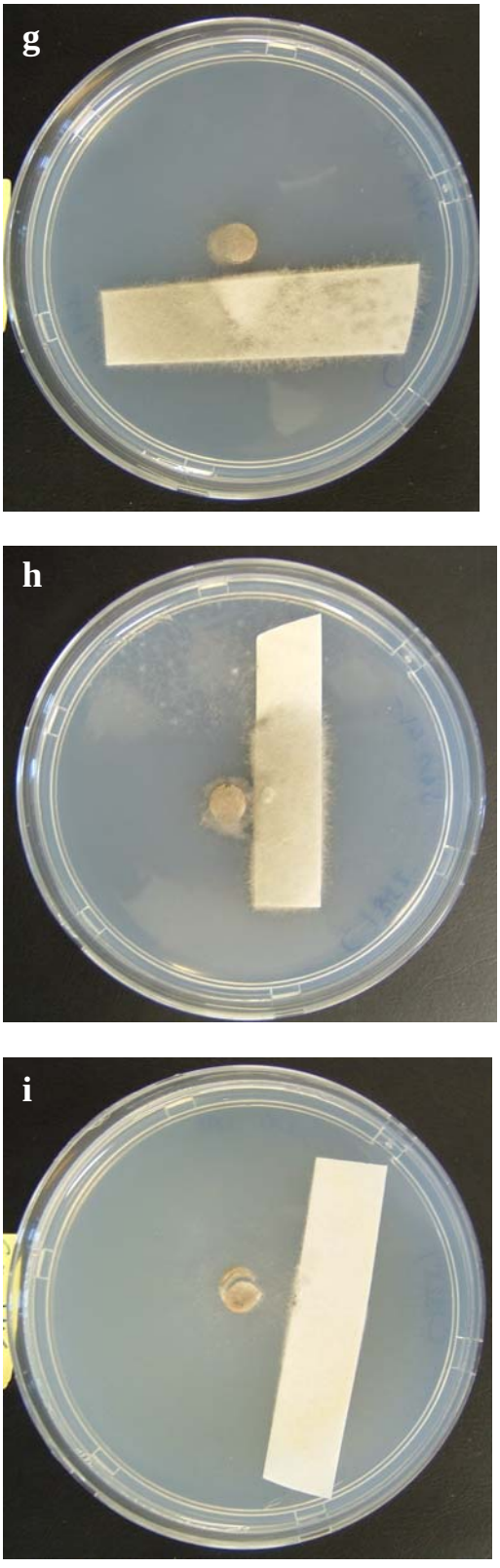
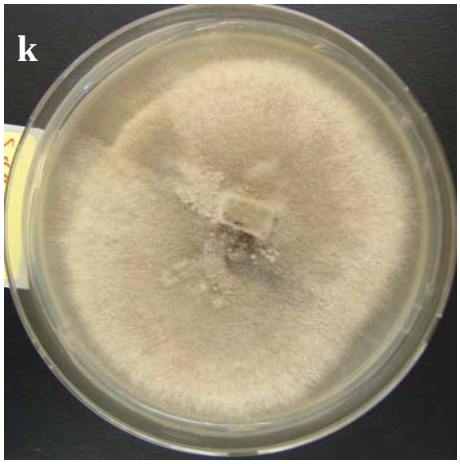
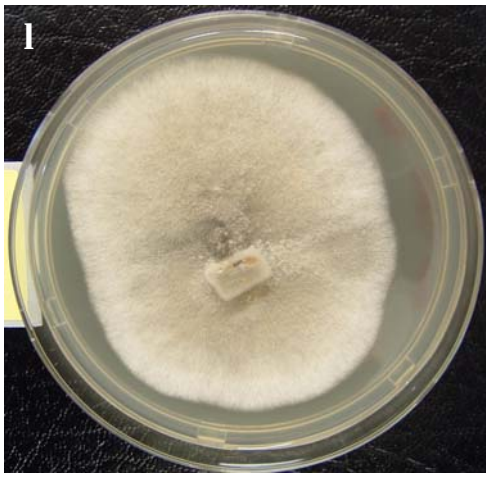


Fig. 2.4 Culture morphology displayed when *Colletotrichum* SHK 2148 (g) and *Colletotrichum lupini* var. *setosum* (h) and *Colletotrichum lupini* var. *lupini* (i) were grown on SNA, with strips of filter paper, under constant light at 20 °C (top view of plates).

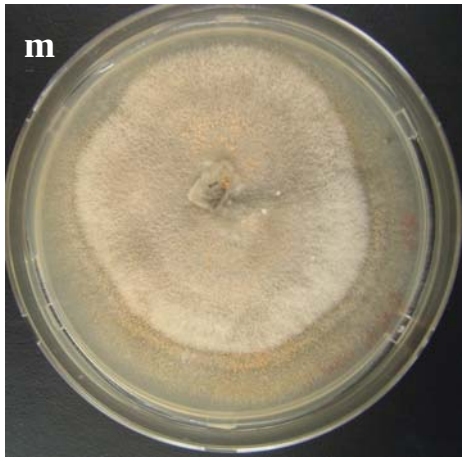
14 Days



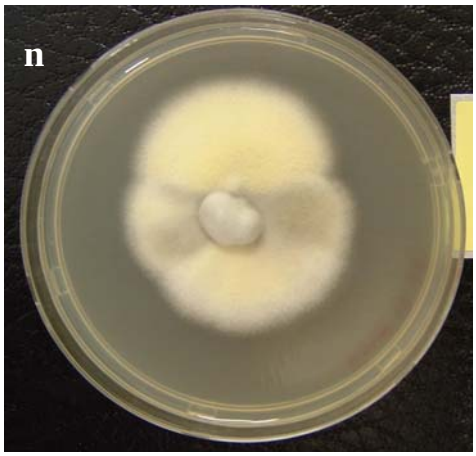
21 Days



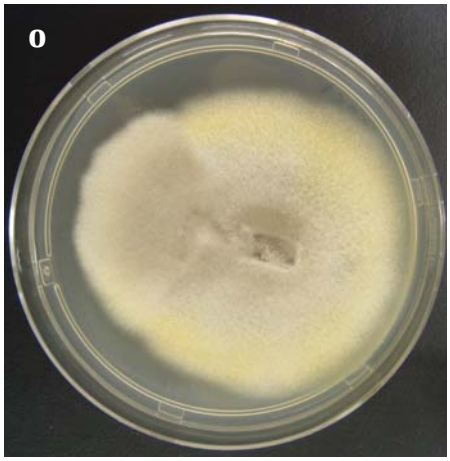
14 Days



21 Days



14 Days



21 Days

Fig. 2.5 Culture morphology displayed when *Colletotrichum* SHK 2148 (j, k) and *Colletotrichum lupini* var. *setosum* (l, m) and *Colletotrichum lupini* var. *lupini* (n, o) grown on PDA under natural day night rhythms after14 and 21days (top view of plates).

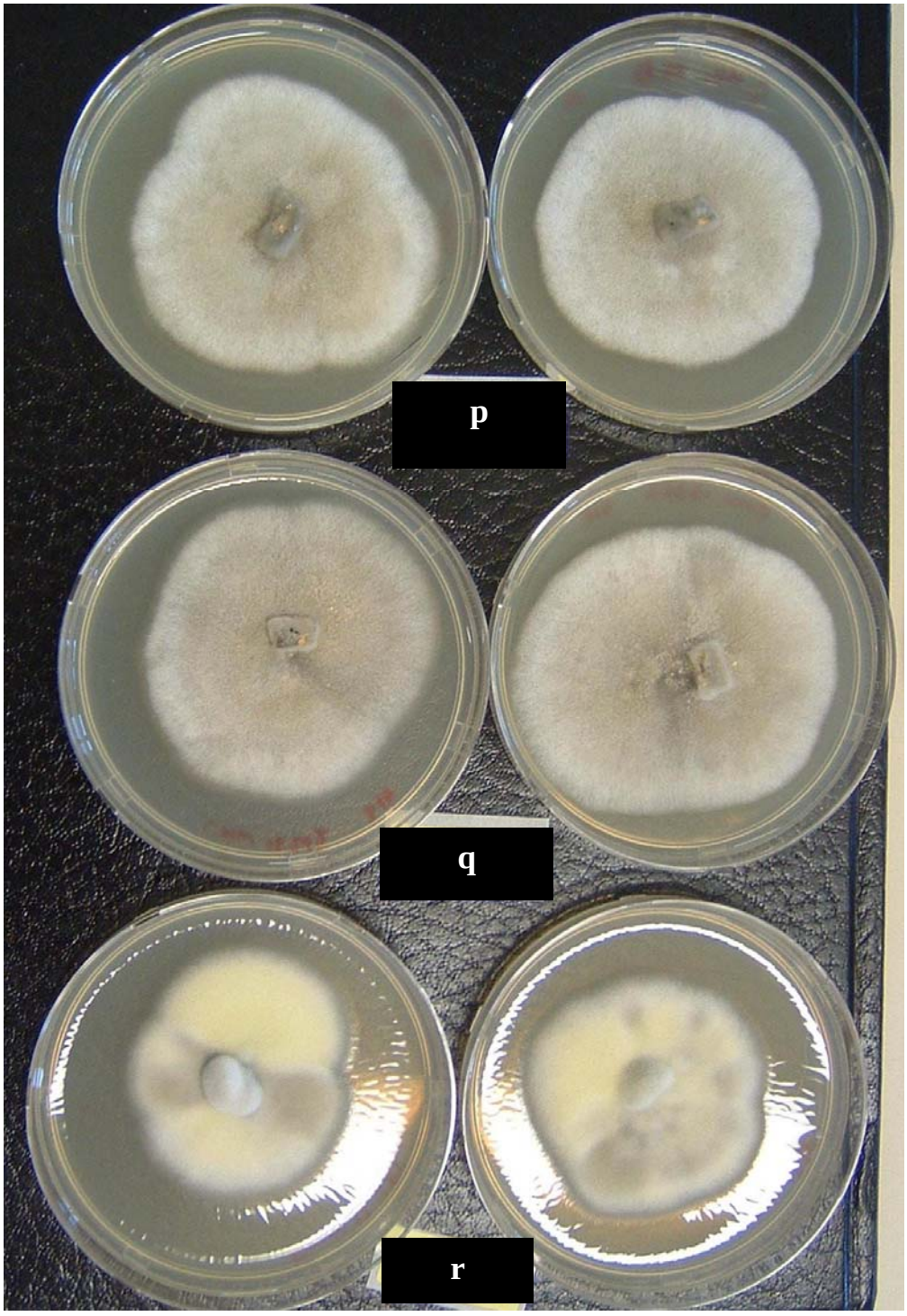


Fig. 2.6 Culture morphology displayed when *Colletotrichum* SHK 2148 (p) and *Colletotrichum lupini* var. *setosum* (q) and *Colletotrichum lupini* var. *lupini* (r) grown on PDA under natural day night rhythms after 14 days (top view of plates).

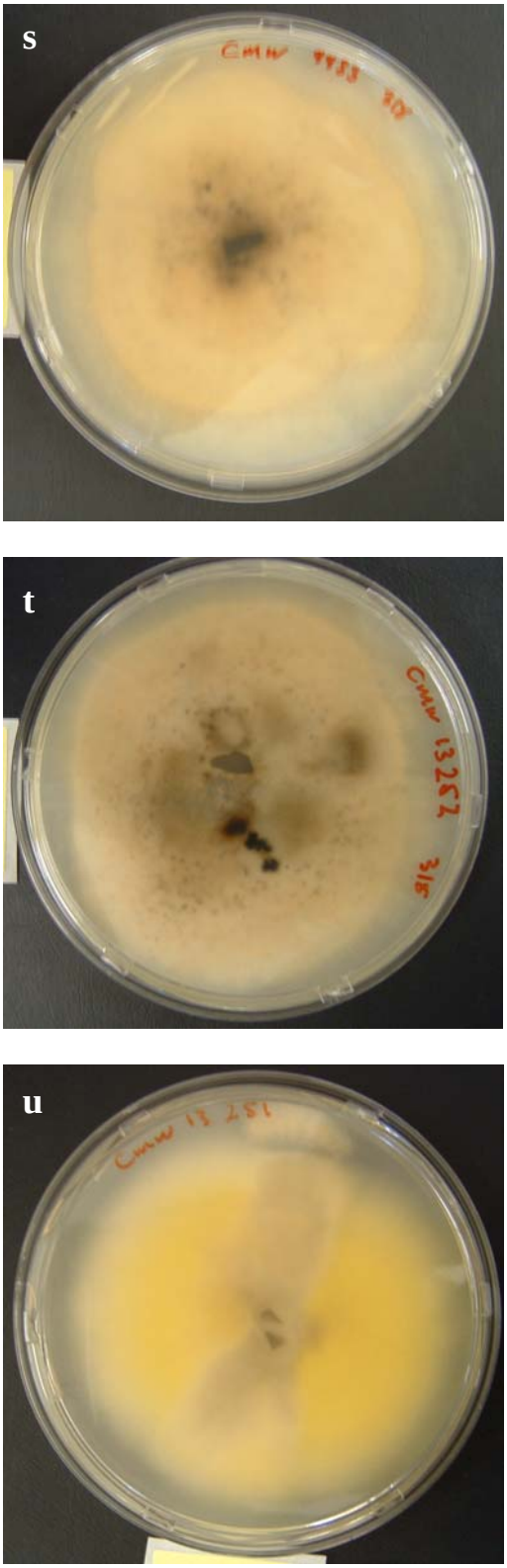
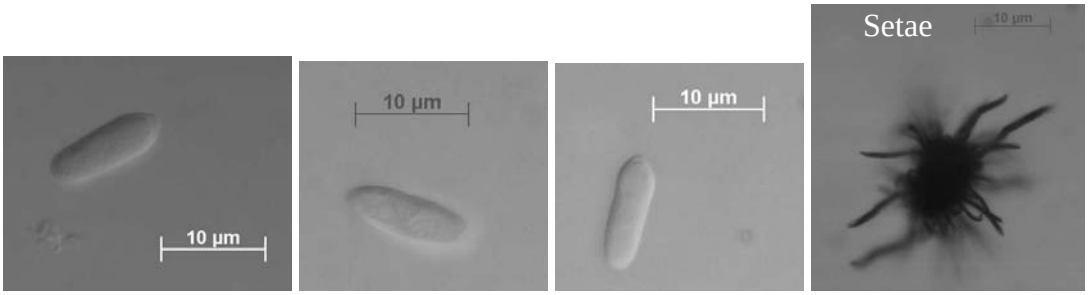


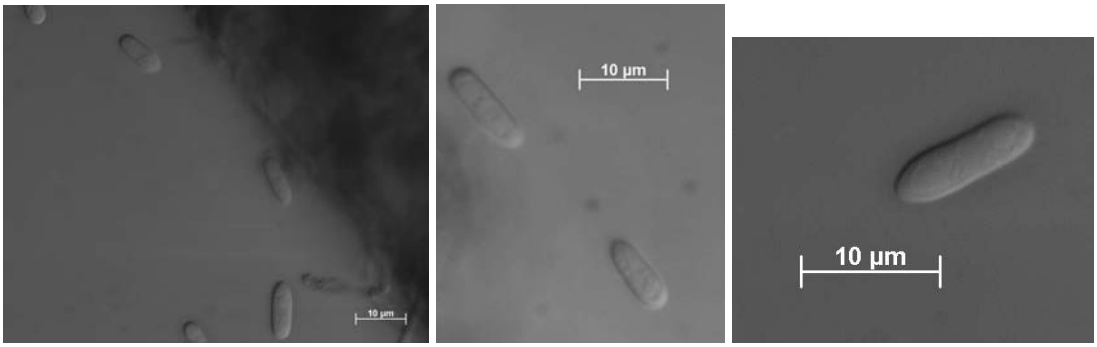
Fig. 2.7 Culture morphology displayed when *Colletotrichum* SHK 2148 (s) and *Colletotrichum lupini* var. *setosum* (t) and *Colletotrichum lupini* var. *lupini* (u) grown on PDA under natural day night rhythms after 21days (bottom view of plates).

3.2.2 Spore characteristics

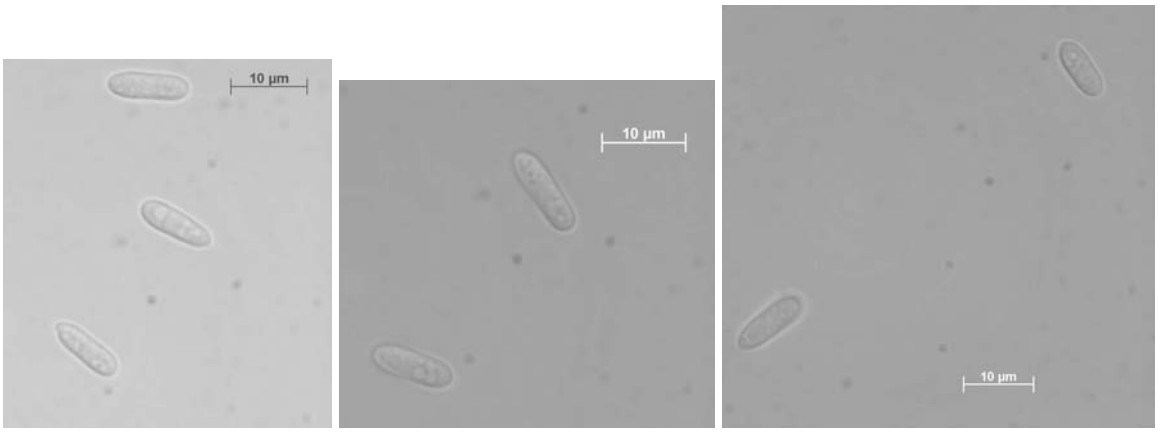
There was no significant difference between the size and shape of the conidia of the different isolates or under different growth conditions. The majority of them displayed the description of Nirenberg *et al* (2002) as being pointed at one end and rounded at the other end (Fig. 2.8, Fig. 2.9 and Fig. 2.10). Setae were observed for *Colletotrichum* SHK 2148, grown on PDA in total darkness (Fig. 2.8 A). No setae have been observed for *C. lupini* var. *lupini* and *C. lupini* var. *setosum* in this study, however it was reported for *C. lupini* var. *setosum* (Nirenberg *et al*, 2002). The setae of *Colletotrichum* SHK 2148 resembled that of *C. lupini* var. *setosum.* The main characteristics of the conidia are summarised in Table 2.9.



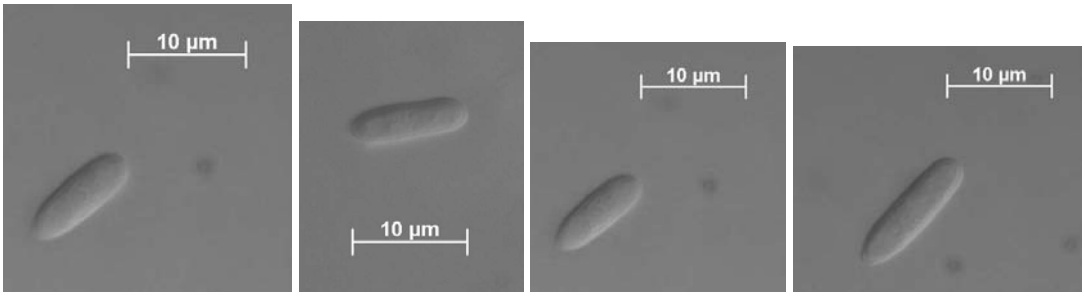
A: PDA, Darkness, 20°C



B: SNA, Darkness, 20°C

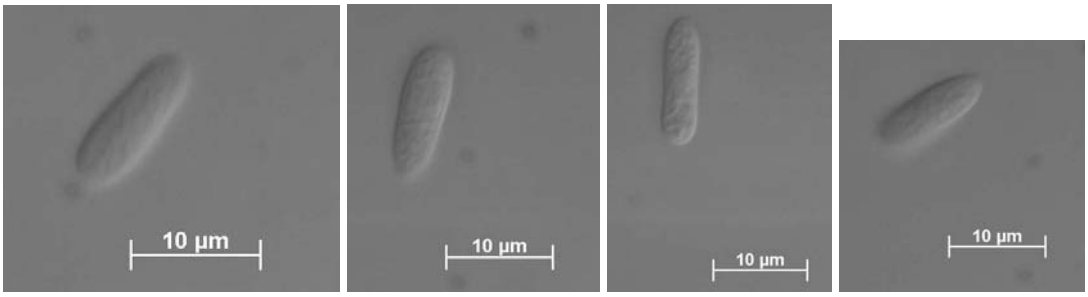


C: SNA, Constant light, 20°C

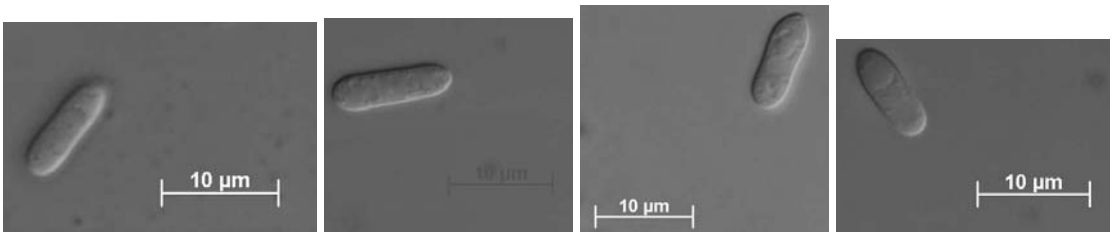


D: PDA, Natural day night rhythm, 20- 22°C

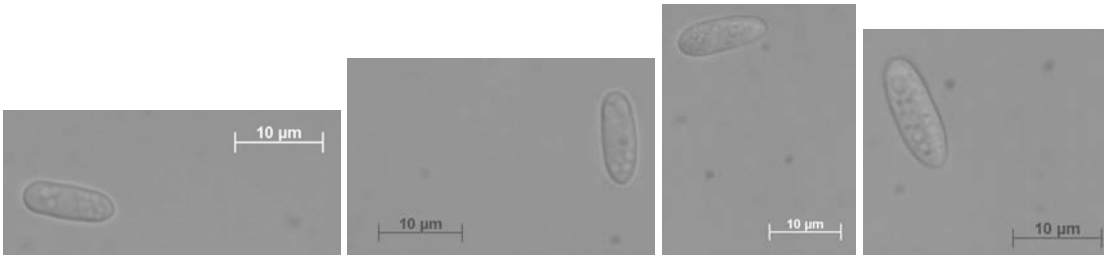
Fig. 2.8 Spores presented for *Colletotrichum* SHK 2148 grown under conditions A, B, C and D.



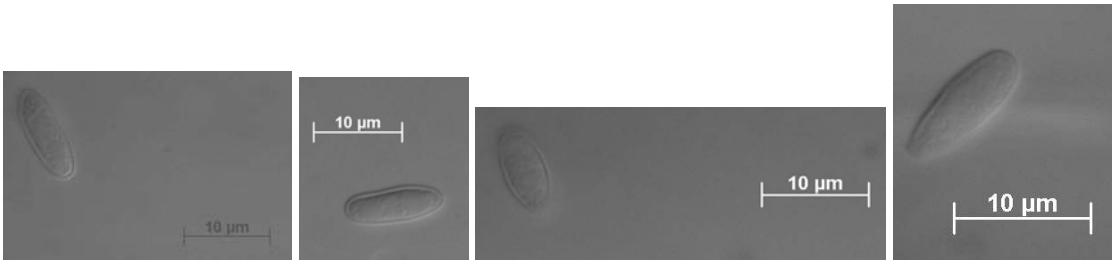
A: PDA, Darkness, 20°C



B: SNA, Darkness, 20°C

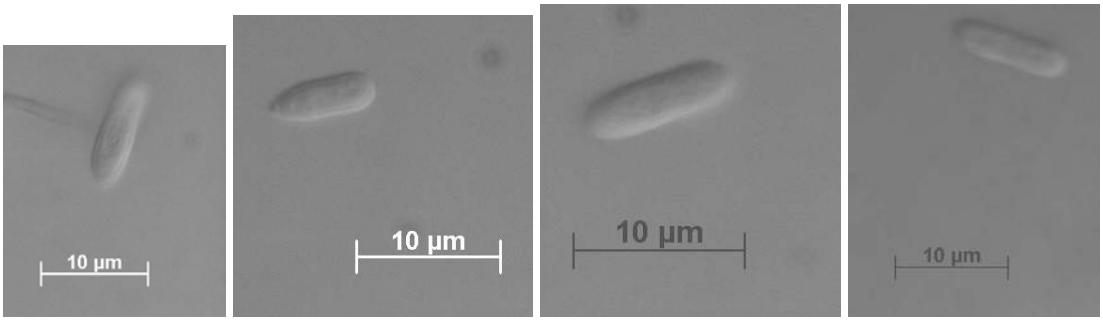


C: SNA, Constant light, 20°C

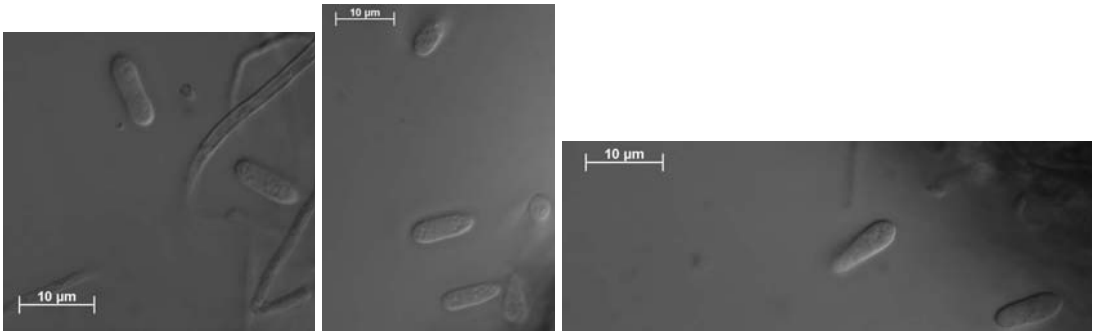


D: PDA, Natural day night rhythm, 20- 22°C

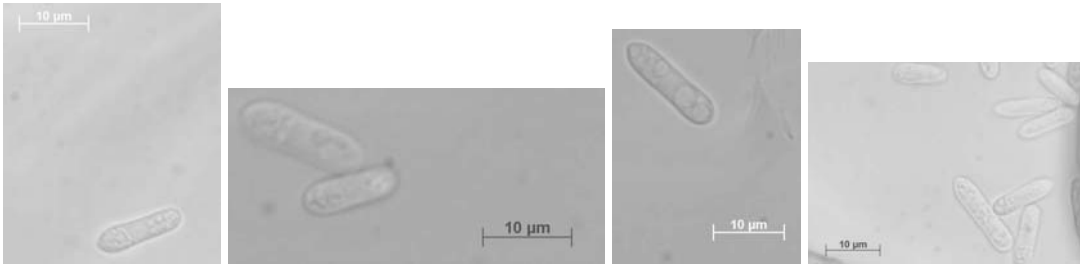
Fig. 2.9 Spores presented for *C. lupini* var. *setosum* grown under conditions A, B, C and D.



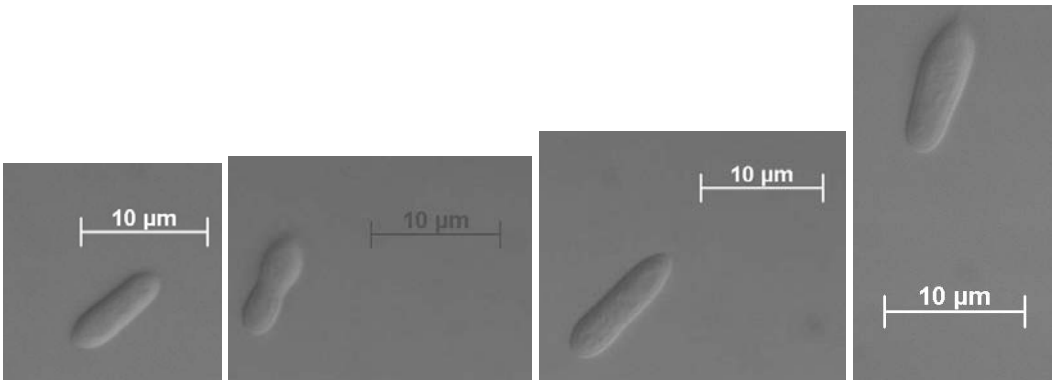
A: PDA, Darkness, 20°C



B: SNA, Darkness, 20°C



C: SNA, Constant light, 20°C



D: PDA, Natural day night rhythm, 20- 22°C

Fig. 2.10 Spores presented for *C. lupini* var. *lupini* grown under conditions A, B, C and D.

2.3.3 PCR amplification of ITS and β-tubulin regions

The ITS regions from *Colletotrichum* SHK2148 (Fig. 2.11) *C. lupini* var. *lupini* and *C. lupini* var. *setosum* (Fig. 2.12) as well as the β-tubulin regions of isolates *Colletotrichum* SHK2148, *C. lupini* var. *lupini* and *C. lupini* var. *setosum* (Fig. 2.13) were successfully amplified from the genomic DNA of the isolates. All the fragments were in the 500 bp size range (Fig. 2.3.11, Fig. 2.3.12 and Fig. 2.3.13).

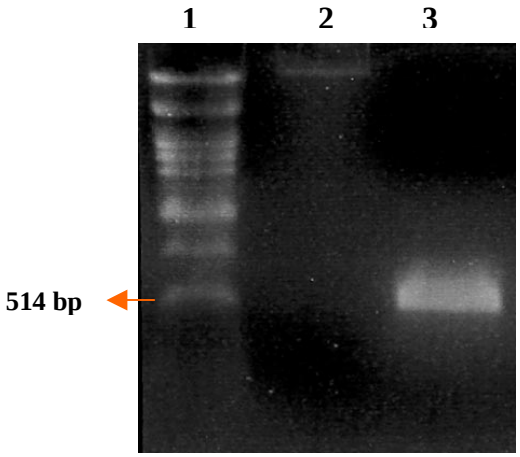


Fig. 2.11 PCR amplification of the ITS regions from *Colletotrichum* SHK 2148
Amplified ITS fragment from the genome of *Colletotrichum* SHK 2148 (lane 3), electrophoresed on a 1%TAE agarose gel together with 20ng λ DNA (lane 2) and aλ *PstI* generated molecular marker (lane 1, Appendix A).

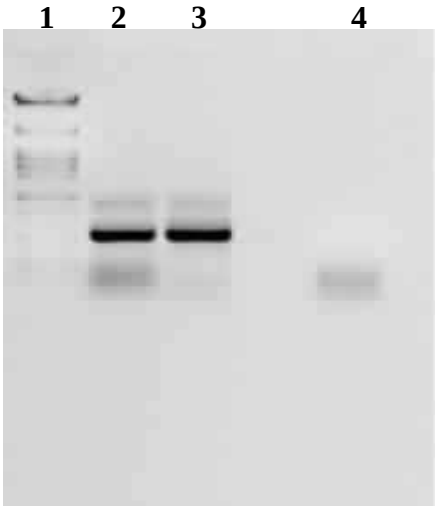


Fig. 2.12 PCR amplification of the ITS regions from isolates *C. lupini* var. *lupini* and *C. lupini* var. *setosum.*
ITS fragments were amplified from the genomic DNA of isolates *C. lupini* var. *lupini* (lane 2) and *C. lupini* var. *setosum* (3) and electrophoresed with the PCR water control (lane 4) and a λ *PstI* molecular marker (lane 1).

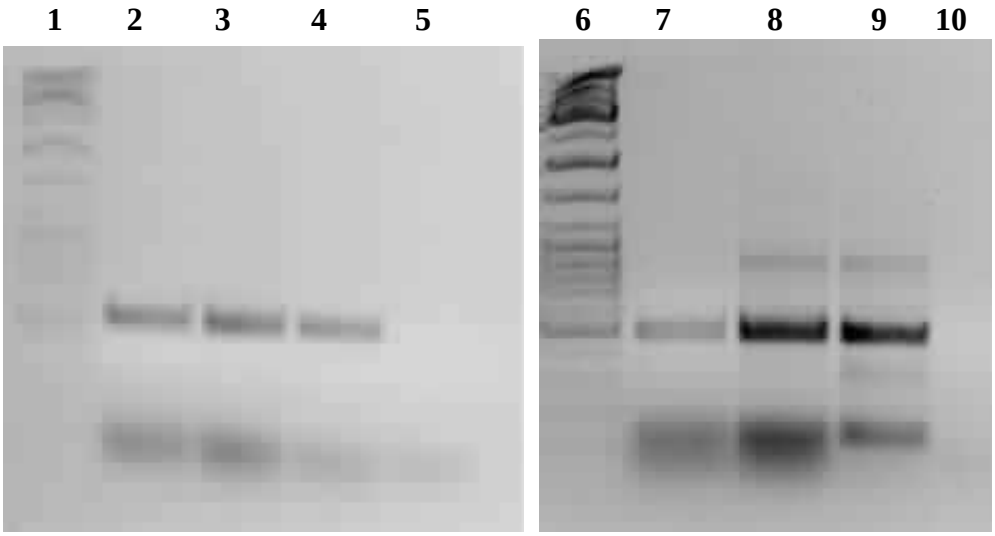


Fig. 2.13 PCR amplification of β-tubulin regions from test isolates *Colletotrichum* SHK 2148, *C. lupini* var. *lupini* and *C. lupini* var. *setosum*.
PCR amplification of β-tubulin region-2 from isolates *Colletotrichum* SHK 2148 (lane2), *C. lupini* var. *setosum* (lane 3) and *C. lupini* var. *lupini* (lane 4). PCR amplification of β-tubulin region-1 from isolates *Colletotrichum* SHK 2148 (lane7), *C. lupini* var. *setosum* (lane 8) and *C. lupini* var. *lupini* (lane 9). Water controls (lanes 5 and 10) and a 100bp molecular marker (lanes 1 and 6, Appendix A) were included on the agarose gel.

Sequence alignment of the ITS regions from *Colletotrichum* SHK 2148, *C. lupini* var *setosum* and *C. lupini* var *lupini* were used to confirm the identity of the CBS cultures and perform a quick identification of *Colletotrichum* SHK 2148 (Fig. 2.14). Nirenberg *et al* (2002) reported that 1 bp difference in the ITS2 region differentiated the two variants. Although not specified by Nirenberg *et al* (2002), it seems that the *C. lupini* var. *setosum* variant contains a T while the *C. lupini* var *lupini* contains a C (Fig. 2.14). Based on this preliminary comparison, it appears if *Colletotrichum* SHK2148 is similar to *C. lupini* var. *setosum* (Fig. 2.14).

```
C. lupini var lupini     (420) CCTCCCGGAGCCTCCTTTGCGTAGTAACTAACGTCTCGCAC C GGGATCCG
C. lupini var. setosum   (415) CCTCCCGGAGCCTCCTTTGCGTAGTAACTAACGTCTCGCAC T GGGATCCG
Colletotrichum SHK 2148 (414) CCTCCCGGAGCCTCCTTTGCGTAGTAACTAACGTCTCGCAC T GGGATCCG
```

Fig. 2.14 Partial nucleotide alignment of a section of the ITS2 region of isolates *Colletotrichum* SHK 2148, *C. lupini* var. *setosum* and *C. lupini* var. *lupini* displaying a single base pair difference between the variants (the single base pair difference is indicated in the block).

2.3.4 Phylogenetic analysis

The ITS analysis were performed with 25 taxa and one outgroup, *Neurospora crassa*. 439 base pairs were aligned for this analysis (Appendix D) of which 143 characters were variable (Fig. 2.15) and only 61 were parsimony informative. The same groupings of Talhinas *et al* (2002) were obtained. The distance matrix (Table 2.5) revealed that the average percentage of variation in group A1 was 0.10%, 0.11% in group A2, and 4.28% in B, while no variation existed in group A3, A4, *Neurospora crassa* or the group consisting of the *Colletotrichum coccodes* and *Colletotrichum* cf. *truncatum* isolates. Based on the percentage of sequence divergence, the groups differed from each other as outlined in Table 2.6. *Colletotrichum* SHK 2148, which grouped in A1, differed from the rest of the members in this group by 0.07%, there was no average distance variance between *Colletotrichum* SHK 2148 and the *C. lupini* var. *setosum* (BBA70352 and BBA71310) isolates as well as *C. lupini* (BBA71330). Data generated support the grouping of *Colletotrichum* SHK 2148 with the rest of these isolates. The average variation between the *Neurospora crassa* outgroup and all the other groups was 25.4%, which indicated that the groups were more related to each other than the outgroup.

Table 2.6 The percentage sequence difference between the groups obtained in the ITS neighbour joining analysis.

	A1	**A2**	**A3**	**A4**	**B**	***Colletotrichum truncatum/ Colletotrichum coccodes* group**	***Neurospora crassa***
A1	0	0.8	1.2	3.2	9.9	9.7	24.0
A2		0	1.0	2.9	10.2	9.2	24.2
A3			0	1.9	9.6	9.3	24.4
A4				0	10.3	9.7	25.4
B					0	9.1	25.9
***Colletotrichum truncatum/ Colletotrichum coccodes* group**						0	28.5

From the neighbour joining analysis, a phylogenetic tree was generated (Fig. 2.16) consisting of two main branches, one containing the *Colletotrichum* isolates from *citrus* and the other containing *Colletotrichum* isolates from lupin and other hosts. The *Colletotrichum* isolates from *citrus* form group B, as described by Talhinas *et al* (2002), however instead of all the isolates contained in the one group; they were split into two sub-groups (Fig. 2.16). The second main branch lead to a group that resembles the A group of Talhinas *et al* (2002), in which the four described sub-groups, A1, A2, A3 and A4 were distinguished and supported by strong bootstrap values (Fig. 2.16). Isolate BBA71292, a *C. acutatum* isolate (Nirenberg *et al*, 2002) groups with the *C. acutatum* isolates that correspond to the A2 group described by Talhinas *et al* (2002) (Fig. 2.16). The rest of the lupin isolates formed a separate group, which correlates to the A1 group described by Talhinas *et al* (2002). This group could be subdivided into two sub-groups. This is consistent with the observation of Nirenberg *et al* (2002) that the two *C. lupini* variants could be distinguished from each other based on a single base pair difference in their ITS2 region. *Colletotrichum* SHK 2148, which had identical ITS sequence data to SHK 788 and SHK 1033, grouped with *C. lupini* var. *setosum* isolates such as the extype BBA70352 (Fig. 2.16). *C. acutatum* CMG12, which had an identical sequence to isolates JR15, PT29, PT30 and CR02 (Talhinas *et al*, 2002) also grouped with *C. lupini* var. *setosum*. Furthermore, other isolates identified as *C. lupini* var. *setosum* (BBA70344, BBA70346, BBA70317, BBA70358, BBA68334 and BBA70385) had an identical sequence to BBA70352 (data not shown). In contrast, *C. lupini* var. *lupini* isolates fell into a different group that included *C. acutatum* HY09 (Fig. 2.16). An attempt was made to determine the maximum parsimony between the taxa, however this analysis was impeded by too few numbers of informative characters.

The β-tubulin analysis, of 19 taxa and a *Neurospora crassa* outgroup, was performed with 993 base pairs aligned between the taxa (Appendix E) of which 472 were variable (Fig. 2.17) and 162 informative. The resulting neighbour joining tree supported the main groups, described by Talhinas *et al* (2002), except for isolates CR46 (A3) and JG05 (A4), which grouped together in this study. Distances between the different taxa, displayed in table 2.7, showed that the average variation was 0.39% in group A1 and 5.54% in B. The average variation of the other groups was zero.

The average variation between the groups is displayed in Table 2.8. The distance between the *Neurospora* outgroup and the other groups were 89.4%, indicating that the groups are closer related to each other than to the outgroup. *Colletotrichum* SHK 2148 grouped as a member of the A1 group. It differed with the other members of this group by 0.31% and had the smallest average distance value with *C. lupini* var. *setosum* (0.13%) and the largest with *C. lupini* var. *lupini* (0.68%). This strongly supports the grouping of *Colletotrichum* SHK 2148 with this group, especially *C. lupini* var. *setosum.*

Table 2.8 The percentage sequence difference between the groups obtained in the β-tubulin phylogenetic analysis.

	A1	**A2**	**A3 and A4**	**B**	***Colletotrichum falcatum***	***Neurospora crassa***
A1	0	2.8	5.3	16.8	16.1	85.7
A2		0	4.7	16.2	15.9	85.6
A3 and A4			0	16.9	16.4	92.5
B				0	18.4	90.3
Colletotrichum falcatum					0	93.1

With the uninformative characters excluded, a parsimony heuristic search was performed, which resulted in two evolutionary trees, which were reduced to one after computing a strict consensus tree (Fig. 2.19). The tree topology differed for the neighbour joining (Fig. 2.18) and parsimony analysis (Fig. 2.19). However the main groups, described by Talhinas *et al* (2002), were supported by both analyses. In both trees *Colletotrichum* SHK 2148 grouped in the A1 group closer to the *C. lupini* variants than with the *C. acutatum* isolates (Fig. 2.18 and Fig. 2.19). According to the neighbour joining analysis the *C. lupini* variants formed a separate group in the A1 group with a strong bootstrap support of 84% (Fig. 2.18). The parsimony analysis did not support this separate grouping very strongly, however it did indicate that *Colletotrichum* SHK 2148 groups closer to *C. lupini* var. *setosum* (Fig. 2.19).

```
                                        1111111122222222233333333344444444555555555667777778
Taxon/Node                              1256789012346791234567890234567890123467901234567891302356 71
-------------------------------------------------------------------------------------------------------------
C. gloeosporioides (AF090855)           ------------TAGTCTCCGCGACCTCCCGGCCTCCCGCTCCGGGCGGGTGCCGAGATA   [48]
C. gloeosporioides (HO19)               ------------.....................-.C.G.CT.........................   [47]
C. gloeosporioides (CR45)               ---TAGGGTCTCGCA----.C.TC.GG..TCCG.CT...G---...TC..--.........   [48]
C. gloeosporioides (CR21)               TAG-----TCTCGCA----.C.TC.GG..TC--.CG..T.GGGC..GTC.-..GAGAT-.   [47]
C. coccodes (BBA71527)                  --GACAACCCCC.C.GGG.G.TCC....-.C.G.CG.GC...AC..GC.T-........T   [55]
C. cf. truncatum (BBA71528)             CGCAGAGGTTCC.CCGAA..C.TC.GGTGAC.-.C.T.A.-----..GC.T-C......T   [53]
C. acutatum (AF081292)                  CAGGAAGCCTCTGC.C....C.TC.GG.G.C.G.C...A.A.G...AC..-........-.   [58]
C. acutatum (AF090853)                  CAGGAAGCCTCTGC.C....C.TC.GG.G.C.G.C...A.A.G...AC..-........-.   [58]
C. acutatum (BBA71292)                  CAGGAAGCCTCTGC.C....C.TC.GG.G.C.G.C...A.A.G...AC..-........-.   [58]
C. acutatum (C2897)                     CAGGAAGCCTCTGC.C....C.TC.GG.G.C.G.C...A.A.G...AC..-........-.   [58]
C. acutatum (CMG12)                     CAGGAAGCCTCTGC.C....C.TC.GG.G.C.G.C...A.A.G...AC..-........-.   [58]
C. lupini var. setosum (BBA70073)       CAGGAAGCCTCTGC.C....C.TC.GG.G.C.G.C...A.A.G...AC..-........-.   [58]
Colletotrichum SHK2148                  CAGGAAGCCTCTGC.C....C.TC.GG.G.C.G.C...A.A.G...AC..-........-.   [58]
C. lupini var. setosum (BBA70352)       CAGGAAGCCTCTGC.C....C.TC.GG.G.C.G.C...A.A.G...AC..-........-.   [58]
C. lupini (BBA71330)                    CAGGAAGCCTCTGC.C....C.TC.GG.G.C.G.C...A.A.G...AC..-........-.   [58]
C. lupini var. setosum (BBA71310)       CAGGAAGCCTCTGC.C....C.TC.GG.G.C.G.C...A.A.G...AC..-........-.   [58]
C. lupini var. lupini (BBA63879)        CAGGAAGCCTCTGC.C....C.TC.GG.G.C.G.C...A.A.G...AC..-........-.   [58]
C. lupini var. lupini (BBA70884)        CAGGAAGCCTCTGC.C....C.TC.GG.G.C.G.C...A.A.G...AC..-........-.   [58]
C. lupini var. lupini (BBA71249)        CAGGAAGCCTCTGC.C....C.TC.GG.G.C.G.C...A.A.G...AC..-........-.   [58]
C. acutatum (HY09)                      CAGGAAGCCTCTGC.C....C.TC.GG.G.C.G.C...A.A.G...AC..-........-.   [58]
C. acutatum (BBA71370)                  CAGGAAGCCTCTGC.C....C.TC.GG.G.C.G.C..-A.A.G...AC..-........-.   [57]
C. acutatum (BBA71371)                  CAGGAAGCCTCTGC.C....C.TC.GG.G.C.G.C..-A.A.G...AC..-........-.   [57]
C. acutatum (JG05)                      CAGGAAGCCTCTGT.CGGA.C.TC.GG.G.C.G.C..-.TA.G...GC..-A......-.   [57]
C. acutatum (TN47)                      CAGGAAGCCTCTGT.CGGA.C.TC.GG.G.C.G.C..-.TA.G...GC..-A......-.   [57]
C. acutatum (CR46)                      CAGGAAGCCTCTGC.C....C.TC.GG.G.C.G.C...A.A.G...AC..-........-.   [58]
Neurospora crassa                       CCAAAGGCCTT--C.C....CG..T--.T...GTCT..----.GCTCGC..C-T...TGC.   [52]

                                        1111111111111111122222333333333333333333333333333333
                                        8999999900111222233333449567890011222223333344444555666666 77
Taxon/Node                              4234678925678123412378297300996856346791346901367012135678 01
-------------------------------------------------------------------------------------------------------------
C. gloeosporioides (AF090855)           CGATTAACCTGGTAAGCTCACT-CACCGCCTTTGCCTAACCGTAGGCCAGGTACTCCCGA   [107]
C. gloeosporioides (HO19)               ......................-G.....................................   [106]
C. gloeosporioides (CR45)               ......................-G.....................................   [107]
C. gloeosporioides (CR21)               .................C-...-G....-.....................................   [104]
C. coccodes (BBA71527)                  .AT.........C.....T...-G......C.......GT..C....T...........   [114]
C. cf. truncatum (BBA71528)             .AT.........C.....T...-G.............GTAAC....T........T...   [112]
C. acutatum (AF081292)                  .AT.AC...C..C.....T...TG.T.A..AC.T..C.G.ACCG...T...........   [118]
C. acutatum (AF090853)                  .AT.AC...C..C.....T...-G.T.AA.AC.T..C.G.ACCG...T...........   [117]
C. acutatum (BBA71292)                  .AT.AC...C..C.....T...-G.T.A..AC.T..C.G.ACCG...T...........   [117]
C. acutatum (C2897)                     .AT.AC...C..C.....T...-G.T.A..AC.T..C.G.ACCG...T...........   [117]
C. acutatum (CMG12)                     .AT.AC...C..C.....T...-G.T.A..AC.T..C.G.ACCG...TG..........   [117]
C. lupini var. setosum (BBA70073)       .AT.AC...C..C.....T...-G.T.A..AC.T..C.G.ACCG...TG..........   [117]
Colletotrichum SHK2148                  .AT.AC...C..C.....T...-G.T.A..AC.T..C.G.ACCG...TG..........   [117]
C. lupini var. setosum (BBA70352)       .AT.AC...C..C.....T...-G.T.A..AC.T..C.G.ACCG...TG..........   [117]
C. lupini (BBA71330)                    .AT.AC...C..C.....T...-G.T.A..AC.T..C.G.ACCG...TG..........   [117]
C. lupini var. setosum (BBA71310)       .AT.AC...C..C.....T...-G.T.A..AC.T..C.G.ACCG...TG..........   [117]
C. lupini var. lupini (BBA63879)        .AT.AC...C..C.....T...-G.T.A..AC.T..C.G.ACCG...TG..........   [117]
C. lupini var. lupini (BBA70884)        .AT.AC...C..C.....T...-G.T.A..AC.T..C.G.ACCG...TG..........   [117]
C. lupini var. lupini (BBA71249)        .AT.AC...C..C.....T...-G.T.A..AC.T..C.G.ACCG...TG..........   [117]
C. acutatum (HY09)                      .AT.AC...C..C.....T...-G.T.A..AC.T..C.G.ACCG...TG..........   [117]
C. acutatum (BBA71370)                  .AT.AC...C..C.....T...-G.T.A..AC.T..C.G.ACCG...T...........   [116]
C. acutatum (BBA71371)                  .AT.AC...C..C.....T...-G.T.A..AC.T..C.G.ACCG...T...........   [116]
C. acutatum (JG05)                      .AT.AC...C..C.....T...-G.T.A..AC.T..C.G...CG...T...........   [116]
C. acutatum (TN47)                      .AT.AC...C..C.....T...-G.T.A..AC.T..C.G...CG...T...........   [116]
C. acutatum (CR46)                      .AT.AC...C..C.....T...-G.T.A..AC.T..C.G...CG...T...........   [117]
Neurospora crassa                       TTGA.TTTTCAA-TTTTGTCACCGGTTA.A..C.ATCGG.---GTCG..ATCGTGT.ATC   [108]

                                        33333333333444444444444
                                        77777889999000000112233
Taxon/Node                              24578582369245789150409
-----------------------------------------------------------------------
C. gloeosporioides (AF090855)           GCTCTAATTGTCCTGGACACTAC   [130]
C. gloeosporioides (HO19)               .......................  [129]
C. gloeosporioides (CR45)               .......................  [130]
C. gloeosporioides (CR21)               .......................  [127]
C. coccodes (BBA71527)                  ........A.............A  [137]
C. cf. truncatum (BBA71528)             .......A-........T.TA.A  [134]
C. acutatum (AF081292)                  .......A-........T....A  [140]
C. acutatum (AF090853)                  .......-A........T....A  [139]
C. acutatum (BBA71292)                  .......A-........T....A  [139]
C. acutatum (C2897)                     .......A-........T....A  [139]
C. acutatum (CMG12)                     .......A-...............  [139]
C. lupini var. setosum (BBA70073)       .......A-...............  [139]
Colletotrichum SHK2148                  .......A-...............  [139]
C. lupini var. setosum (BBA70352)       .......A-...............  [139]
C. lupini (BBA71330)                    .......A-...............  [139]
C. lupini var. setosum (BBA71310)       .......A-...............  [139]
C. lupini var. lupini (BBA63879)        .......-A....C.........  [139]
C. lupini var. lupini (BBA70884)        .......A-....C.........  [139]
C. lupini var. lupini (BBA71249)        .......A-....C.........  [139]
C. acutatum (HY09)                      .......A-....C.........  [139]
C. acutatum (BBA71370)                  .......A-...............  [138]
C. acutatum (BBA71371)                  .......A-...............  [138]
C. acutatum (JG05)                      .......A-............TA  [138]
C. acutatum (TN47)                      .......A-............TA  [138]
C. acutatum (CR46)                      .......A-............TA  [139]
Neurospora crassa                       AACGAGGC.AGTTGTCGGCT...  [131]
```

Fig. 2.15 Nucleotide alignment of the ITS1, 5.8S and ITS2 region from different taxa included in the study, which displays the variable positions.

Table 2.5 Percentage sequence divergence between different *Colletotrichum* isolates based on the ITS1, ITS2 and 5.8S rDNA region.

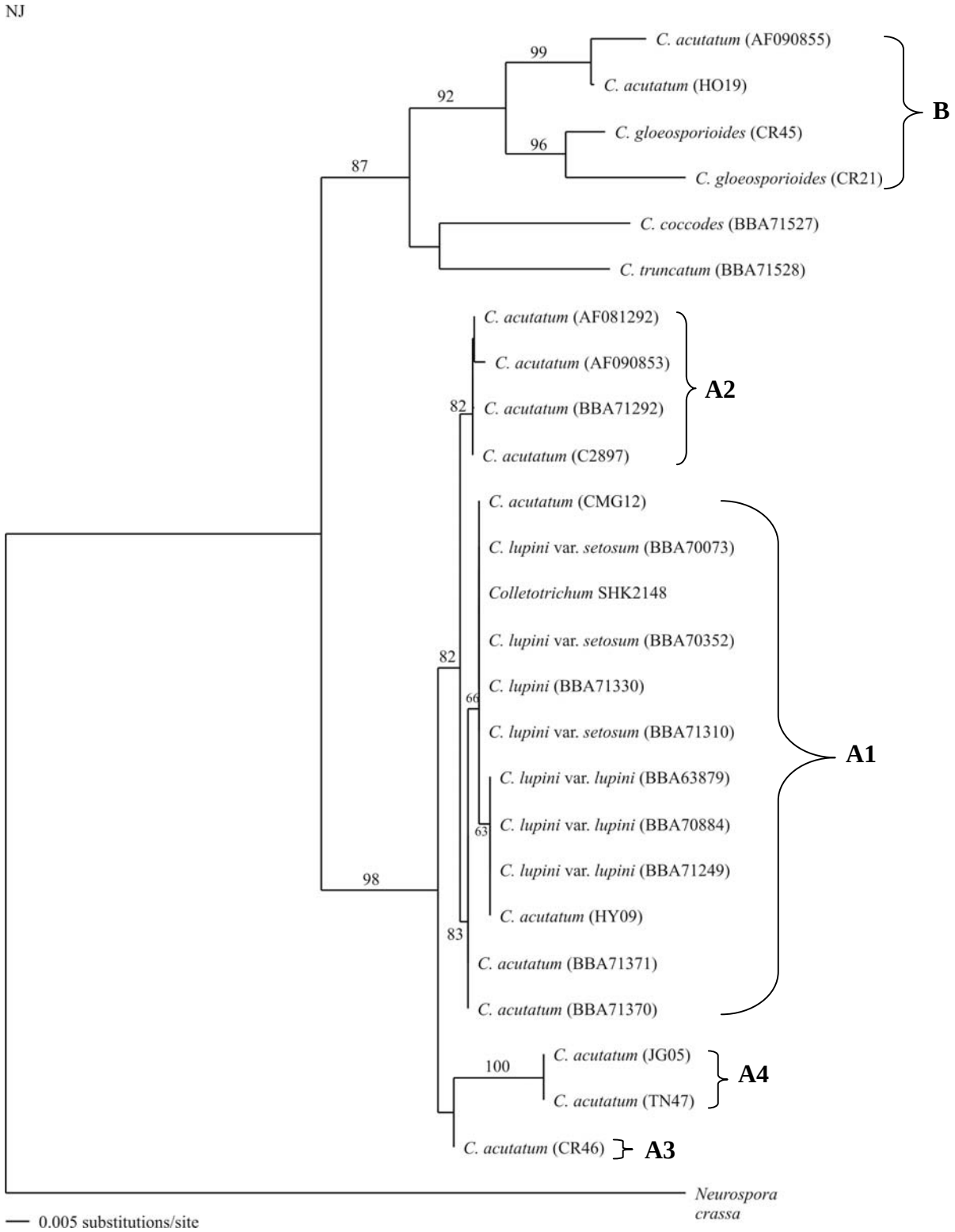


Fig. 2.16 Neighbour joining tree showing the reconstruction of the molecular phylogeny of different *Colletotrichum* isolates based on the ITS1, ITS2 and 5.8S rDNA operon. *Neurospora crassa* was included as the outgroup. (Bootstrap values are indicated on the tree). The grouping of Talhinas *et al* (2002) is indicated in brackets.

```
                                                                             1111111111111111
                                        1111111122222222233333334445678999990000000011111111
Taxon/Node                              234567890124578901234678934567890173813234560345678901234567
---------------------------------------------------------------------------------------------------
C. acutatum(PT30)                       ?????????????????????????CCTTGATACGCTCCCACTTTTCCAGTGCGGCATCC   [60]
C. acutatum(PT29)                       TTTCGATATTTACCGATCTC-GAAC...................................   [59]
C. acutatum(JR15)                       TTTCGATATTTACCGATCTC-GAAC...................................   [59]
C. acutatum(CR02)                       TTTCGATATTTACCGATCTC-GAAC...................................   [59]
C. acutatum(96A4)                       TTTCGATATTTACCGATCTC-GAAC...................................   [59]
C.lupini var setosum(CW52)              TTTCGATATTTACCGATCTC-GAAC...................................   [59]
C.lupini var lupini(CW51)               TTTCGATATTTACCGATCTC-GAAC.....GC............................   [59]
Colletotrichum SHK2148                  TTTCGATATTTACCGATCTC-GAAC...................................   [59]
C. acutatum(JR03)                       TTCCGACATTTACCGATCTC-GTAC.....G...A........C........T...T...   [59]
C. acutatum(HO01)                       TTCCGACATTTACCGATCTC-GTAC.....G...A........C........T...T...   [59]
C. acutatum(CR20)                       TTCCGACATTTACCGATCTC-GTAC.....G...A........C........T...T...   [59]
C. acutatum(C2897)                      TTCCGACATTTACCGATCTC-GTAC.....G...A........C........T...T...   [59]
C. acutatum(JG05)                       TTTCGACATTTACCGATCTC-ATAC...C..C..A.....T............A..TA..   [59]
C. acutatum(CR46)                       TTTCGACATTTATCGATTTC-GTAC.....G...A.....................TG..   [59]
C. gloeosporioides(CR45)                TATC-ATACTTCGGGACGGCAATGT.GA..GA.TATCAA..TG--C.TTA.CT.T.CA.A   [57]
C. gloeosporioides(CR21)                TATC-ATACTTCGGGACGGCAATGT.GA..GA.TATCAA..TG--C.TTA.CT.T.CA.A   [57]
C. gloeosporioides(315)                 TAT--ATACTTCGGGACGGCAATGT.GA.AGAGTATC.A..TG-CCTT.TCTG.C..CAT   [57]
C. gloeosporioides (HO19)               ATATCATACTTCGGGACGGCAATGT.GA..GA.TATCAA..T--CCTT.TCTGTC..CAT   [58]
C. falcatum(CFA12)                      GATCGGTCTCGAAGACTAGATATATTACGAGC..A.C..AG.C-AC.AGA.CT..TCA.T   [59]
Neurospora crassa(M13906)               ????????????????????????????????????????????????????????????   [60]


                                        111111111111111111111111111111111111111111111111111111111222
                                        112222222222333333444444444455555666666677777778888888999000
Taxon/Node                              890123456789126789012345678901579012456901345791245678478067
----------------------------------------------------------------------------------------------------
C. acutatum(PT30)                       TCATGGACCCAGCAAATCACACCACAGGTACGACTTCGATCCGCTGGGCTGAGCTCACTT   [120]
C. acutatum(PT29)                       ............................................................   [119]
C. acutatum(JR15)                       ............................................................   [119]
C. acutatum(CR02)                       ............................................................   [119]
C. acutatum(96A4)                       ............................................................   [119]
C.lupini var setosum(CW52)              ............................................................   [119]
C.lupini var lupini(CW51)               ...................T........................................   [119]
Colletotrichum SHK2148                  ............................................................   [119]
C. acutatum(JR03)                       C.G................T....T.............................C.....   [119]
C. acutatum(HO01)                       C.G................T....T.............................C.....   [119]
C. acutatum(CR20)                       C.G................T....T.............................C.....   [119]
C. acutatum(C2897)                      C.G................T....T.............................C.....   [119]
C. acutatum(JG05)                       ..G................T..........T.......................C.....   [119]
C. acutatum(CR46)                       C........T..T....T.T............................T.....C.....   [119]
C. gloeosporioides(CR45)                .TGGT.GTTG.-.C..A.T.G-A....C......C.T.................C.....   [115]
C. gloeosporioides(CR21)                .TGGT.GTTG.-.C..A.T.G-A....C......C.T.................C.....   [115]
C. gloeosporioides(315)                 .G....--TTGA.C..A.T.G-A....C......C.T.................C.....   [114]
C. gloeosporioides (HO19)               .GG...--TTGA.C..A.T.GA-....C......C.T.................C.....   [115]
C. falcatum(CFA12)                      .AC.CA.GTTTA.C...TT.T.A....T......C.T.................C.....   [119]
Neurospora crassa(M13906)               ?????????????GTTCGG.C.GGGGTCGG.ACACCAAGCGGAAGTTCAACTCG.AGGAA   [120]


                                        222222222222222222222222222222222222222222222222222222222333
                                        111111122222223333334444455555566666777777788888889999999000
Taxon/Node                              234567902467891235671346701345705689013467801234580135689145
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C. acutatum(PT30)                       TATCCTGTCCCAGTTGCGGCTCTTTCGATGTCCTACCCACAGGCTCCGGAAATCGTCTCG   [180]
C. acutatum(PT29)                       ............................................................   [179]
C. acutatum(JR15)                       ............................................................   [179]
C. acutatum(CR02)                       ............................................................   [179]
C. acutatum(96A4)                       ............................................................   [179]
C.lupini var setosum(CW52)              ............................................................   [179]
C.lupini var lupini(CW51)               ............................................................   [179]
Colletotrichum SHK2148                  ............................................................   [179]
C. acutatum(JR03)                       .........................................................C..   [179]
C. acutatum(HO01)                       .........................................................C..   [179]
C. acutatum(CR20)                       .........................................................C..   [179]
C. acutatum(C2897)                      .........................................................C..   [179]
C. acutatum(JG05)                       .........T..................A............................C..   [179]
C. acutatum(CR46)                       .........................................................C..   [179]
C. gloeosporioides(CR45)                ..C.T.TA..A..A...A.AAA.....G.A...GT..TC....T...........G.CT.   [175]
C. gloeosporioides(CR21)                ..C.T.TA..A..A...A.AAA.....G.A...GT..TC....T...........G.CT.   [175]
C. gloeosporioides(315)                 ..C.T.TA.....A...A.AAA.....G.A...GT..TC....T...........G.CT.   [174]
C. gloeosporioides (HO19)               ..C.T.TA..A..A...A.AAA.....G.A...GT..TC....T...........G.CT.   [175]
C. falcatum(CFA12)                      ............TGC.....C.C..T...A..T...G.T....T.........T.C.C..   [179]
Neurospora crassa(M13906)               CG.AACAGT..GT.GTGAC.GGGGGTCT.AAG-.-G.A.ATCCACAAACTGCATC.ACTA   [178]


                                        333333333333333333333333333333333333333333334444444444444444
                                        012222233444444455555566666677777778888999990000011111266666
Taxon/Node                              771356835123457801247802368912367891356234680235656789024568
---------------------------------------------------------------------------------------------------
C. acutatum(PT30)                       TCTGACTCACGCCTCGTGCTCCTTCAGTTTCGCCCAAATCTTGCGTCGAACAACCTGCC   [240]
C. acutatum(PT29)                       ...........................................................   [239]
C. acutatum(JR15)                       ...........................................................   [239]
C. acutatum(CR02)                       ...........................................................   [239]
C. acutatum(96A4)                       ...........................................................   [239]
C.lupini var setosum(CW52)              ................................................GTT.C......   [239]
C.lupini var lupini(CW51)               ...........................................................   [239]
Colletotrichum SHK2148                  .......................................G.----------.C......   [229]
C. acutatum(JR03)                       ...........................................................   [239]
C. acutatum(HO01)                       ...........................................................   [239]
C. acutatum(CR20)                       ...........................................................   [239]
C. acutatum(C2897)                      ...........................................................   [239]
C. acutatum(JG05)                       ..................T....................G...................   [239]
C. acutatum(CR46)                       ...........................................................   [239]
C. gloeosporioides(CR45)                .T...................T.CT...C............C....T............   [235]
C. gloeosporioides(CR21)                .T...................T.CT...C............C....T............   [235]
C. gloeosporioides(315)                 .T...................T.C....C............C....T............   [234]
C. gloeosporioides (HO19)               .T...................T.CT...C............C....T........A.GT   [235]
C. falcatum(CFA12)                      .........T..T...C......C..A.C..........T...................   [239]
Neurospora crassa(M13906)               G.GCGTAAC.TT.GGC.C.AT.CGTTCCGCGCGGTCCGGAG-CGAATA...TCTTGAA.   [237]
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                          4444444444444455555555555555555555555555555555566666666666666
                          6777777899999900000000001111122222222233333999000000000011111
Taxon/Node                901245903567890123456789012380123456789012347890134567890123
-------------------------------------------------------------------------------------------------
C. acutatum(PT30)         GCCTCT-AGTGCCACA----CGACGA-TACCCTAG-AATATAAGCAAAGCTAACTTTGT   [293]
C. acutatum(PT29)         ......-.........----......-........-.......................   [292]
C. acutatum(JR15)         ......-.........----......-........-.......................   [292]
C. acutatum(CR02)         ......-.........----......-........-.......................   [292]
C. acutatum(96A4)         ......-.........----......-........-.......................   [292]
C.lupini var setosum(CW52)......-.........----......-........-.......................   [292]
C.lupini var lupini(CW51) ......-.........----......-........-.......................   [292]
Colletotrichum SHK2148    ......-.........----......-........-.......................   [282]
C. acutatum(JR03)         ......-.........----T.....-........-.....G.............G.A.   [292]
C. acutatum(HO01)         ......-.........----T.....-........-.....G.............G.A.   [292]
C. acutatum(CR20)         ......-.........----T.....-........-.....G.............G.A.   [292]
C. acutatum(C2897)        ......-.........----T.....-........-.....G.............G.A.   [292]
C. acutatum(JG05)         ......-.......-.----ATT...-...TT.T.G...GCG.A..C.A.......GT.   [290]
C. acutatum(CR46)         ......-...T---G.-----.T..A.-.C......-.G.GCG.ATG.............   [289]
C. gloeosporioides(CR45)  ......-G..T---G.-----.ACGA.-..TT.CTT--T.CC.T.ATTT.......CC--   [282]
C. gloeosporioides(CR21)  ......-G..T---G.-----.ACGA.-..TT.CTT--T.CC.T.ATTT.......CC--   [282]
C. gloeosporioides(315)   ......-G..T----GACCCGA.T..T--..T.--TTCCG.G.--TTT.......CA--   [281]
C. gloeosporioides (HO19) T.G.G.CG.G..TG.GACTG.AG.AGAC....C.AG..C..G.A.G...A.TC....TC   [295]
C. falcatum(CFA12)        ......-....T.--.TTTC.AG.C.---.....T-...GACTAT.TTA.....AGC-.   [291]
Neurospora crassa(M13906) TTGA.CACT..----------..CTCG----..-..T.T.C.GG.AGC...CTGT.CGAG   [283]

                          6666666666666666666666666666666666777777777777777777777777
                          1113333344555556667777888888899999000001222233334444445555555
Taxon/Node                4563567926034564793578123457814589012345346917893456890456 78
-------------------------------------------------------------------------------------------------
C. acutatum(PT30)         GT-CCCGGTGGCCATTACAATGCAACTTGCAAACTCGTCTTCAACCACGCCTTTCCATT   [352]
C. acutatum(PT29)         ..-.....................................................   [351]
C. acutatum(JR15)         ..-.....................................................   [351]
C. acutatum(CR02)         ..-.....................................................   [351]
C. acutatum(96A4)         ..-.....................................................   [351]
C.lupini var setosum(CW52)..-.....................................................   [351]
C.lupini var lupini(CW51) ..-.....................................................   [351]
Colletotrichum SHK2148    ..-.....................................................   [341]
C. acutatum(JR03)         ..-.T.........C.........................................   [351]
C. acutatum(HO01)         ..-.T.........C.........................................   [351]
C. acutatum(CR20)         ..-.T.........C.........................................   [351]
C. acutatum(C2897)        ..-.T.........C.........................................   [351]
C. acutatum(JG05)         ..-T.T.........C..........C..............................   [349]
C. acutatum(CR46)         ..-............C.........T.C.....................................C   [348]
C. gloeosporioides(CR45)  CC-T.T..C......C..........C........C...C..........C..T...   [340]
C. gloeosporioides(CR21)  CC-T.T..C......C..........C........C...C..........C..T...   [340]
C. gloeosporioides(315)   T.-T....C...T..C..........C........C.T.C..........C......   [339]
C. gloeosporioides (HO19) A.CT.T..C......C..........C........C...C..........C..T...   [355]
C. falcatum(CFA12)        CC-.T.........C.........................C..........C..T...   [350]
Neurospora crassa(M13906) ..ATT.AACCTG.TC.GTCCGTGTC.C.ATCTGGCG.G.G.ACGGGTTCTAA.CATGG.   [343]

                          7777777777777777777777778888888888888888888888888888888888888
                          6666777777888888899999990000011122222233333344455566666666 7
Taxon/Node                0124014578123456901245679023790120124673456783484790124567 80
-------------------------------------------------------------------------------------------------
C. acutatum(PT30)         CTCCCCAAATCTTCCATTTGCGGTACCTGCATCCTCTAATCGGTGGTCCTTTCCTCGCAG   [412]
C. acutatum(PT29)         .....................................................   [411]
C. acutatum(JR15)         .....................................................   [411]
C. acutatum(CR02)         .....................................................   [411]
C. acutatum(96A4)         .....................................................   [411]
C.lupini var setosum(CW52).....................................................   [411]
C.lupini var lupini(CW51) .....................................................   [411]
Colletotrichum SHK2148    .....................................................   [401]
C. acutatum(JR03)         ..........................C..........................   [411]
C. acutatum(HO01)         ..........................C..........................   [411]
C. acutatum(CR20)         ..........................C..........................   [411]
C. acutatum(C2897)        ..........................C..........................   [411]
C. acutatum(JG05)         ...........C................................C..........   [409]
C. acutatum(CR46)         .C.........C..............C....................T.....   [408]
C. gloeosporioides(CR45)  ...........C......C........C....T..G.....................   [400]
C. gloeosporioides(CR21)  ...........C......C........C....T..G.....................   [400]
C. gloeosporioides(315)   ...........C......C........C.......G.....................   [399]
C. gloeosporioides (HO19) ...........C......C........C....T..G.....................   [415]
C. falcatum(CFA12)        ...........C..T.............................C....T........T..   [410]
Neurospora crassa(M13906) G.GGGTTTCCAAAA.CCCATTCTAGGTGTGGATGAAACCAACT.ATAAGGAAAAAAA.CA   [403]
```

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                          8888888888889999999999999999999999999999999999999999
                          7777778889990000000011111111122222333333334444455556
Taxon/Node                123456124148023567912345678901245902345789135681247 6
-------------------------------------------------------------------------------------
C. acutatum(PT30)         GCTTTCATGTTGTTGACGGTGGAGTTCACTGGGAGCCAAATGAGTCCCTCGA  [464]
C. acutatum(PT29)         .................................................  [463]
C. acutatum(JR15)         .................................................  [463]
C. acutatum(CR02)         .................................................  [463]
C. acutatum(96A4)         .................................................  [463]
C.lupini var setosum(CW52)  ..........................??????????????????????  [463]
C.lupini var lupini(CW51)  ..............................CT.....A.C....A???  [463]
Colletotrichum SHK2148    ..........................??????????????????????  [453]
C. acutatum(JR03)         .................................................  [463]
C. acutatum(HO01)         .................................................  [463]
C. acutatum(CR20)         .................................................  [463]
C. acutatum(C2897)        .................................................  [463]
C. acutatum(JG05)         ................T................................  [461]
C. acutatum(CR46)         .........C.....................................A.  [460]
C. gloeosporioides(CR45)  ........................T.................CTGT....  [452]
C. gloeosporioides(CR21)  ........................T.................CTGT....  [452]
C. gloeosporioides(315)   ..........................................TG.....  [451]
C. gloeosporioides (HO19)  ........................T.................CTGT....  [467]
C. falcatum(CFA12)        ..........C.....T............C......T.......CTG..T.G  [462]
Neurospora crassa(M13906)  CAGGGACCA-CTAGCG.ACGCTCAAATTTGAATGCT.GGCC.G?????????  [454]
```

Fig. 2.17 Aligned nucleotide sequence of the β-tubulin region1 and β-tubulin region2 from *Colletotrichum* isolates and a *Neurospora crassa* outgroup displaying the variable positions.

Table 2.7 Percentage sequence divergence between different *Colletotrichum* isolates based on the β-tubulin region.

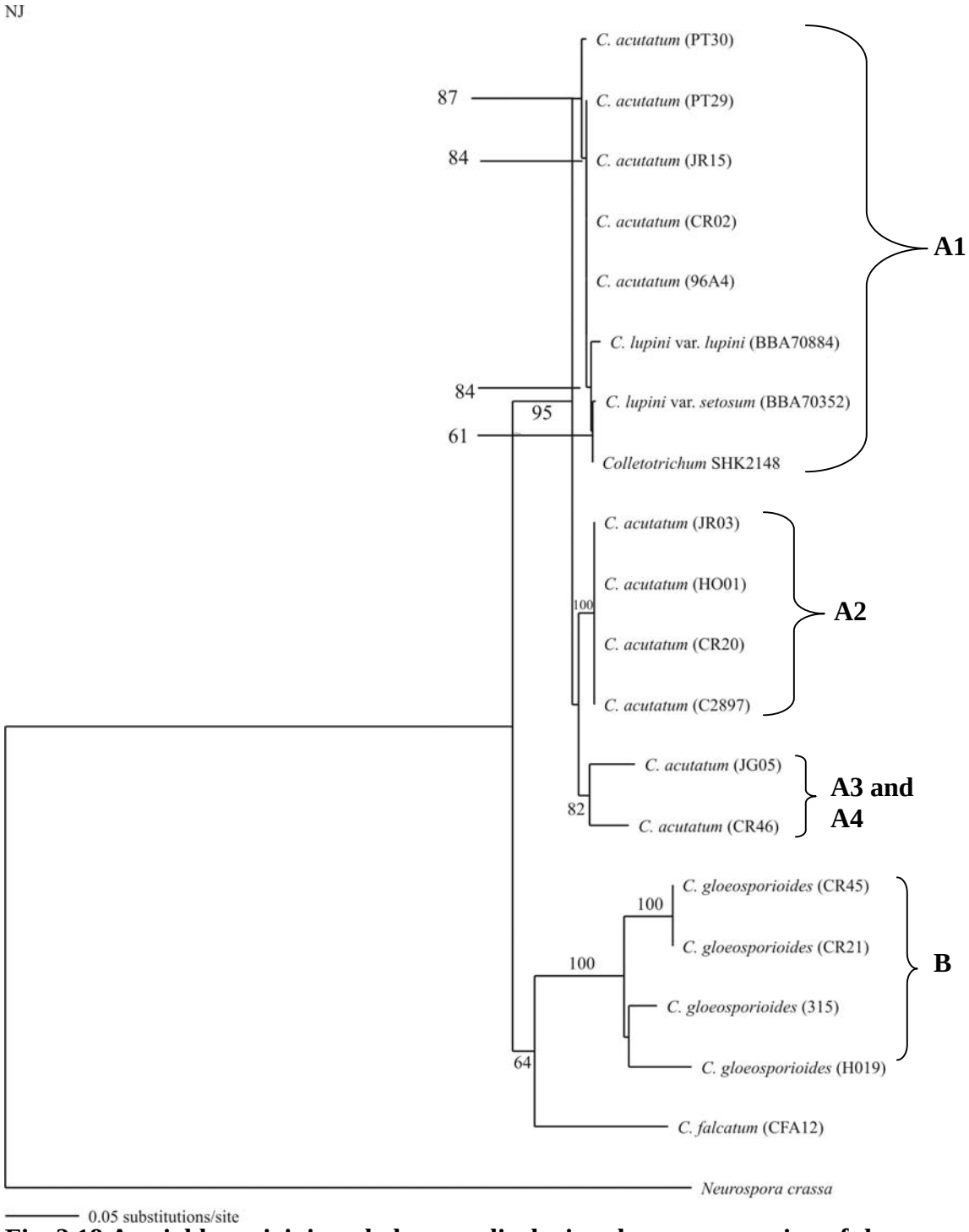


Fig. 2.18 A neighbour joining phylogram displaying the reconstruction of the molecular phylogeny of *Colletotrichum* isolates based on the β-tubulin region using *Neurospora crassa* as outgroup. (Bootstrap values are indicated on the tree). The brackets indicate the groupings from Talhinas *et al* (2002).

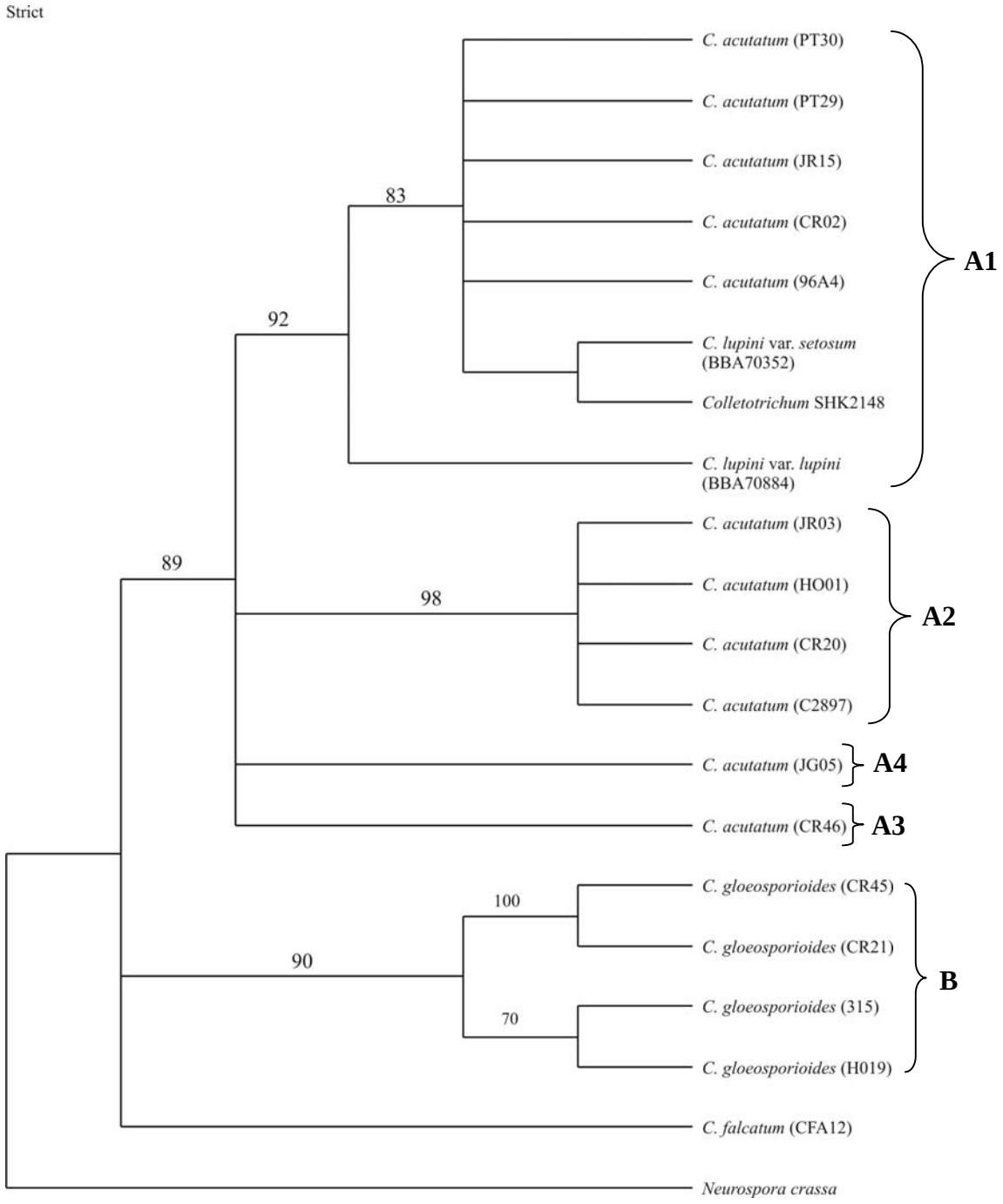


Fig. 2.19 The parsimony strict consensus tree, with bootstrap values, displays the reconstruction of the molecular phylogeny of different *Colletotrichum* isolates, based on the analysis of the β-tubulin region. (Bootsrap values are indicated on the tree).

Table 2.9 Summary of the main morphological and molecular characteristics of *Colletotrichum* SHK 2148, *C. lupini* var. *setosum* and *C. lupini* var. *lupini*.

	SHK2148	***C. lupini* var. *setosum***	***C. lupini* var. *lupini***	**Conclusion**
Colony morphology	Growth on SNA limited, more vigorous growth on and around filter paper.	Growth on SNA limited, more vigorous growth on and around filter paper.	Growth on SNA limited, more vigorous growth on and around filter paper. However, growth less than SHK 2148 and *C. lupini* var. *setosum*.	Based on the morphology and the production of setae, SHK 2148 is apparently more similar to *C. lupini* var. *setosum* than to *C. lupini* var. *lupini*
	Setae observed on PDA, grown in total darkness.	Setae not observed. Setae produced under black light (Nirenberg *et al*, 2002)	Setae not observed. No setae produced (Nirenberg *et al*, 2002)	
	On PDA: white margin with a darker centre that displays light pale olivaceous grey and light salmon coloured patches. Bottom view displays light saffron colour interrupted with small black spots	On PDA: white margin with a darker centre that displays light pale olivaceous grey patches and light salmon coloured patches. Bottom view displays light saffron colour interrupted with small black spots	On PDA: displays patches of light smoke grey and straw yellow. Bottom view a pure yellow colour is observed with no black spots.	
	Growth rate of 3mm/day	Growth rate 3mm/day	Growth rate 2.5mm/day	
Spore characteristics	**A:** 9.7 × 3.8 µm **B:** 10.2 × 3.4 µm **C:** 11.0 × 4.3 µm **D:** 10.4 × 3.5 µm	**A:** 11.3 × 4.2 µm **B:** 10.2 × 3.4 µm **C:** 11.8 × 4.1 µm **D:** 10.4 × 3.4 µm	**A:** 8.9 × 3.3 µm **B:** 10.4 × 3.5 µm **C:** 12.1 × 3.5 µm **D:** 11.6 × 3.0 µm	Spores shape as described by Nirenberg *et al* (2002). SHK 2148 contains conidia that are rounded at one end and pointed at the other like the majority of the spores of the two *C. lupini* variants. The spore sizes are smaller under growth condition A. For growth condition B, there was no difference between the isolates. For growth condition C and D it seems that the spores for isolate *C.lupini* var. *lupini* and *C.lupini* var. *setosum* are slightly larger. Nevertheless SHK 2148 are similar to *C. lupini*.
	A, B, C and D: Cylindrical conidia, pointed at one end.	**A, B, C and D:** Cylindrical conidia, pointed at one end.	**A, B, C and D:** Cylindrical conidia, pointed at one end.	
ITS and β- tubulin	In the ITS region, *Colletotrichum* SHK 2148 and *C. lupini* var. *setosum* contains the same bp (T), while *C. lupini* var. *lupini* contains a C at position 405 (Fig. 2.15). The phylogenetic trees, generated from the ITS and β-tubulin sequence data, groups *Colletotrichum* SHK 2148 with *C. lupini*, more specifically with *C. lupini* var. *setosum*.			The *Colletotrichum* SHK 2148 isolate groups closer to isolate *C. lupini* var. *setosum* than to *C. lupini* var. *lupini*

2.3 Discussion

The identity of lupin anthracnose-associated *Colletotrichum* isolates of South Africa has been re-assessed. The morphology and conidial properties as well as ITS and β-tubulin sequence analysis of *Colletotrichum* SHK 2148 were compared to two type cultures of the recently described *C. lupini* (Nirenberg *et al*, 2002).

Colletotrichum SHK 2148 and *C. lupini* var. *setosum* had very similar morphological characteristics especially those observed on PDA under normal day night rhythm; they displayed the same growth rate and mycelium colour on PDA, while *C. lupini* var. *lupini* grew slower and had a distinctly different mycelium colour. The shape and size of the conidia observed were typical of *C. lupini* (Nirenberg *et al*, 2002) and similar between the three isolates. In this study, setae have been observed for *Colletotrichum* SHK 2148 but not for the other two isolates. However it has been reported that *C. lupini* var. *setosum* produced setae (Nirenberg *et al*, 2002).

Molecular analysis was performed on the ITS1, 5.8 S rDNA and ITS4 region as well as the β-tubulin1 and β-tubulin2 regions. An alignment of the ITS sequence data of these three isolates confirmed that the two *C. lupini* variants could be distinguished from each other by a single base pair difference in the ITS2 region (Nirenberg *et* al, 2002) and indicated that *Colletotrichum* SHK 2148 contained the same base pair as *C. lupini* var. *setosum*. The ITS and β-tubulin sequence data obtained from *Colletotrichum* SHK 2148, *C. lupini* var. *setosum* and *C. lupini* var. *lupini* was phylogenetically compared to other sequences used by Talhinas *et al* (2002) and Nirenberg *et al* (2002). In both the ITS and β-tubulin analysis, *Colletotrichum* SHK 2148 grouped with the *C. lupini* isolates, more specifically with *C. lupini* var. *setosum* (Table 2.9). Furthermore the groupings of Talhinas *et al* (2002) were supported in both analyses with the *C. lupini* isolates falling into the A2 subgroup (This group correlated to the *C. gloeosporioides* VCG-2 group of Yang *et al* (1998)). From the additional data of Nirenberg *et al* (2002) it seems that the A1 group could be subdivided further into two groups corresponding to the two *C. lupini* variants, which differ by a single base pair. Interestingly Talhinas *et al* (2002) mentioned a HY09 isolate in the A2 group which differed from the other isolates in this group by one

base pair, this isolate grouped with the *C. lupini* var. *lupini* while the other representative of the A2 group CMG12 (Talhinas *et al*, 2002) grouped with *C. lupini* var. *setosum*.

From this study it could be concluded that *Colletotrichum* SHK 2148 can be classified as *C. lupini*. The analyses also indicated that *Colletotrichum* SHK 2148 is more closely related to *C. lupini* var. *setosum* and for the purpose of this study will be referred to as *C. lupini* SHK 2148. Furthermore, *Colletotrichum* SHK 1033 and *Colletotrichum* SHK 788 are very similar to *Colletotrichum* SHK 2148. This was evident from their colony morphology as well as their identical ITS sequence data (data not shown). Moreover, in a recent study, there was no significant difference observed in the overall virulence of these isolates (Koch *et al*, 2002). Thus, even though these isolates were obtained from different regions in South Africa, it is possible that the isolates spread from one region to another and that they could be identical and thus all be classified as *C. lupini*.

Finally, although the different studies used lupin anthracnose isolates from different countries that not always correlated between studies, it does appear as if all these lupin anthracnose-associated isolates from all over the world are similar. This similarity, as well as their host specificity for lupin plants, amongst them, led Nirenberg *et al* (2002) to propose a new species with two variants (of which the type culture of one of these variants –var. *lupini* is the oldest strain of *Colletotrichum* on lupins). This species is very closely related to *C. acutatum* on other hosts (Talhinas *et al*, 2002), yet displays significant differences that distinguish them from the latter species as well as other *Colletotrichum* species.

Chapter 3

Cloning and characterization of a full-length Polygalacturonase gene from *Colletotrichum lupini*

Abstract

Several plant pathogenic fungi produce endopolygalacturonases (endoPGs) to facilitate the degradation of the plant cell wall and many of the encoding genes of these enzymes have been isolated and characterized. *Colletotrichum lupini* SHK 2148 is the causal agent of lupin anthracnose in South Africa. PG activity has previously been reported for this fungus and was confirmed with an agarose diffusion assay. The highest PG activity was observed when the fungus was grown for three days on pectin. An internal fragment of a PG encoding gene was isolated from the genome of *C. lupini* SHK 2148, while inverse PCR was used to resolve the remaining sequences of the gene. The complete PG gene was subsequently isolated and characterized from genomic DNA of *C. lupini* SHK 2148. The isolated PG gene displayed typical filamentous fungal gene characteristics, it was 1153 bp in size and was interrupted by a single intron of 59 base pairs. Furthermore, the PG gene of *C. lupini* SHK 2148 was present as a single copy in the genome and had the highest similarity to the *CmpgII* and *ClpgII* genes of *Colletotrichum gloeosporioides* f.sp. *malvae* and *Colletotrichum lindemuthianum*, respectively. The encoded protein sequence of the PG gene from *C. lupini* SHK 2148 contained all four conserved regions reported for PGs. The cDNA copy of the PG gene confirmed the predicted intron position and splice sites and *in vitro* expression studies revealed that the gene was expressed after 3, 4, 5, and 7 days after inoculation in the pectin media.

3.1 Introduction

Endopolygalacturonases (EndoPGs) are ubiquitous in plants, fungi, bacteria and insects (Markovič *et al*, 2001). In these organisms they have different functions of which many have not been resolved yet. Therefore, several PG genes have been isolated and characterized to elucidate their biological function (Markovič *et al*, 2001). Characterizations of EndoPG genes from various organisms have shown that several residues are conserved amongst all PGs, while other residues are only conserved in a species relationship (Markovič *et al*, 2001).

Endopolygalacturonases are one of the first of a series of cell wall degrading enzymes (CWDEs) that are produced in fungal pathogens when the pathogen contacts the host cell wall (Albersheim and Anderson, 1971; Collmer and Keen, 1986; Cooper *et al*, 1981; Esquerré-Tugayé *et al*, 2000). Fungal EndoPGs along with some other CWDEs, are being studied for their role as pathogenicity factors. However, while several studies have addressed this issue; only pectic enzymes appear to be key role players as pathogenicity factors (reviewed by D'Ovidio *et al*, 2004). One of the aspects of EndoPGs as pathogenicity factors that has been studied, is their interaction with plant polygalacturonase inhibiting proteins (PGIPs). Plant PGIPs are leucince rich proteins that are associated with plant cell walls (De Lorenzo *et al*, 2001; De Lorenzo *et al*, 2001). PGIPs are thought to be part of the plant's defense response against pathogens, since they inhibit pathogen EndoPGs that are produced to degrade the host cell wall. Studies investigating the specific interactions between PGIPs and PGs have indicated that certain domains and residues present in both play an important role in their interaction (Federici *et al*, 2001; Di Matteo *et al*, 2003; Leckie *et al*, 2003).

Several EndoPGs have been cloned and characterized from fungi. In fungi, EndoPGs are present as gene families of various sizes, ranging from as little as one family member in *Colletotorichum gloeosporioides* f.sp. *malvae*, two members in *C. lindemuthianum* (Centis *et al*, 1996 and 1997), six members in *Botrytis cinerea* (Ten Have *et al*, 1998; Ten Have, 2000) and up to seven family members in *Aspergillus niger* (Bussink *et al*, 1990 and 1992; Pařenicová *et al*, 2000 a, b). Most of

these EndoPG genes display characteristics common to filamentous fungal genes as described by Gurr *et al* (1987).

Lupin, an important crop in the animal feed, the flower and numerous other industries, are threatened by lupin anthracnose, which is considered to be the most devastating disease of lupin world-wide. Lupin anthracnose is caused by *Colletotrichum* and has been recorded in several countries including South Africa (Koch, 1996).

The aim of this study was to first establish whether *Colletotrichum lupini* isolates obtained from lupin plants with anthracnose symptoms had PG activity. The presence of PG activity would suggest that the *Colletotrichum* isolates also contain the genes encoding these enzymes. After establishing PG activity a putative EndoPG gene was cloned from one of the *Collectotrichum* isolates. The expression of the mRNA EndoPG copy was investigated by northern and reverse transcriptase polymerase chain reaction (RT-PCR) analyses.

3.2 Materials and Methods

3.2.1 Determining PG activity

C. lupini SHK 2148 was inoculated into Czapek Dox medium (Difco, Detroit, USA) and grown for a period of 7 days. The mycelia was harvested; resuspended in pectin containing media (Appendix A), whereafter 2.5ml of the suspension was inoculated into 25ml of similar pectin-containing media. This was performed in three replicates. Mycelia and supernatant were collected on day 3, 4, 5, 6, 7 and 12 after inoculation by vacuum filtration. Protein was precipitated from the filtrate with ammonium sulphate at a final concentration of 80%. Precipitation proceeded overnight followed by a 30 minute centrifugation step at 4 °C. The pellet was resuspended in 40mM Sodium-acetate pH 5 (1/10 of the original volume). Protein concentrations were determined for a dilution series of each day of each replicate using the Bradford assay method, which is based on the binding of an acidic dye, to the basic and aromatic amino acid residues of the protein, which absorbancy is measured at 595nm. BSA was used as a standard to construct a BSA curve to facilitate protein concentration determination (Appendix C). PG activity was evaluated with the agarose diffusion assay (ADA) (Taylor and Secor, 1988). The medium consisted of 100mM citrate, 200mM Na_2HPO_4 (pH 5.3), 0.5% ammonium oxalate, 0.01% polygalacturonic acid (PGA) and 1% molecular grade agarose. The mixture was dissolved in a microwave, whereafter 20ml aliquots were poured into petri dishes (9cm diam.). 30µl of precipitated protein sample from each day of each replicate was pipetted into wells (punched with a #1 cork borer) in the ADA plates. The plates were incubated overnight at 25 °C, stained with 0.05% Ruthenium red (Sigma) for 1h at 37 °C whereafter the zone diameters were determined. The PG activity of each day was expressed as zone diameter/ng protein.

3.2.2 Genomic DNA extraction

Isolation of genomic DNA was performed according to the method of Raeder and Broda (1985). Mycelium-covered agar blocks were transferred to 250ml visualized flasks containing 25ml Czapek-Dox media amended with 50mg/ml Streptomycin and 50mg/ml Chloramphenicol. The flasks were shake-incubated for approximately 7

days at 25 °C and 150 rpm. Mycelium was collected on Whatman qualitative filter paper (# 1) and frozen in liquid nitrogen. Frozen mycelia were ground to a powder and transferred to 15ml PP-Tubes (Greiner Labortechnik, Germany) to which 10µl/mg genomic DNA extraction buffer (Appendix A) was added. An equal volume of phenol: chloroform: isoamylalcohol (25:24:1) was added to the suspension and mixed. The phases were separated by centrifuging for approximately 40 minutes at 10 000 rpm. The upper aqueous phase was transferred to a new sterile Polypropylene-Tube to which 20 mg/ml RNAseA (Roche Diagnostics, Mannheim, Germany) was added and tubes incubated for 10min at 37 °C. Two chloroform extractions were performed, whereafter the phases were separated during a centrifugation period of 10min at 10 000 rpm. The clear upper phase was transferred to a new Polypropylene –tube in which DNA was precipitated with 0.54 volume of isopropanol and pelleted by centrifuging for 10 min at 10 000 rpm. The pellet was washed with 70% Ethanol, centrifuged for 5 min, air-dried and resuspended in 100µl TE, pH 8. The concentration of the DNA samples was determined with a Hoefer® DyNA Quant ® 200 Fluorometer (Hoefer, Germany). The fluorometer was calibrated with 1× TNE buffer, pH 7.4 (Appendix A) containing 1µg/ml Hoechst 33258 DNA binding buffer, 100ng/µl Calf thymus DNA (Sigma) was included as a DNA standard. Alternatively, concentrations were determined using agarose gel-electrophoresis with a λ DNA standard.

3.2.3 Cloning an internal fragment of a *Colletotrichum* PG gene

PCR amplification on the internal PG gene fragment

PCR reactions were performed in a final PCR volume of 50µl, using Biotaq (1.25U/µl) (Bioline, Germany), 1.5mM $MgCl_2$, 1× PCR reaction Buffer, dNTPs (200µM of each), DNA template (30ng) and two PCR primer pairs (information of primers presented in Table 3.1); PCR primer set1 containing PG-COLL-F1 and PG-COLL-R and PCR primer set 2 containing PG-COLL-F2 and PG-COLL-R, at a concentration of 0.2µM each. PCR reactions were performed with genomic DNA from all three *Collectotrichum* isolates as DNA template. The PCR cycle were performed as previousy described (Chapter 2, section 2.2.4). PCR products were

separated on an EtBr bromide containing 1% (w/v) agarose gel (TAE) together with a λ- Pst generated molecular marker. Bands were visualized on a UV transimmulator. Where multiple bands were present, the desired band was excised and gel purified with the Gene Clean kit from Southern Cross Biotechnology, and single PCR bands were purified using the Qiagen PCR purification kit (Qiagen, Germany). The concentration of the purified samples was determined via agarose gel electrophoresis on a 1% (w/v) agarose EtBr containing gel with a λ DNA standard.

Table 3.1 Information of primers used in this chapter.

Primer	Sequence	Annealing Temp
PG-COLL-F1 PG-COLL-F2 PG-COLL-R	5' CGC GCC AGC TGC ACC TTC 3' 5' CGC GCC TCG TGC ACC TTC 3' 5' GCT GAC GCC GGA CCA CTT CCA 3'	55 °C
InversePCR_FII InversePCR_RII	5' TCCATCGGTTCCGTTGGTGGAACGTAG 3' 5' AGGTCTTCTTGGACATGGCCGTCTTG 3'	58 °C
C. lupini_Compl F C. lupini_Compl R	5' CCACGTGTTGATCACATACC 3' 5' CCCACCATCAAGCATTTAGC 3'	55 °C
C. lupini_Compl FII C. lupini_Compl RII	5' CGATGAAGTTCCTCTCCGTTGTC 3' 5' TTTAGCACTTGGCACCGGAGCCGGAAGG 3'	65 °C
C. lupini_Int F I C. lupini_Int R I	5' CCTTCGGCTACAAGGAGT 3' 5' AACGGAACCGATGGACAG 3'	55 °C
T7 SP6	5' TAATACGACTCACTATAGGG 3' 5' ATTTAGGTGACACTATAG 3'	55 °C
M13 R	5' TGAGCGGATAACAAT TTCACA CAG 3'	55 °C

Cloning and sequencing of the internal PG fragments

The purified putative internal PG fragments, obtained by PCR amplification of genomic DNA from the three *Colletotrichum lupini* islolates, SHK 788, SHK 1033 and SHK 2148, were ligated separately into pGEM-T-Easy (Promega, Madison,USA), in a 15µl reaction volume containing 1µl of T4 ligase (1U/ µl) (Roche), 40ng – 50ng of insert and 50ng pGEM-T-Easy, to construct pPGint788, pPGint1033 and pPGint2148 respectively. Ligations were incubated overnight at 5 °C. A positive control (50ng pGEM-T-Easy vector with 8ng control insert, supplied by the manufacturer) a background control (50ng pGEM-T Easy vector) as well as 50ng of a pUC plasmid were included in the transformation reactions. Competent *E. coli* JM 109 cells (Promega) were prepared according to the $CaCl_2$ method (Dugert *et al*

Ehrlich, 1974; Seideman *et al*, 1997) and transformed with pUC-vector, positive control, the background control as well as with the vector constructs containing the putative internal PG fragments (half of the volume of each ligation mix was used), by heat shocking cells for 40 sec. at 42 °C. Cells were incubated in LB for 1h at 37 °C. Transformants were plated onto Luria agar plates containing 100mg/ml Ampicillin (Sigma) as well as 40µl of 100mM IPTG and 40 µl X-gal. The plates were incubated overnight at 37 °C. Positive colonies (white) were inoculated into 5ml LB broth with 100mg/ml ampicillin, grown for 16h, whereafter plasmid DNA was isolated from the clones according to the method of Sambrook *et al* (1989). The plasmid constructs were digested in a reaction volume of 10µl using 1µl *EcoRI* (10U/µl) and 1× restriction buffer for 1h at 37 °C. The result of the restriction reaction was visualized on a 1% (w/v) TAE agarose gel.

Positive plasmid clones as well as PCR products that were not cloned, were sequenced using the PCR BigDye Terminator v 3.1 cycle sequencing kit (Applied Biosystems) and 10µM each of the universal primers T7 and SP6 (Table 3.1). All sequence reactions were performed in a 10 µl reaction volume, containing 4 µl Big Dye v 3.1, 1µl primer (10µM) and DNA template (50 –100ng). The PCR sequencing cycles consisted of a denaturation step at 96 °C for 1min., followed by 25 cycles consisting of three steps; 96°C for 10 sec., and annealing step carried out at 50°C for 5 sec. and an elongation step at 60°C for 4 min. The sequencing reactions were purified in 0.5ml Eppendorf tubes by adding 2µl NaOAc (3M, pH 4.6) and 50µl ice cold Ethanol (96%) to the sequencing mixture and centrifuging it at maximum speed for 30 minutes. The pellet was washed twice with 250µl Ethanol (70%) and air-dried. The sequencing reactions were performed in an ABI prism 3000 sequencer (ABI Advanced Biotechnological Institute, Perkin-Elmer Corporation, Foster City, USA).

3.2.4 Southern blot analysis of *Colletotrichum lupini* SHK 2148

Probe and marker labeling and quantification

The cloned internal PG fragment of SHK 788 was used for labeling with DIG, and subsequent Southern analysis. The PG fragment was first excised from pPGint788 by restriction digestion of the plasmid with 10U *EcoRI* (1µl) in 1× reaction buffer in a total a reaction volume of 25µl. The digestions were carried out at 37 °C for 1h. In total approximately 1.3ug of pPGint788 were restriction digested and run on a 1% (w/v) TAE agarose gel. The 950 bp internal PG fragments were excised from the gel and purified using the Qiagen gel purification kit. The concentration of the gel-purified sample was determined via electrophoresis with a λ DNA standard as well as the Fluorometer as previously described. For the labeling reaction approximately 360ng excised purified internal PG gene fragment and 1µg of lambda *PstI* marker were heat denatured, for 10 minutes, snap frozen on ice, whereafter 4µl of the DIG-High prime was added (DIG High Prime DNA Labeling and Detection Starter Kit I, Roche Biochemicals). The probe and marker mix were incubated overnight at 37 °C. The reaction was terminated with 2µl EDTA (0.2M, pH 8.0).

The quantity of labeled probe and molecular marker was determined by the spot test. A series of dilutions, of known DIG labeled concentrations (DIG High Prime DNA Labeling and Detection Starter Kit I, Roche Biochemicals), were prepared and 1µl of each dilution was spotted onto a nitrocellulose membrane (Hybond, Amersham Biosciences, Buckinghamshire, UK) together with 1µl of a dilution series of the samples. The DNA was fixed onto the membrane via UV-cross linking. The membrane was washed in maleic acid buffer (Appendix A) for 2min at room temperature and transferred into blocking solution (Elite 2% fat-free milk powder, added to 50ml Maleic acid buffer) for 30 min at room temperature. A 1:5000 dilution of anti-DIG-alkaline phosphatase antibiotics (Roche) was added to the blocking solution and the membrane was incubated for 30 min at room temperature. The membrane was washed twice for 15 min with washing buffer (Appendix A) and incubated for 2 minutes in detection buffer (Appendix A). The color development proceeded in the dark after the detection buffer was replaced with 10ml fresh detection buffer containing 45µl NBT and 35µl BCIP. Sterile distilled water was

added to terminate the reaction. Spot intensities were compared to determine the probe and marker concentrations.

Southern membrane preparation

Approximately 3.6µg of genomic DNA from isolate *C. lupini* SHK 2148 were each digested with 2µl *AvaI* (Roche, 5U/µl), *BamHI* (Roche, 10U/µl), *EcoRI* (Roche, 10U/µl), *EcoRV* (Roche, 10U/µl) and *HindIII* (Roche, 10U/µl) in separate reactions. The restricted DNA was separated on a 1% TAE agarose gel together with an aliquot (100ng) of the pPGint788 internal fragment construct to serve as a positive control. A non-labeled λ Pst marker was included on the gel. The gel was visualized under a UV transilluminator whereafter it was subjected to two 15 minute denaturing steps with 1.5M NaCl and 0.5M NaOH buffer, followed by two 15 minute neutralization washes with 1M Tris (pH 7.8), 1.5M NaCl. The DNA was fixed to the membrane on a UV transilluminator (Vacutec) for 5minutes.

Prehybridisation, hybridisation and detection of Southern membrane

The blot was set up according to the method in Current protocols, first described by Southern (1975). The membrane was prehybridised for 3-4 h in a 50% formamide buffer (Appendix A). Subsequently, the membrane was visualized overnight at 42 °C with the labeled probe (10ng/µl) in a LASEC hyb-oven. Following hybridization, the membrane was subjected to two stringency washes for 5 minutes each with a 2 × wash solution (2 X SSC/0.1% SDS) at room temperature and three washes for 15minutes with a 0.5 × wash solution (0.5 X SSC/0.1% SDS) at 65 °C. The membrane was rinsed in a wash buffer (Appendix A) and subjected to the blocking buffer (Maleic acid buffer containing 2% fat-free milk powder) for 45min at room temperature. The antibody was added to the blocking buffer in a dilution ratio of 1: 20 000 and allowed to interact with the hybridized probe during an incubation period of 30 minutes at room temperature. The unbound antibody was removed by washing the membrane twice for 15 minutes with a washing buffer (Appendix A). The membrane was equilibrated with the detection buffer (Appendix A) for 2 minutes whereafter the color development step proceeded with the NBT/ BCIP solution (200µl/10ml Detection

buffer) in the dark for 60 hours. The reaction was terminated by the addition of distilled water.

3.2.5 Resolving the complete PG gene sequence through inverse PCR

The complete PG gene sequence was obtained through inverse PCR. A schematic representation of the inverse PCR method using genomic DNA is presented in Fig. 3.1. The inverse PCR method was done using genomic DNA of *C. lupini* isolate SHK 2148, as well as pPGint778 that was used as a positive control. The steps used in the inverse PCR method for genomic DNA and the positive plasmid control is summarized in Table 3.2.

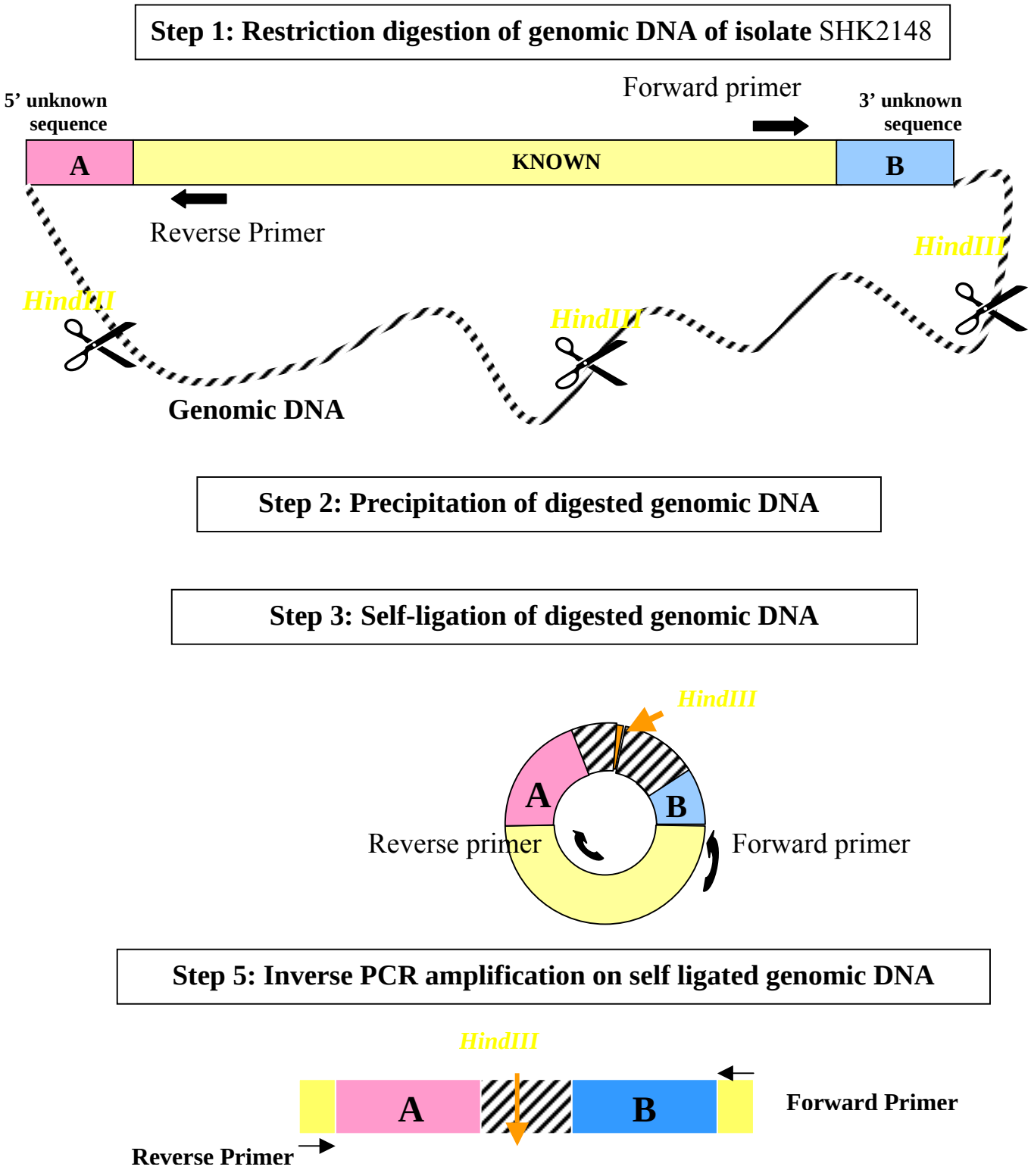


Fig. 3. 1 Schematic presentation of the inverse PCR method used for isolation of a full-length genomic PG copy from *C. lupini* SHK2148.

Table 3.2 Steps used in the inverse PCR method for isolation of the full-length genomic PG copy from *C. lupini* SHK 2148.

	Positive Control	Genomic DNA
Source	pPGint788	Genomic DNA isolated from SHK2148
Inverse PCR step1	Restrict construct with *SalI*	Restrict genomic DNA with *AvaI*, *HindIII*, *SacI* or *ClaI*
Inverse PCR step2	Precipitation of restricted DNA construct	Precipitation of DNA fractions
Inverse PCR step3	Self ligation of vector construct	Self ligation of DNA fragments
Inverse PCR step4	Linearise construct, using restriction enzyme with unique cutting site in the PG gene fragment (*BstEII*)	Bypassed this step, proceeded with the inverse PCR reaction
Inverse PCR step5	Proceed with Inverse PCR	Proceed with inverse PCR

Restriction digestion and religation of inverse PCR positive control

The vector pPGint788, was used as positive control during the inverse PCR reaction and subjected to all the steps of an inverse PCR reaction (Table 3.2). At first, an aliquot of 120ng of the positive control was restricted with 2.5µl *SalI*, which cuts outside the internal PG fragment to linearise the construct. The restriction was confirmed with agarose gel electrophoresis on a 1% (w/v) TAE agarose gel with EtBr. The restricted construct was subjected to the same clean-up procedure used for the genomic DNA samples as described by Arendse *et al.* (1999). The linearised construct (40ng) was religated overnight, using T4 Ligase (1U/µl) and an aliquot was used in the inverse PCR reaction. The rest of the religated sample was restricted with 2.5µl *BstEII*, which restricts the PG fragment only once to linearise the construct for the inverse PCR reaction. The pPGint788 positive controls were used as templates in the inverse PCR; the uncut pPGint788 template (12ng), pPGint788 *SalI* linearised and relegited (15ng) and pPGint788 *SalI* linearised, religated and *BstEII* linearised (25ng).

Restriction digestion and religation of *C. lupini* SHK 2148 genomic DNA

Genomic DNA extraction and concentration determination were performed as previously described. Approximately 10µg of genomic DNA from *C. lupini* SHK 2148 were restriction digested in a 110µl reaction volume with 1× restriction buffer

using 5 – 10U of the following enzymes that had no restriction sites in the internal PG fragment: *AvaI*, *BamHI*, *HindIII*, *SalI*, *SacI*, *EcoRV*, *ClaI* and *BssHII*. Restriction reactions were carried out at 37 °C for 1h. Aliquots of the restricted DNA samples were evaluated on a 1% agarose gel to determine if the restrictions were successful. Digested DNA samples were precipitated by using 3 M Sodium acetate (1/10 volume) and 2.5 volumes absolute Ethanol (as performed by Arendse *et al*, 1999). Restricted fragments were religated overnight, at 5 °C in a 50µl volume, using 1U of T4 DNA ligase (Roche).

Inverse PCR components and parameters

The inverse PCR reaction was set up with primers InversePCR_FII (0.2µM) InversePCR_RII (0.2µM) (Table 3.1), dNTPs (0.2mM), $mgCl_2$ (1.5mM), PCR buffer (1×) and 0.3µl Biotaq in a total volume of 50µl. Aliquots of the ligation mixes were used as template, the concentration of the DNA used in each PCR varied and were as follow:

AvaI Inverse PCR	4.8ng/µl
BamHI Inverse PCR	4.1ng/µl
ClaI Inverse PCR	13ng/µl
EcoRV Inverse PCR	2.9ng/µl
BssHI Inverse PCR	1.7ng/µl
HindIII Inverse PCR	4.4ng/µl
SalI Inverse PCR	4.8ng/µl
SacI Inverse PCR	0.7ng/µl

The inverse PCR cycle consisted of an initial denaturation step of 96 °C for 2min., followed by 25 cycles that each contained a denaturation step, 94 °C for 20 sec., an annealing step, 65 °C for 45 sec. and an elongation step of 72 °C for 45 sec. The final PCR cycle was completed with two hold steps, one at 4 °C and another final hold step at 25 °C. A small aliquot of the PCR reactions were visualized on a 1% agarose gel. PCR DNA bands were excised from the agarose gel and gel purified using the Qiagen gel purification kit. Purified fragments (50ng) were ligated overnight at 5 °C into

pGEM-T-Easy (50ng) using 1U of T4 ligase (Roche) in a 10µl reaction volume. An aliquot (2µl) of each of the ligation mixtures as well as 10ng pPGint788 were electroporated into *E. coli* DH10β in a BIORAD electroporator at 1.8kV, 25m Faradys and 200Ω. Transformants were selected as previously described.

Screening of positive transformants

Positive transformants were inoculated into 5ml LB containing 100mg/ml ampicillin and incubated with shaking overnight at 37 °C. Plasmid DNA was isolated as described previously. An aliquot of 28ng of the plasmid sample was restricted with *EcoRI* (1µl) in a 10µl reaction volume to verify the presence of an inverse PCR product. Uncut plasmid as well as the restriction reaction was electrophoresed on a 0.8% agarose gel. The cloned inverse PCR fragment was sequenced with T7, SP6 and M13 primers (10µM each, Table 3.1) using the Big Dye system as described before.

3.2.6 Isolation and characterization of the complete PG gene using sequence data obtained from inverse PCR analyses

The inverse PCR data were used to compile a composite PG gene sequence. This sequence was used to design primers for the isolation of the complete PG gene from the genome of isolate *C. lupini* SHK 2148 using 10ng of template together with primer pair, C. lupini_Compl F and C. lupini_Compl R (Table 3.1) at a final concentration of 1µM, 1.5mM $mgCl_2$, 0.2mM dNTPs and 0.2µl Biotaq. The cycle included a 94 °C denaturation step for 2 minutes, followed by a three-step cycle, consisting of 94 °C for 20 sec., 55 °C for 45 sec. and 72 °C for 45 sec., this cycle was repeated thirty-three times. A final elongation step at 72 °C for 4 minutes completed the PCR step. 10µl of each reaction were electrophoresed on an EtBr containing, 1% (w/v) TAE agarose gel. The PCR bands were excised and gel purified as described previously. The purified fragment's concentration was determined whereafter it was cloned into pGEM-T-Easy as previously described using approximately 50ng of purified insert, 50ng pGEM-T-Easy and 1µl T4 ligase (Roche, 1U/µl). The ligation reaction proceeded overnight at 5 °C. A third of the ligation solution was chemically transformed into DH5α competent cells as well as a pUC positive control (300ng) as previously described. Positive colonies were inoculated into 5ml Luria broth with

100mg/ml Ampicillin, plasmid isolations were performed after 18h of incubation using the Qiagen plasmid isolation kit. The plasmid constructs (3µl) were restricted with *EcoRI* (1µl) for 1h at 37 °C to verify the presence of an insert with the correct size. Positive clones were sequenced with T7 and Sp6 primers as described before.

3.2.7 RNA extractions

RNA was extracted from mycelia grown in pectin media for 12 days using the TRI reagent (Chomczynski *et al*, 1987) (Sigma). Approximately 50mg of fungal tissue, from day days 3, 4, 5, 6, 7 and 12 after the inoculation in the pectin media (described previously), was crushed in liquid nitrogen. TRI reagent was added to the fungal starting material (1ml per 50mg of tissue), mixed and the homogenate was centrifuged for 10 minutes, 12 000 × g at 4 °C. The clear supernatant was transferred to a sterile 15ml falcon tube (Greiner Bio-One); left for 5 minutes at room temperature whereafter chloroform was added (0.2ml/ml TRI reagent used). The samples were mixed vigorously for 15 seconds and left at room temperature for 10 minutes, samples were then centrifuged for 15 minutes at 4 °C and 12 000 × g. The resulting upper phase was transferred to a sterile falcon tube to which isopropanol (0.5ml per ml of TRI reagent used) was added. The samples were mixed and left at room temperature for 8 minutes. The RNA was precipitated by a centrifugation step of 10 minutes at 12 000 × g and 4 °C. The pellet was washed with 75% ethanol (1ml per 1ml TRI reagent used) vortexed and centrifuged for 5 minutes at 4 °C and 7500 × g. The washed RNA pellet was briefly air dried for 5 minutes and resuspended in RNAse free water. The concentration of the RNA samples was determined with a photometer (Eppendorf BioPhotometer).

3.2.8 Northern Blot analysis

Probe labeling

The complete PG gene was PCR amplified from plasmid pPGcompl1 using primers C. lupini_Compl F II and C. lupini_Compl R II (Table 3.1) (20µM of each). The PCR product was cleaned up using the Qiagen PCR clean-up kit according to manufacturer's instructions. The purified PCR product was used as a probe for the

Northern blot analysis on RNA isolated from mycelia of isolate *C. lupini* SHK 2148 grown for 12 days in pectin media. The mycelia were harvested on days 3, 4, 5,6,7 and 12 after inoculation. The probe was labelled with the HexaLabel Plus DNA labeling Kit (Fermentas Inc. 7520 Connelley drive, Unit A, Hanover, MP 21076, USA); approximately 100ng of probe was mixed with the hexanucleotide 5× reaction buffer and Millipore water in a reaction volume of 40µl. The mixture was incubated for 10 minutes in a boiling water bath and cooled on ice whereafter 4µl [α^{-32} P] labeled dATPs and 5U of Klenow fragment were added and the reaction was incubated at 37 °C for 60 minutes. The unincorporated labelled nucleotides were removed by eluting the solution through a PCR clean-up column (Qiagen) in a final volume of 50µl Millipore water using manufacturer's instructions.

Northern gel preparation and blotting

A 1.2% agarose formaldehyde (FA) gel (Appendix A) was prepared. 20µg of RNA, isolated from mycelia grown for 3, 4, 5, 6, 7 12 days in pectin containing media, was resuspended in 1 × RNA loading buffer (Appendix A), denatured at 65 °C for 10 minutes and snap frozen. RNA concentrations were determined with a spectrophotometer. The denatured RNA samples were loaded on the formaldehyde gel and run in 1 × FA gel running buffer (Appendix A) at 120V until the loading dye front was two thirds from the top of the gel. After electrophoresis, the gel was removed and washed three times for 10 minutes in DEPC treated water. The gel was then washed in 10 × SSC, pH 7 (DEPC prepared) for 45 minutes. Subsequently, RNA on the gel was transferred overnight onto a Hybond nitrocellulose membrane (Amersham) in 10 × SSC. After transfer the membrane was rinsed in 2 × SSC and cross-linked in the UV cross linker (Whitehead Scientific, Brakenfell, Cape Town, South Africa) at 0.167 joules.

Prehybridisation, hybridization and detection

The Northern membrane was prehybridised in 10ml HYB-9 hybridisation solution (Gentra system) at 65 °C for 10 minutes in the TECHNE Hybridiser HB-1D hybridization chamber hybridization oven. Salmon sperm (Sigma) was denatured for

5 minutes and immediately transferred to ice and added to the prehyb. solution to a final concentration of 100µg/ml. The prehybridisation proceeded for another hour. The probe was denatured, immediately cooled on ice and added to the prehyb. solution. The hybridisation continued overnight at 65 °C. The membrane was first washed for 20 minutes in a 2 × SSC/ 0.1% SDS solution, then for 10 minutes in a 1 × SSC/ 0.1% SDS solution and finally for 10 minutes in a 0.1× SSC/ 0.1% SDS solution. The membrane was exposed to an intensifying screen and X-ray film (Hyperfilm, Amersham Biosciences, Buckingshire, England) at –80 °C in an X-ray cassette (Amersham) for three weeks.

3.2.9 RT-PCR amplification

RNA was isolated from day 4 after inoculation, using the Qiagen RNeasy kit. From the total RNA, mRNA was isolated with the Oligotext mRNA kit (Qiagen) according to the manufacturers guidelines. Isolated mRNA samples from day 4 were used for cDNA synthesis using an M-MLV Reverse Transcriptase kit (Sigma); 1µg of mRNA was used with 10mM dNTP mix and 1µl oligo (dT) primer. The mixture was incubated at 70 °C for 10 min. and immediately cooled on ice. M-MLV Reverse Transcriptase buffer (1×), 1µl M-MLV Reverse Transcriptase (200U/µl) and 0.5µl RNAse inibitor (40U/µl) were added to the mixture. The sample was incubated at room temperature for 10min prior to the cDNA synthesis step at 37 °C for 50 min. The M-MLV Reverse Transcriptase was inactivated at 80 °C for 10 min. A PCR reaction was set up using primer set C. lupini_Compl F I and C. lupini_Compl R I (20µM of each), 10mM $MgCl_2$, 10mM dNTPs and 0.5µl Biotaq (25U/µl) with 10ng genomic DNA and 1ng pPGcompl1 as positive controls as well as 10ng cDNA template. The PCR reaction was initiated with a denaturing step at 95 °C for 5 minutes, followed by a 30 cycle repeat of a 94 °C for 1 minute, 60 °C for 1 minute and 72 °C elongation of 2 minutes steps. The cycle was finalized with an elongation step at 72 °C for 5 minutes. The result of the PCR reaction was evaluated on a 2% TAE agarose gel containing EtBr. The bands obtained were excised and gel-purified with the gel purification kit from Qiagen. The purified fragments were subjected to concentration determination and 10ng were ligated to 12.5ng pGEM-T-Easy vector

using 1U T4 ligase (Roche). The ligation mixes were transformed into *E.coli* JM 109 as described previously and selected on LB agar plates containing 100mg/ml Ampicillin (Sigma) as well as 40µl of 100mM IPTG and 40µl X-gal. Colony PCR was performed on 5 clones from each ligation mix using the PCR parameters as previously described with the primer sets C. lupini_Compl F I and C. lupini_Compl R I as well as C. lupini_Int F I and C. lupini_Int R I (20µM of each). Clones containing the desired fragments were subjected to restriction enzyme analysis with 1µl *EcoRI*, 3µl plasmid preparation in a final volume of 10µl at 37 °C for 1 hour. The restriction digestion was evaluated on an EtBr containing 2% TAE agarose gel together with *EcoRI* digested pPGcompl1. Sequence analysis was also performed on selected clones using the universal primer set T7 and SP6 as previously described.

3.3 Results

3.3.1 Polygalacturonase (PG) activity

Culture filtrates of *C. lupini* SHK 2148 grown in pectin media over time produced clear zones in the Agarose diffusion assay (Fig. 3.2). The zones indicated the presence of PG specific activity, which was expressed as zone diameter/ng protein (Fig. 3.3). The PG activity was at its highest on day 3 and 4. The activity decreased for days 5, 6, 7 and 12, however it seems that some basal level of PG activity might be present indicating either a constitutive expression of one or more PGs or different PGs being activated subsequently providing constitutive PG activity.

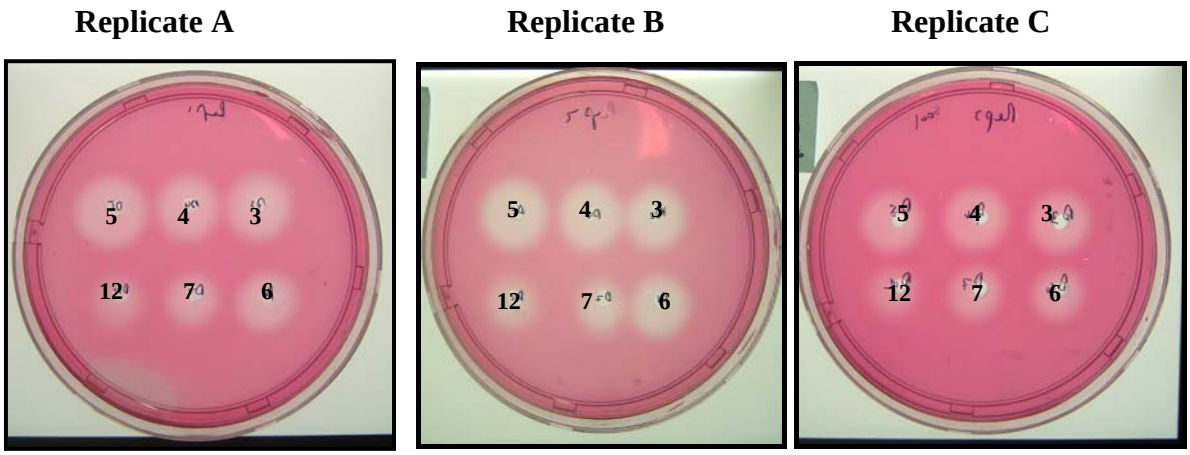


Fig. 3.2 Agarose diffusion assay displaying the PG activity obtained for three replicates A, B and C. The zones indicate PG activity (numbers within zones indicate the number of days that the isolates were grown in the pectin media).

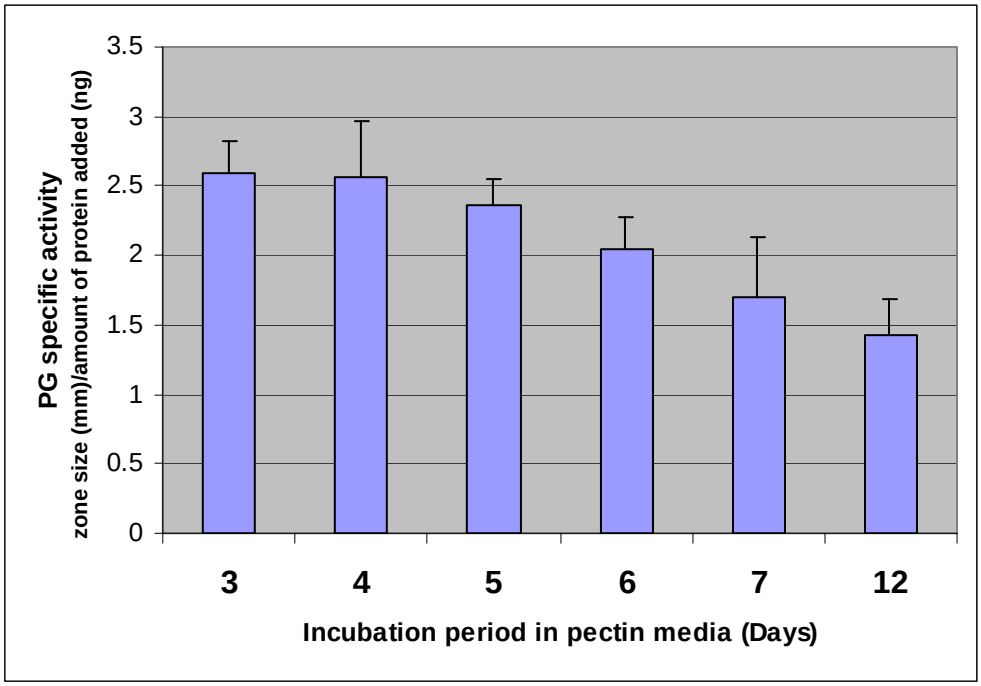


Fig. 3.3 PG specific activity of *C. lupini* SHK 2148, grown in pectin media, expressed as zone size/ amount of protein added. The PG specific activity was initially very high and decreased as the growth period was prolonged.

3.3.2 Genomic DNA Extraction

Genomic DNA was extracted from *Colletotrichum* isolates SHK788, SHK1033 and SHK2148. The concentration of the DNA samples from isolates SHK 788, 1033 and 2148 were 193ng/µl, 78ng/µl and 970ng/µl respectively, according to Fluorometer analyses.

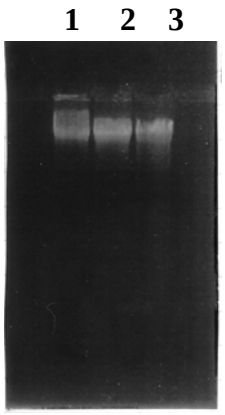


Fig. 3.4 Genomic DNA isolated from *Colletotrichum* strains. Genomic DNA isolated from *Colletotrichum* SHK 788 (lane 1), *Colletotrichum* SHK 1033 (lane 2) and *C. lupini* SHK 2148 (lane 3) was evaluated on a 1% TAE agarose gel

(approximately 5 µg of genomic DNA, isolated from *Colletotrichum* was loaded per lane).

3.3.3 PCR amplification of an internal fragment of the PG gene

PCR primers were designed from alignments of three published *Collectotrichum* PG gene sequences. The PG sequences of *Colletotrichum lindimuthianium clpg1*, *clpg2* and *Colletotrichum gloeosporioides* f. sp *Malvae cgmpg2***,** accession numbers X89370, X95457 and AJ291494 respectively, were downloaded from Genbank. The coding sequences of the PGs were translated and aligned with each other (Fig. 3.5). Three PCR primers, two forward (PG-COLL-F1 and PG-COLL-R) and one reverse primer (PG-COLL-R) (Table 3.1), were designed from conserved regions, RASCTF and WKWSGVS, within the alignment. Subsequently, these primers were for used for PCR amplification of the internal PG gene fragment from three *Colletotrichum* isolates (Fig. 3.6).

The two PCR primer sets both yielded PCR products. Internal PCR primer set 1, consisting of PG-COLL-F1 and PG-COLL-RV, resulted in non-specific amplification, while internal PCR primer set 2, PG-COLL-F2 and PG-COLL-RV, produced a single PCR fragment in all three *Colletotrichum* isolates (Fig. 3.6). The sizes of the internal PG fragments were approximately 950 bp.

Fig. 3.5 Amino acid sequence alignment of the PG genes from *C. gloeosporioides* f.sp. *malvae* (*cmpgII*) and the two PG genes from *C. lindemuthianum* (*clpgII* and *clpgI*). The blocked sequences indicate the conserved region from which the primers were designed. (The DNA sequences of *CmpgII* and *clpgII* are indicated underneath the aminoacid sequence). PG-COLL-R was designed from the *clpgII* sequence.

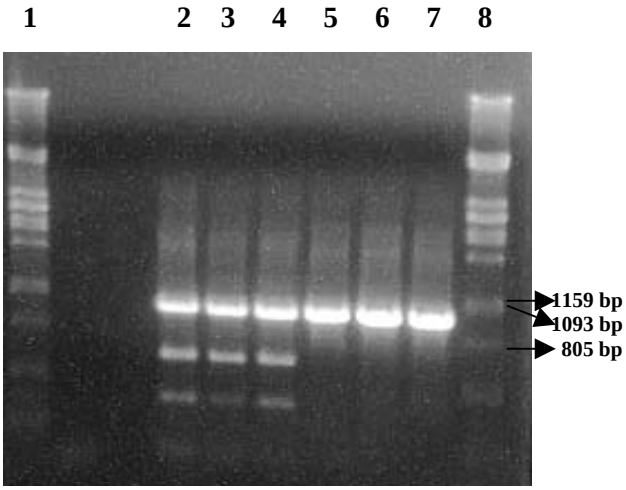


Fig. 3.6 PCR amplification of the internal PG gene fragment from *C. lupini* isolates SHK 788, SHK 1033 and SHK 2148.
Internal PG fragments isolated via PCR with internal PCR primer set 1 for isolates SHK 788, 1033 and 2148 (lanes 2-4 respectively) and the PCR fragments obtained with internal PCR primer set 2 for isolates SHK 788, 1033 and 2148 (lanes 5 –7 respectively). Lane 1 and 8 represents the λ Pst molecular marker.

The 950 bp PCR products obtained from amplifications with internal PCR primer set 2 (Fig 3.6 lanes 5,6 and 7) were successfully gel purified (Fig. 3.7).

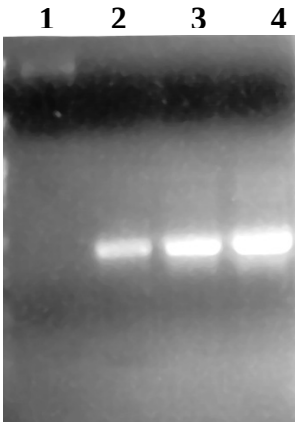


Fig. 3.7 Gel purified internal PG PCR fragments.
The purified PG internal fragment 788, (6 µl), PG internal fragment 1033 (8 µl) and PG internal fragment 2148 ((8 µl) were evaluated on a 1% TAE agarose gel (lane 2,3 and 4 respectively) to determine the quality and quantity with a λ standard 100ng (lane 1).

Ligation of the gel purified PG PCR fragments, PG internal fragment 788, PG internal fragment 1033 and PG internal fragment 2148 (Fig. 3.7) into the pGEM-T Easy vector, followed by transformation of *E. coli* with the ligation mixes, yielded two white colonies each for pPGint788 (pPGint788-1 and pPGint788-2) and pPGint2148 (pPGint2148-1 and pPGint2148-2) (Fig. 3.8) and no white colonies for pPGint1033. No colonies were observed in the background control transformation. Transformation with pUC gave a transformation result of 3.8×10^{7} cfu/ug DNA. Even though the transformation efficiency for pUC was high, only a few transformants (21 colonies) were obtained for the positive control (constructed according to the manufacturers guidelines). The low transformation efficiencies might not be a result of competency as the pUC transformation showed, but rather due to an inefficient ligation reaction, since the pUC vector was not subjected to a ligation reaction, while the others were.

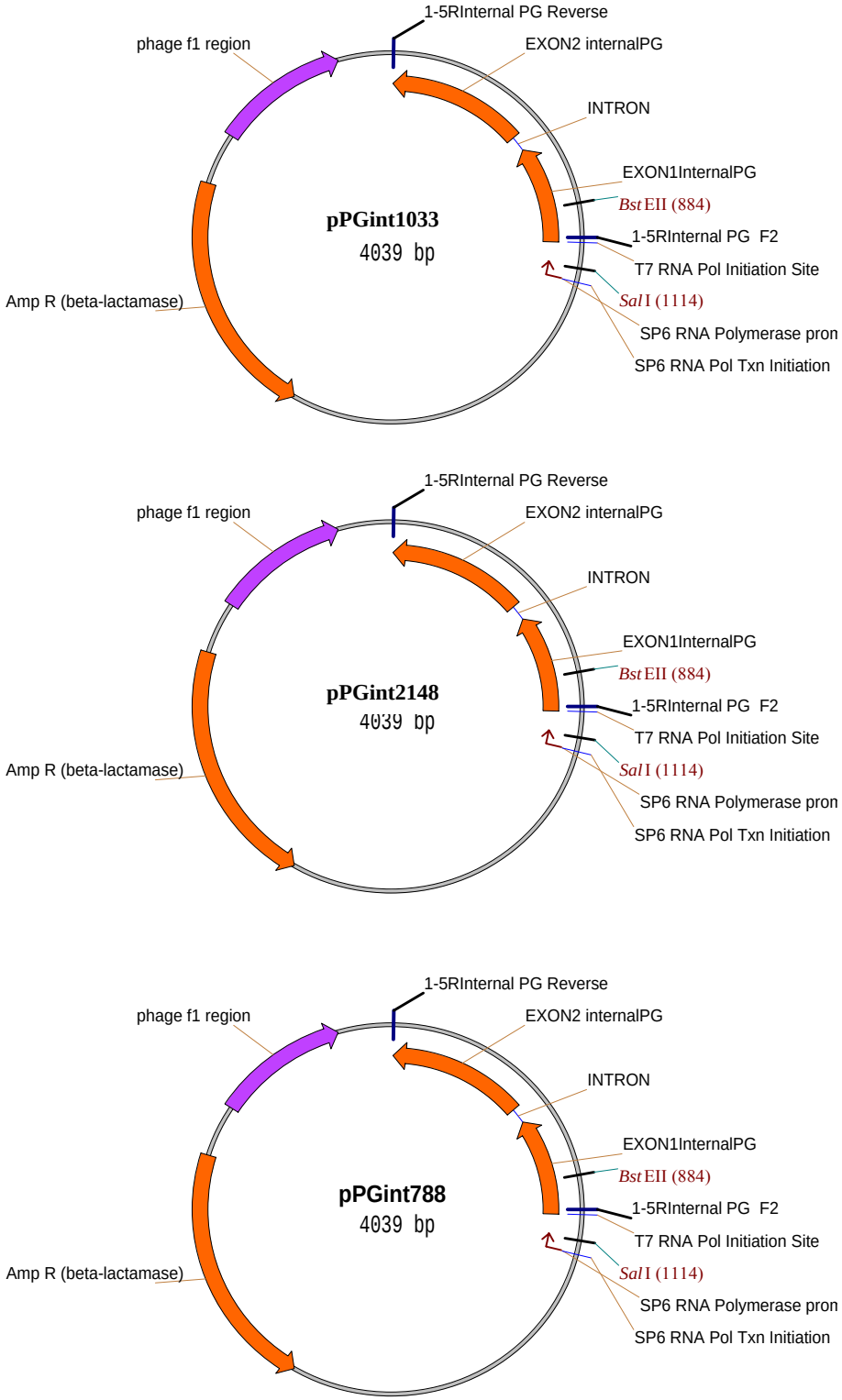


Fig. 3.8 Plasmid maps of pPGint788, pPGint1033 and pPGint2148. (Insert could be cloned in any orientation)**.**

Plasmids were extracted from positive *E. coli* clones containing pPGint788 and pPGint2148. Restriction digestion of the plasmids with *EcoRI* revealed that both clones of pPGint788 (Fig. 3.9, lanes 5 & 6) as well as both clones of pPGint2148 harbored inserts of approximately 900 bp, although the samples ran differently due to overloading (Fig. 3.9, lanes 7 & 8).

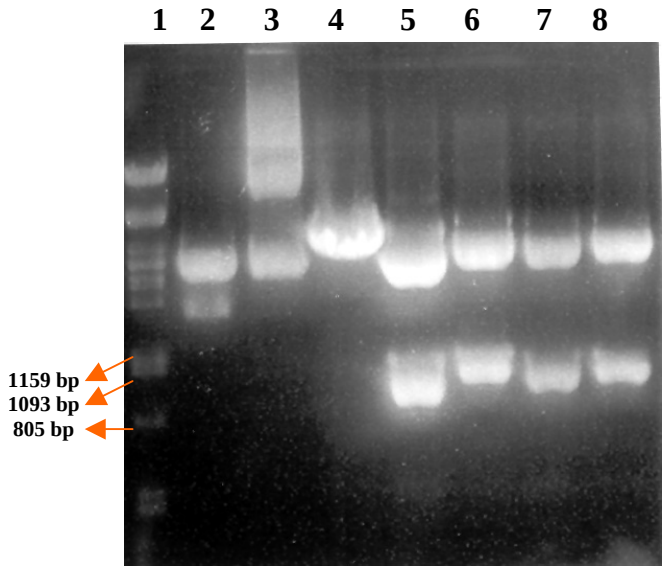


Fig. 3.9 *EcoRI* restriction analysis of both clones from pPGint788 and pPGint2148.
Uncut vectors pUC (lane 2) and pGEM-T-Easy (lane 3) were analysed on a 1% TAE agarose gel with *PstI* digested pGEM-T-Easy, (lane4), *EcoRI* digested pPGint788-1, pPGint788-2, pPGint2148-1 and pPGint2148-2 (lane 5, 6, 7 and 8 respectively). A λ *PstI* molecular marker was included in the electrophoresis (lane 1).

The putative PG fragments were sequenced directly as PCR products obtained from the genomic DNA, as well as from two pPGint788 & two pPGint2148 plasmid clones. Sequence data obtained with PCR primer set 2 were of better quality that that obtained with the first primer pair. All sequences were edited according to their relative chromatograms. Comparison of sequence data obtained directly from PCR products showed that 917 bp of the internal fragment was identical for all three isolates. This core region of 917 bp was aligned to sequence data from the plasmid clones (pPGint778 & pPGint2148) (Fig. 3.10). The alignments showed that a region of 469 bp of the cloned fragments had a good homology to the 917 bp core region. The alignment of the cloned PG sequences and PCR PG sequences differed upstream from this 469 bp region, since the cloned sequences could be more reliable, the front

part of the internal PG fragment was compiled from the alignment of the clones, using the additional sequence data (50 bp) from the forward primer (PG-COLL-F2) to the 469 bp region in the core fragment (Fig. 3.10). The end part of the internal PG fragment was obtained from pPGint788 clone2, which was the only clone providing clear downstream sequence data (48 bp) starting from the reverse primer (PG-COLL-R) to the end of core fragment (Fig. 3.10). The sequence of the compiled internal PG fragment is presented in Fig. 3.11.

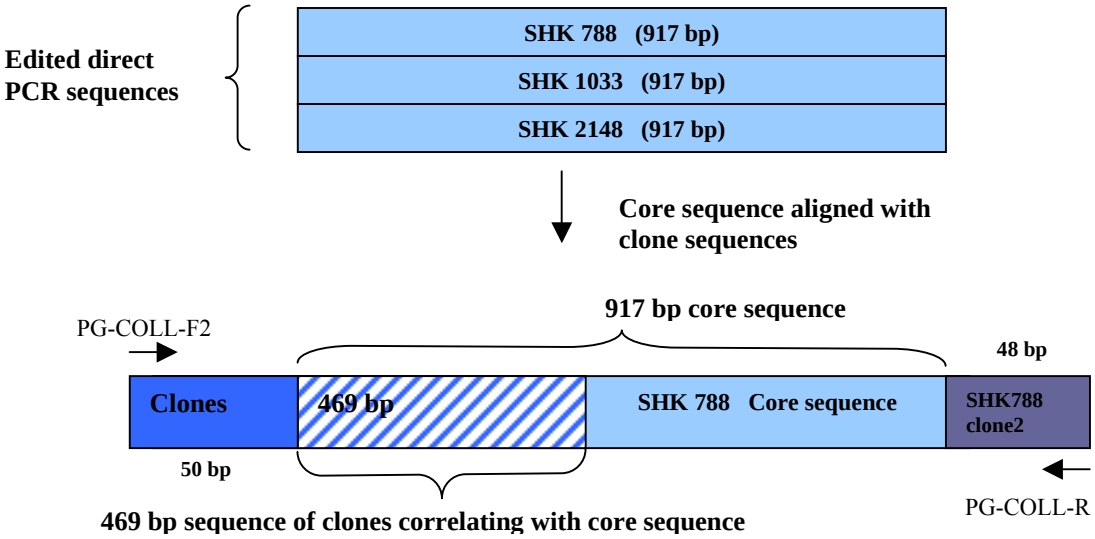


Fig. 3.10 Schematic representation of how sequence data from cloned PG fragments and directly sequenced PCR products were used to obtain the internal PG sequence.

5’ PG-COLL-F2 3’

```
   1  CGCGCCTCGT GCACCTTCAC CGACGCCAAG ACGGCCATGT CCAAGAAGAC
  51  CTCCTGCACC GACATCGTCC TCAACGGCAT CAAGGTTCCC GCCGGCGAGA
 101  CCCTCGACCT CACCGGCCTC AAGGACGGAA CCAAGGTTAC CTTCAAGGGC
 151  ACCACCACCT TCGGCTACAA GGAGTGGGAG GGCCCTCTCA TCGCCATCGG
 201  CGGTAAGAAG GTCAGCGTTG TCGGTGCTTC CGGCAACTCC ATCTCCTGCC
 251  AGGGTGAGCG CTGGTGGGAC GGCAAGGGAG GCAACGGTGG CAAGAAGAAG
 301  CCCAAGTTCT TCAAGGTCAA GATCAACGAC GGTTCCATCT CTGGCCTGAA
 351  CGTCAAGAAC ACCCCGGCCC ACGGATTCTC CATCAGCGGC GTTTCTGGCC
 401  TCAAGGTGTA AGTCACAACT CAATGTTGGG TTTTTGGACA GTAATACTGA
 451  CCCGACTATT CTCTAGCTCC AACATCCTTT TCGACAACAA GGATGGTGAC
 501  TCCAAGGGTG GCCACAACAC CGATGCCTTC GATGTCGGTA CGTCTTCTGA
 551  CGTGACCATC TCCGGCGCCA AGGTCTACAA CCAGGACGAC TGCCTGGCTA
 601  TCAACTCTGG CACCGGTATC ACTTTCGAGA ACGGATACTG CTACGGCTCC
 651  CACGGCCTGT CCATCGGTTC CGTTGGTGGA CGTAGCGACA ACACCGTCCA
 701  GAACGTCATC ATCCGTGACT CCACCATCGA GAAGGCCGAC AACGGTATCC
 751  GCATCAAGAC CATTGCCAAC AAGACTGGCA AGGTCAACGG TATCACCTTC
 801  GAGAACATCA CCCTCAAGAA CATCAACAAG AAGGGCATTG TCATCCAGCA
 851  GGATTACGAG AACGGCAGCC CTACCGGCAA GCCCACCGCT GGTATTCCCA
 901  TCACCGACGT CACCGTCAAG AACGTTAAGG GTACCGTCGC CGCGAAGGGC
 951  ACCAACGTGT ACATCCTTTG CAGCCTTTGC GCCAAGGGTG CCTGCTCCAA
1001  CTGGAAGTGG TCCGGCGTCA GC
```

PG-COLL-R (3’ ACCTTCACCAGGCCGCAGTCG 5’)

Fig. 3.11 Nucleotide sequence of compiled internal PG fragment.

3.3.4 Southern blot analysis of the PG gene from *Colletotrichum lupini*

Gel analysis showed that the genomic DNA of *C. lupini* SHK 2148 and SHK 788 used for Southern analysis were successfully digested with the various restriction enzymes, since a smear was evident after digestion (Fig. 3.12).

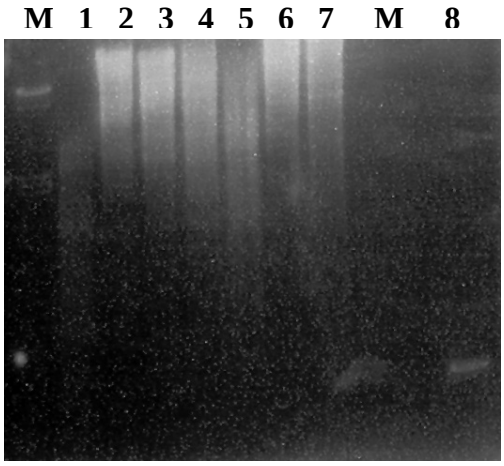


Fig. 3.12 Restriction enzyme digestion and electropoheretic separation of genomic DNA in preparation for Southern blot analysis.
Electrophoretic separation of genomic DNA of *C. lupini* SHK 2148 digested with *Ava1* (lane 1), *BamHI* (lane2)**,** *EcoRI* (lane 3), *EcoRV* (lane 4) and *HindIII*(lane 5) and separation of genomic DNA of *Colletotrichum* SHK 788 digested with *BamHI* (lane 6) and *PstI* (lane 7). As a control 100ng pPGint788 (Lane 8) was included. A λ PstI generated molecular marker (M) was electrophoresed with the samples.

Hybridization of the membrane with the internal PG fragment revealed the presence of a single band for each restriction enzyme used (Fig. 3.13). The positive control was used as a point of reference to determine the sizes of the hybridizing bands. The size of the positive control was approximately 950 bp adding to this an additional 18 bp to accommodate pGEM-T-Easy sequences added after the *EcoRI* digestion. Thus the size of the positive control was 970 bp. The predicted sized of the PG gene fragments were as follows, 1. 8 Kb for *AvaI*, 2.8 Kb for *BamHI*, 10.5 Kb for *EcoRI*, 4.9 Kb for *EcoRV*, 1 Kb for *HindIII* and 2.5 Kb for *Pst*I. Fragments from the *BamHI* generated digestion from both isolates (lanes 2 and 6) appear to be identical in size. The Southern blot provided information to facilitate the inverse PCR, for which one needs an indication of a fragment, that is not too large to amplify, yet is still large enough to provide the missing upstream and downstream sequence data. From the Southern blot results, the fragment from the *HindIII* digestion, which is approximately 1 Kb, falls into a suitable size range for the Inverse PCR. *HindIII* and *EcoRV* were chosen for the inverse PCR analysis as well as additional restriction enzymes, *ClaI*, *BssHI*, *SalI* and *SacI*, which did not digest the internal PG fragment.

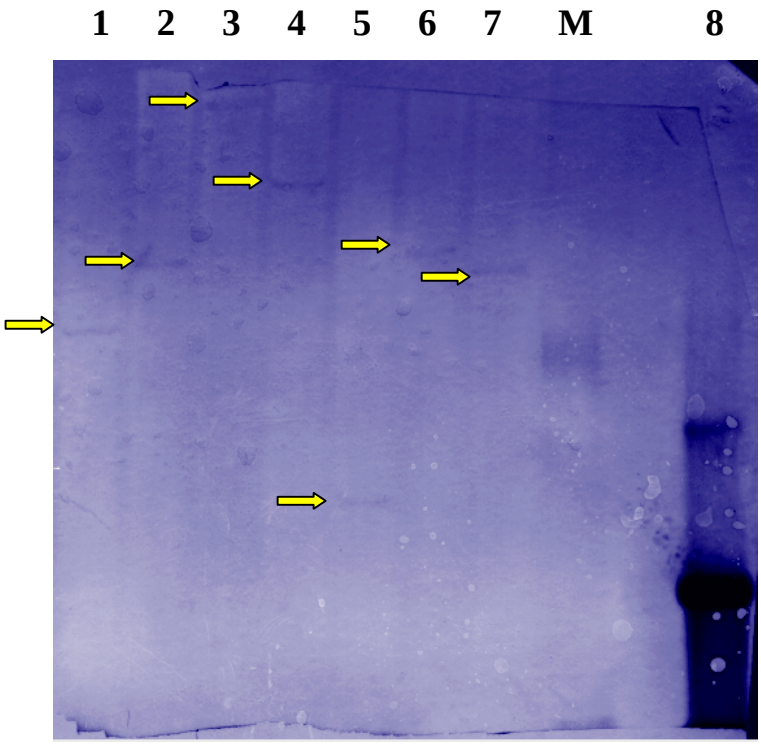


Fig. 3.13 Southern blot analysis of the PG gene from *C. lupini* SHK 2148 and *C. lupini* SHK 788.
Southern blot analysis of *C. lupini* SHK 2148 restricted with *AvaI* (lane 1), *BamHI* (lane2), *EcoRI* (lane 3), *EcoRV* (lane 4), *HindIII* (lane 5) and *C. lupini* SHK 788 restricted with *BamHI* (lane 6) and *PstI* (lane 7) using a 950 bp internal PG fragment, excised from pPGint788, as probe. The same plasmid, pPGint788, was included in the analysis as positive control (lane 8). M represents non-labeled *PstI* generated molecular marker.

Since it was reported that *C. lupini* SHK 2148 was a super virulent strain (Personal communication Dr. S. H. Koch) and that the PG gene sequences of the isolates seem to be identical, it was decided to focus mainly on *C. lupini* SHK 2148 for future research.

3.3.5 Inverse PCR

Genomic DNA Extraction and restriction enzyme digestion

Approximately 10µg of genomic DNA was available for each restriction enzyme digestion with various restriction enzymes (Fig. 3.14). Initially ligation reactions were extracted as previously described by Arendse *et al* (1999). However too much

DNA was lost during the precipitation step and this step was excluded, proceeding directly with the inverse PCR step.

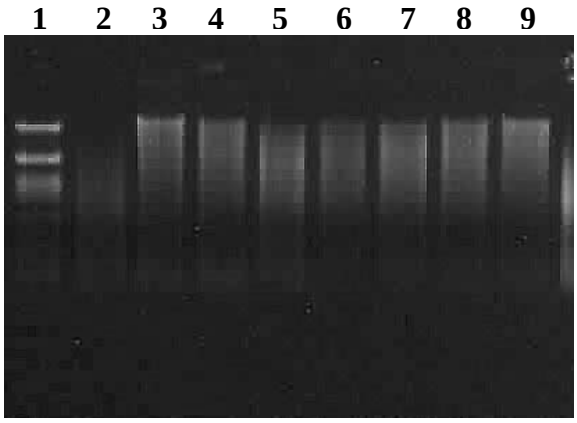


Fig. 3.14 Digestion of genomic DNA from *C. lupini* SHK 2148 in preparation for inverse PCR.
Genomic DNA of isolate *C. lupini* SHK 2148 restricted with *AvaI* (lane 2), *BamHI* (lane 3), *ClaI* (lane 4), *HindIII* (lane 5), *SalI* (lane 6), *SacI* (lane 7), *EcoRV* (lane 8), and *BssHII* (lane 9) electrophoresed with a λ*PstI* molecular marker (lane 1).

A positive control, pPGint788 containing the internal PG fragment, was subjected to all the inverse PCR steps. All three phases of the positive control gave a band signal during the inverse PCR with the primers InversePCR_FII and InversePCR_RII (Fig. 3.15, lanes 8-10), which confirm that the ligation of the genomic fragments should be successful and that the primers used in the inverse PCR were adequate to produce a PCR product. The size of the positive control included the vector size of 3015 bp and some PG gene sequence included by the inverse PCR primers, which is approximately 338 bp. Thus the expected size of the positive control was approximately 3353 bp.

Faint PCR bands were observed for *EcoRV* (Fig. 3.15, lane3), *BssHI* (Fig. 3.15, lane 4) while brighter bands were visible in the *HindIII* (Fig. 3.15, lane 5) and *ClaI* (Fig. 3.15, lane 2) inverse PCR reactions, which were approximately 1 Kb and 3 Kb respectively (Fig. 3.15). No bands were visible for the other reactions with *SalI* (lane 6) and *SacI* (Fig. 3.15, lane 7).

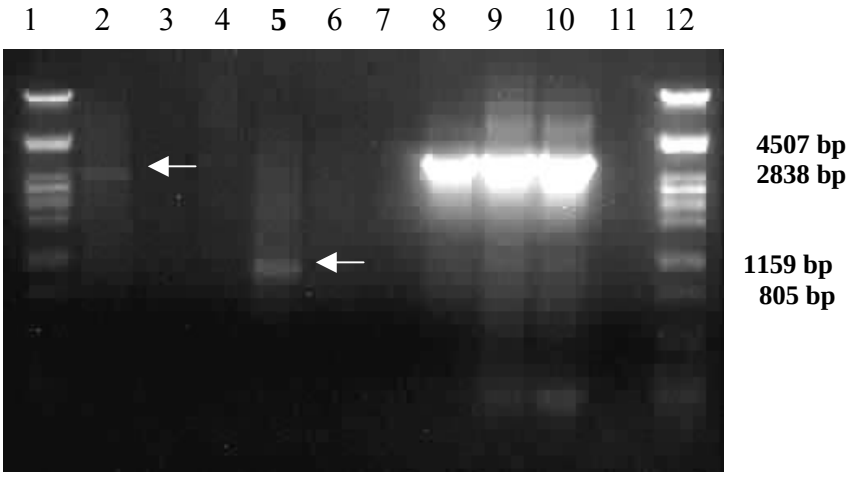


Fig. 3.15 Inverse PCR performed on religated genomic DNA fragments from *Colletotrichum* SHK2148 and pPGint788
Inverse PCR reactions, performed with ligation mixes containing religated molecules that were restricted with *ClaI* (lane 2), *EcoRV* (lane3), *BssHI* (lane 4), *HindIII* (lane 5), *SalI* (lane 6) and *SacI* (lane 7) digestions, were evaluated with agaorse gel electrophoresis. PCR amplification reactions from pPGint788 (lane 8); pPGint788 *SalI* restricted, religated and *BstEII* linearised (lane 9); pPGint788 *SalI* restricted and religated (lane 10) as well as a PCR water control (lane 11) and λ*PstI* molecular marker (lane 1 and 12) were included in the electrophoresis.

Cloning of the inverse PCR *HindIII* fragment

The gel-purified inverse PCR fragments of the *HindIII* sample were ligated into pGEM-T-Easy and electroporated into *E.coli* DH10B cells. pPGint788 was also included in the electroporation event as a positive control. The presence of a lawn of bacteria for the positive control transformation confirmed that the cells were very competent (data not presented). Several colonies were also obtained for the transformation with the *HindIII* inverse PCR-pGEM-T-Easy ligation reaction. An average of 28 colonies were obtained per plate of which approximately 72% were white colonies. Two positive controls, two negative controls and 12 white colonies were selected and inoculated for plasmid isolations and restriction enzyme digestion verification. From the 12 selected positive transformants, 7 contained the plasmid. Restriction digestion with *EcoRI* showed that one clone had the predicted (1.1 kb) sized insert (Fig. 3.16). This clone was designated pPGinvHind10, and was used for a larger scale plasmid preparation that was then sequenced.

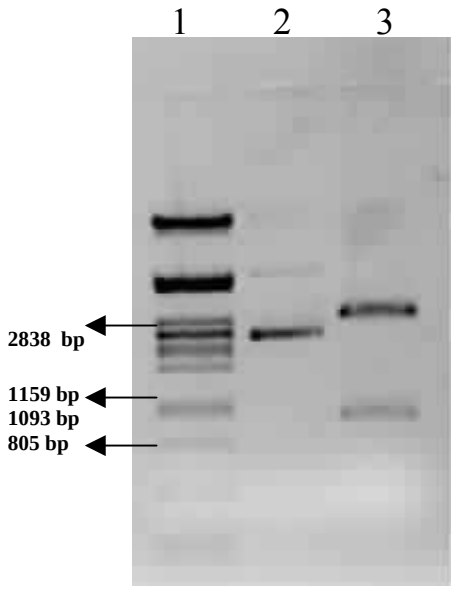


Fig. 3.16 *EcoRI* restriction analysis of plasmid pPGinvHind10
Uncut pPGinvHind10 (lane2) were separated on an agarose gel together with pPGinvHind10 restricted with *EcoRI* (lane3) from which an insert of approximately 1 Kb were excised. A λ*PstI* marker was loaded as well (lane1).

Three sequence data sets obtained from sequencing with the universal primers SP6, T7 and M13, were used to solve the sequence of the complete PG gene cloned in pPGinvHind10 (Fig. 3.17). The reverse compliment of the SP6 and M13 reverse sequence revealed the missing sequence of region A (the 5' region of the PG gene), while the T7 sequence data resolved region B (the 3' region of the PG gene) (Fig. 3.17).

In the T7 sequence data neither the sequence corresponding to the Reverse or the Forward primer could be identified with certainty, however the *HindIII* site was located and used as a point of reference. Sequence data from the start of the T7 sequence to the *HindIII* site were aligned with the internal fragment and with the PG gene sequence from *Colletotrichum gloeosporioides* f. sp *malvae* (the PG gene sequence which showed the highest similarity to the internal PG fragment of *C. lupini* SHK 2148). From the alignments the end part of the gene was resolved, the remaining 3' sequence included the TAA stop codon as well as an additional 21 bp to the *Hind III* site.

The same approach was followed to obtain the front part of the gene by using the Sp6 and M13 R sequence information. From these data, a putative *HindIII* site was determined and confirmed with alignments with a previously sequenced SP6-set1 as well as the T7 sequence. Sequence data from the reverse primer location to the putative *HindIII* site were aligned with a corresponding reverse compliment sequence of the PG gene from *Colletotrichum gloeosporioides* f. sp *malvae.* A very good alignment was obtained and the front part of the gene was resolved. In addition to the ATG start, approximately 400 bp of upstream sequence was also resolved.

Thus a complete PG gene sequence was constructed by using sequence data of B for the end part, the internal fragment (previously constructed) and the A for the front part. Fig. 3.18 represents the sequence of the compiled PG gene sequence.

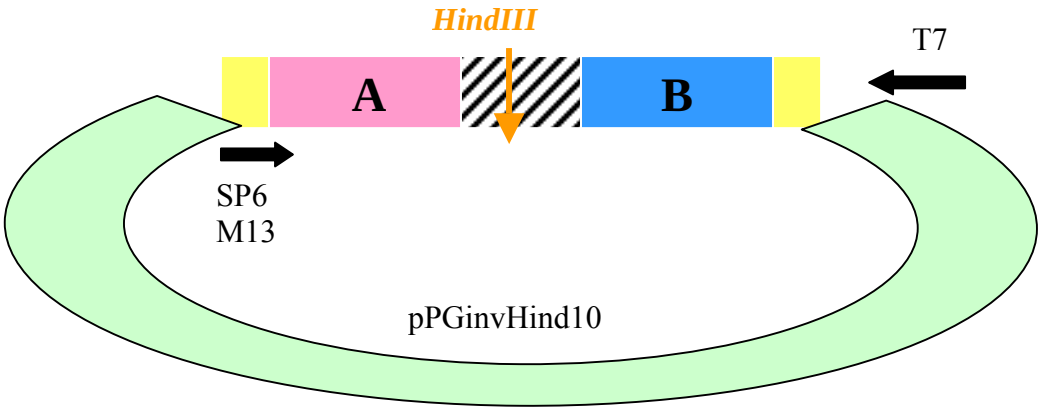


Fig. 3.17 Schematic representation of pPGinvHind10 showing primer annealing sites and the different regions that were resolved by sequence analyses.

Known region of the PG gene

Unknown front region of PG gene

Unknown end region of PG gene

PGEM-T- Easy vector

Additional genomic DNA included from the restriction reaction

HindIII restricted point – relegated

3.3.6 Isolation and characterization of the complete PG gene

PCR primers, C. lupini_Compl F and C. lupini_Compl R, were designed from the compiled PG gene sequence (Fig. 3.18). These primers amplified a product of approximately 1Kb (Fig. 3.19). The size predicted from the composite gene sequence was 1210 bp. The fragment was gel purified and cloned into pGEM-T-Easy and transformed into *E.coli* strain DH5α together with pUC DNA as a positive control. Numerous colonies were obtained for the positive control. Five clones, pPGcompl1 – pPGcompl5, were screened for the presence of the complete PG gene insert via *EcoRI* digestion (Fig. 3.20). One of these clones was sequenced with T7 and SP6 and it was revealed that the clone harboured the complete PG gene; this construct was depicted as pPGcompl1 (Fig. 3.21). Although proofreading Taq was not used for the PCR reactions, it did not seem as if mutations were introduced during subsequent PCR reactions, since the internal PG fragment, the compiled PG sequence as well as the complete PG gene sequence did not display any sequence discrepancies. The

complete PG gene sequence was aligned with the PG cDNA from *Colletotrichum gloeosporioides* f. sp *malvae* since this gene had the highest sequence similarity to the compiled PG gene (Fig. 3.22). This alignment together with the characteristics of filamentous fungal genes described by Gurr *et al* (1987) was used to characterize the complete PG gene and determine the position of a putative intron. Fig. 3.23 displays the complete nucleotide and predicted amino acid sequence of the PG gene from *C. lupini* SHK 2148. Furthermore, the nucleotide sequence of the PG gene from *C. lupini* SHK 2148 was aligned with PG genes from several other fungal sources (results not shown). As mentioned, the PG gene from *C. lupini* SHK 2148 had the highest identity to the PGs of *Colletotrichum gloeosporioides* f. sp *malvae* (*cmpgII*) and *C. lindemuthianum* (*clpgII*), 87% and 86% respectively. The intron positions of these genes were very similar, however the sequence of the introns themselves differed significantly (results not shown).

The predicted amino acid sequence of the PG gene from *C. lupini* SHK 2148 was aligned with amino acid sequences from several other fungal PG genes which was obtained from Genbank using the accession numbers provided by Markovič *et al* (2001) (Fig. 3.24); *ClpgII* and *ClpgI* (*Colletotrichum lindemutianum*), *pecB*, and *pecA* (*Aspergillus flavus*), *PgaII* (*A. niger*), *FmPG* (*Fusarium moniliforme*), *BcPG1*, *BcPG2*, *BcPG3*, *BcPG4*, *BcPG5* and *BcPG6* (*Botrytis cinerea*) and *CmpgII* (*C. gloeosporioides* f. sp. m*alvae*). The predicted PG protein had the highest homology to the *CmpgII* PG protein of *C. gloeosporioides* f. sp. m*alvae* (87. 3%) and *ClpgII* of *C. lindemutianum* (85. 5%) and contained the four domains (Fig. 3.24) that are conserved in all PGs (Markovič *et al*, 2001).

HindIII

AAGCTTAGGGGAATAAATTACCAGGGGGAGGCCTCTTTTGGTCTTGGGGAAACTCGGGGGCAGGCAAATAAGGGTTTGAATCAGGGCCTCTAGTCCTTCAAAGACGGGAGATAACAACGGATGCCGCTATCCGGGTGGAACCGCA

CCAACCCAGTTGGGGAATTCAACGAGCTTCCACAAGTAGCATCGAACGAAAGGGGAGGAGGAGGCCTAGAGTCTTGCATGTTCGA**CAAAT**TGA**TATAA**GTATCATCTTTTCTGCCCACCTCTTTTGCTTCTGTTCTTGAT

C. lupini_Compl F

ACCCCATCGTGCTTCTTCACTCACACTCTTTTCAACCACATTCTTTTTTTCCAACTCTAGTCTCTCATTCGACAATTTTTGTACATACTCGAAGTATTCCACGTGTTGATCACATCCAAAACAAATCAAGAAACG

```
 1    M   K   F   L   S   V   V   A   G   L   S   A   L   A   A   A   A   P   A   E   L   D   T   R   A   S   C   T   F   T
 1    ATG AAG TTC CTC TCC GTT GTC GCG GGC CTC AGC GCC CTG GCT GCC GCC GCC CCC GCC GAG CTC GAT ACC CGT GCC TCG TGC ACC TTC ACC
```

PG-COLL-F1 and F2

```
31    D   A   K   T   A   M   S   K   K   T   S   C   T   D   I   V   L   N   G   I   K   V   P   A   G   E   T   L   D   L
91    GAC GCC AAG ACG GCC ATG TCC AAG AAG ACC TCC TGC ACC GAC ATC GTC CTC AAC GGC ATC AAG GTT CCC GCC GGC GAG ACC CTC GAC CTC
```

InversePCR_RII

```
61    T   G   L   K   D   G   T   K   V   T   F   K   G   T   T   T   F   G   Y   K   E   W   E   G   P   L   I   A   I   G
181   ACC GGC CTC AAG GAC GGA ACC AAG GTT ACC TTC AAG GGC ACC ACC ACC TTC GGC TAC AAG GAG TGG GAG GGC CCT CTC ATC GCC ATC GGC

91    G   K   K   V   S   V   V   G   A   S   G   N   S   I   S   C   Q   G   E   R   W   W   D   G   K   G   G   N   G   G
271   GGT AAG AAG GTC AGC GTT GTC GGT GCT TCC GGC AAC TCC ATC TCC TGC CAG GGT GAG CGC TGG TGG GAC GGC AAG GGA GGC AAC GGT GGC

121   K   K   K   P   K   F   F   K   V   K   I   N   D   G   S   I   S   G   L   N   V   K   N   T   P   A   H   G   F   S
361   AAG AAG AAG CCC AAG TTC TTC AAG GTC AAG ATC AAC GAC GGT TCC ATC TCT GGC CTG AAC GTC AAG AAC ACC CCG GCC CAC GGA TTC TCC

151   I   S   G   V   S   G   L   K   V                    Putative intron of 59 bp
451   ATC AGC GGC GTT TCT GGC CTC AAG GT GTA AGT CAC AAC TCA ATG TTG GGT TTT TGG ACA GTA ATA CTG ACC CGA CTA TTC TCT AG

159   V   S   N   I   L   F   D   N   K   D   G   D   S   K   G   G   H   N   T   D   A   F   D   V   G   T   S   S   D   V
541   C TCC AAC ATC CTT TTC GAC AAC AAG GAT GGT GAC TCC AAG GGT GGC CAC AAC ACC GAT GCC TTC GAT GTC GGT ACG TCT TCT GAC GTG
```

```
189   T   I   S   G   A   K   V   Y   N   Q   D   D   C   L   A   I   N   S   G   T   G   I   T   F   E   N   G   Y   C   Y
631   ACC ATC TCC GGC GCC AAG GTC TAC AAC CAG GAC GAC TGC CTG GCT ATC AAC TCT GGC ACC GGT ATC ACT TTC GAG AAC GGA TAC TGC TAC

219   G   S   H   G   L   S   I   G   S   V   G   G   R   S   D   N   T   V   Q   N   V   I   I   R   D   S   T   I   E   K
721   GGC TCC CAC GGC CTG TCC ATC GGT TCC GTT GGT GGA CGT AGC GAC AAC ACC GTC CAG AAC GTC ATC ATC CGT GAC TCC ACC ATC GAG AAG
                          InversePCR_FII

249   A   D   N   G   I   R   I   K   T   I   N   K   T   G   K   V   N   G   I   T   F   E   N   I   T   L   K   N   I   N
811   GCC GAC AAC GGT ATC CGC ATC AAG ACC ATT AAC AAG ACT GGC AAG GTC AAC GGT ATC ACC TTC GAG AAC ATC ACC CTC AAG AAC ATC AAC

279   A   K   K   G   I   V   I   Q   Q   D   Y   E   N   G   S   P   T   G   K   P   T   A   G   I   P   I   T   D   V   T
991   GCC AAG AAG GGC ATT GTC ATC CAG CAG GAT TAC GAG AAC GGC AGC CCT ACC GGC AAG CCC ACC GCT GGT ATT CCC ATC ACC GAC GTC ACC

                                                                                                PG-COLL-R
309   V   K   N   V   K   G   T   V   A   A   G   T   N   V   Y   I   L   C   A   K   G   A   C   S   N   W   K   W   S   G
1081  GTC AAG AAC GTT AAG GGT ACC GTC GCC GCG GGC ACC AAC GTG TAC ATC CTT TGC GCC AAG GGT GCC TGC TCC AAC TGG AAG TGG TCT GGC

339   K   V   S   V   T   G   G   K   S   S   S   E   C   S   G   I   P   S   G   S   G   A   K   C   *
1171  AAG GTC TCC GTT ACC GGT GGC AAG TCC TCT TCT GAG TGC TCT GGC ATC CCT TCC GGC TCC GGT GCC AAG TGC TAA ATG CTT GAT GGT TGG GAA GCT T
                                                                                                C. lupini_Compl R      HindIII
```

Fig. 3.18 Compiled nucleotide and predicted amino acid PG gene sequence of *Colletotrichum lupini* SHK 2148. The sequence presents 420 bp of the 5' untranscribed region of the gene, the sequence of the compiled PG gene from the inverse PCR data as well as the *HindIII* site that was used during the inverse PCR reaction. The putative signal peptide of the PG gene is indicated in red, while the predicted intron is underlined and the CAAT and TATA boxes are presented in bold. Furthermore the regions from which primers were designed are highlighted.

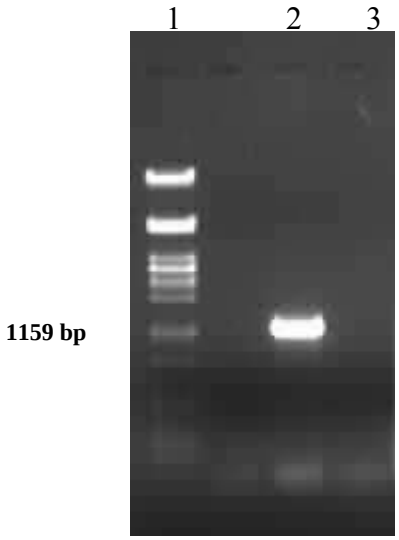


Fig. 3.19 PCR amplification of the complete PG gene from the genome of *C. lupini* SHK 2148.
A band, representing the putative complete PG gene, was amplified using primers C.lupini _Compl F, C. lupini_Compl R and genomic DNA of isolate *C. lupini* SHK 2148 (lane 2). A PCR water control (lane 3) and λ*PstI* marker (lane1) were included on the agarose gel.

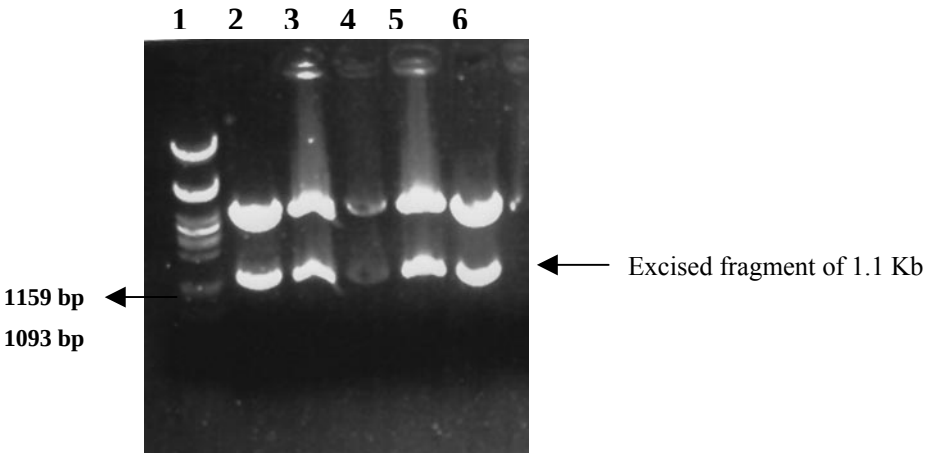


Fig. 3.20 *EcoRI* restriction analysis of pPGcompl1 – pPGcompl5, harboring the putative complete PG gene of *C. lupini* SHK 2148.
Plasmids pPGcomplete1 – pPGcomplete5 were restricted with *EcoRI* (lanes 2 – 6 respectively), revealing the presence of a 1 Kb putative complete PG gene fragment. λ*PstI* generated molecular marker (lane 1) were electrophoresed with the digested plasmids.

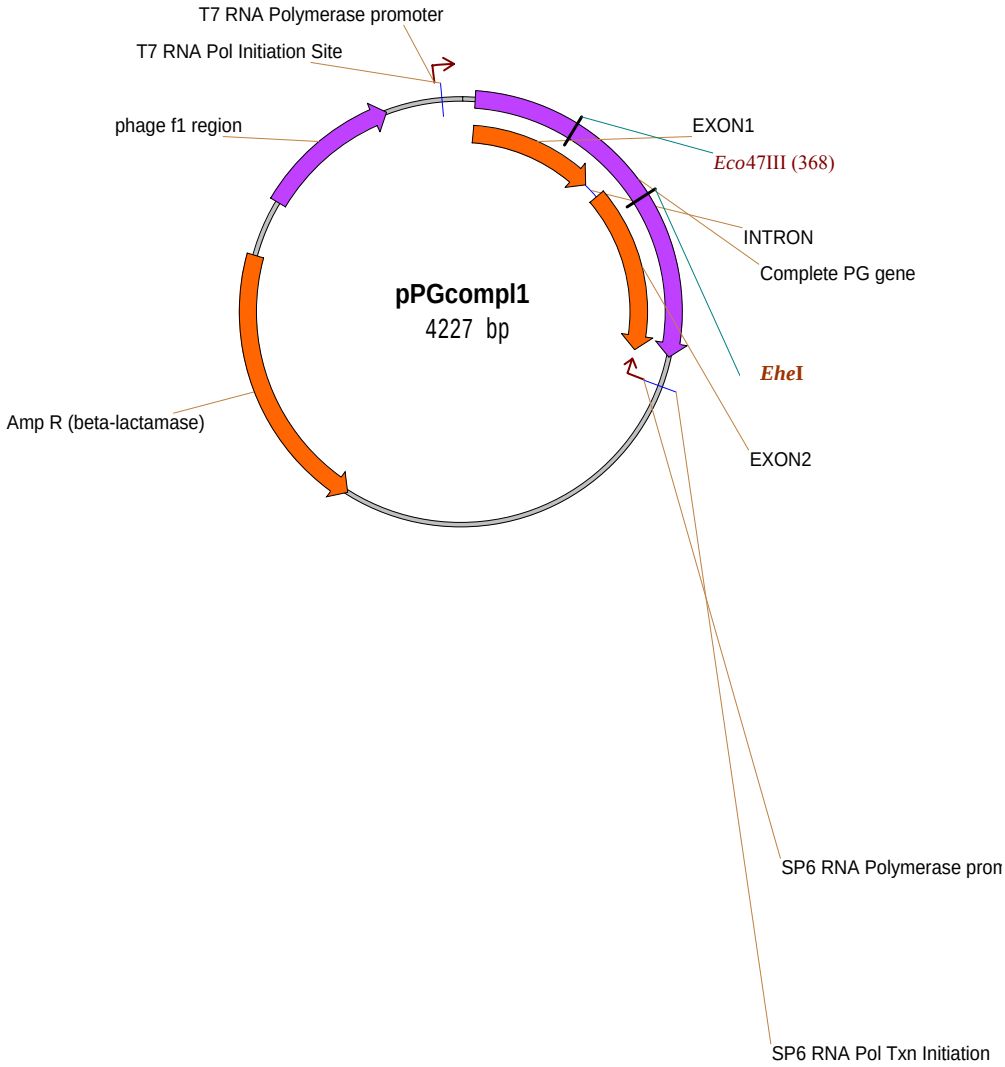


Fig. 3.21 Plasmid map of pPGcompl1 harbouring the complete PG gene.

```
PG COLLETOTRICHUM          (451) ATCAGCGGCGTTTCTGGCCTCAAGGTGTAAGTCACAACTCAATGTTGGGT
C.gloeosporioides(Malvae)(451) ATCAACAGCGTCAAGGGCCTCAAGGT----------------------

PG COLLETOTRICHUM          (501) TTTTGGACAGTAATACTGACCCGACTATTCTCTAGCTCCAACATCCTTTT
C.gloeosporioides(Malvae)(477) ----------------------------------CGTCAACGTCGAGTT

PG COLLETOTRICHUM          (551) CGACAACAAGGATGGTGACTCCAAGGGTGGCCACAACACCGATGCCTTCG
C.gloeosporioides(Malvae) (492) CAACAACAAGGACGGCGACACCAAGGGCGGCCACAACACGGACGTCTTTG
```

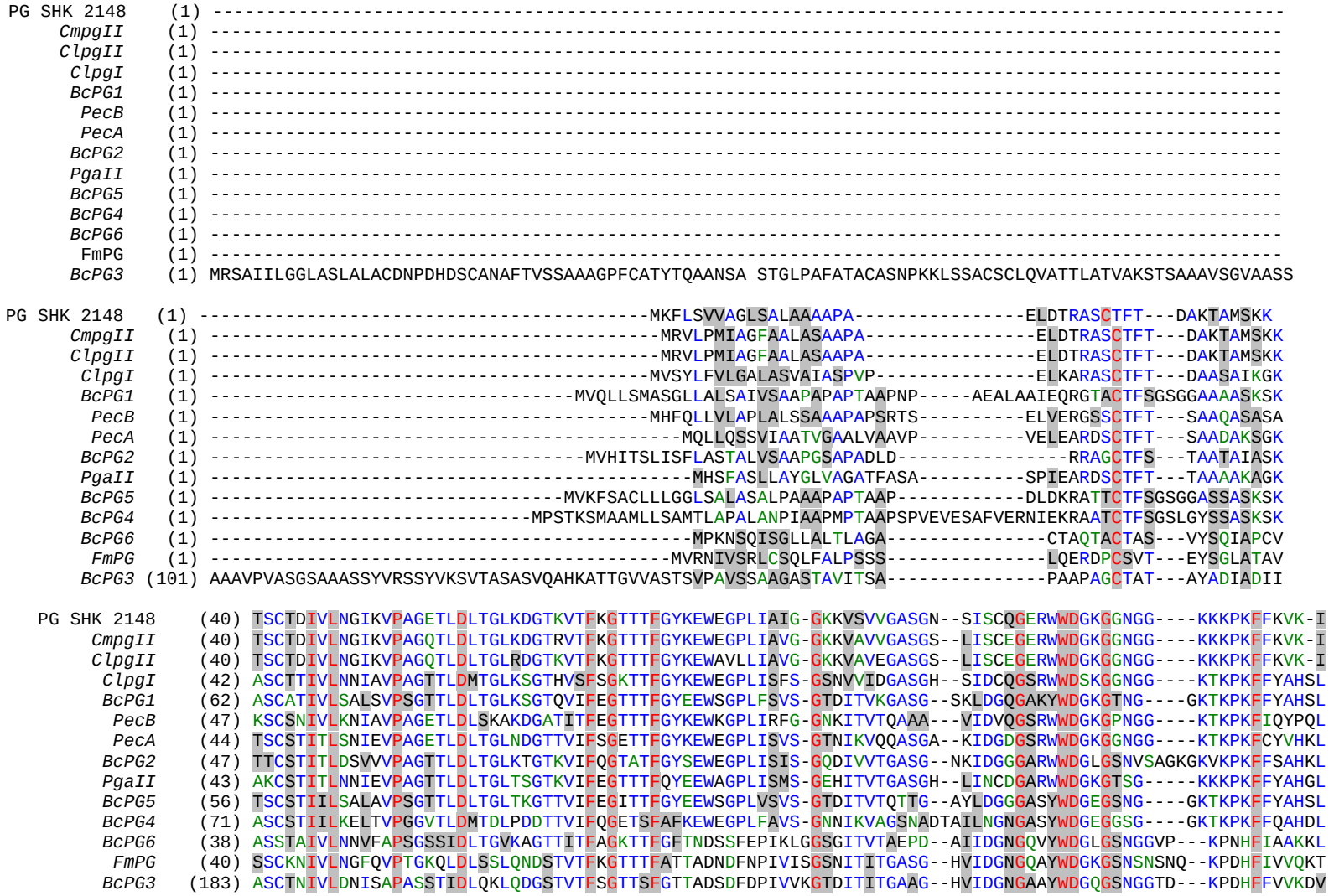
Fig. 3.22 Partial nucleotide alignment between the PG cDNA of *C. gloeosporioides* f. sp. *Malvae* and the complete PG gene cloned from genomic DNA of *C. lupini* SHK 2148. This alignment revealed a predicted intron of 59 bp with typical border sequences, indicated in red bold letters, as described by Gurr *et al* (1987).

```
 1    M  K  F  L  S  V  V  A  G  L  S  A  L  A  A  A  A  P  A  E
 1   ATG AAG TTC CTC TCC GTT GTC GCG GGC CTC AGC GCC CTG GCT GCC GCC GCC CCC GCC GAG
21    L  D  T  R  A  S  C  T  F  T  D  A  K  T  A  M  S  K  K  T
61   CTC GAT ACC CGT GCC TCG TGC ACC TTC ACC GAC GCC AAG ACG GCC ATG TCC AAG AAG ACC
41    S  C  T  D  I  V  L  N  G  I  K  V  P  A  G  E  T  L  D  L
121  TCC TGC ACC GAC ATC GTC CTC AAC GGC ATC AAG GTT CCC GCC GGC GAG ACC CTC GAC CTC
61    T  G  L  K  D  G  T  K  V  T  F  K  G  T  T  T  F  G  Y  K
181  ACC GGC CTC AAG GAC GGA ACC AAG GTT ACC TTC AAG GGC ACC ACC ACC TTC GGC TAC AAG
81    E  W  E  G  P  L  I  A  I  G  G  K  K  V  S  V  V  G  A  S
241  GAG TGG GAG GGC CCT CTC ATC GCC ATC GGC GGT AAG AAG GTC AGC GTT GTC GGT GCT TCC
101   G  N  S  I  S  C  Q  G  E  R  W  W  D  G  K  G  G  N  G  G
301  GGC AAC TCC ATC TCC TGC CAG GGT GAG CGC TGG TGG GAC GGC AAG GGA GGC AAC GGT GGC
121   K  K  K  P  K  F  F  K  V  K  I  N  D  G  S  I  S  G  L  N
361  AAG AAG AAG CCC AAG TTC TTC AAG GTC AAG ATC AAC GAC GGT TCC ATC TCT GGC CTG AAC
141   V  K  N  T  P  A  H  G  F  S  I  S  G  V  S  G  L  K  V
421  GTC AAG AAC ACC CCG GCC CAC GGA TTC TCC ATC AGC GGC GTT TCT GGC CTC AAG GT

481  GTA AGT CAC AAC TCA ATG TTG GGT TTT TGG ACA GTA ATA CTG ACC CGA CTA TTC TCT AG

161   V  S  N  I  L  F  D  N  K  D  G  D  S  K  G  G  H  N  T  D
541   C TCC AAC ATC CTT TTC GAC AAC AAG GAT GGT GAC TCC AAG GGT GGC CAC AAC ACC GAT
181   A  F  D  V  G  T  S  S  D  V  T  I  S  G  A  K  V  Y  N  Q
601  GCC TTC GAT GTC GGT ACG TCT TCT GAC GTG ACC ATC TCC GGC GCC AAG GTC TAC AAC CAG
201   D  D  C  L  A  I  N  S  G  T  G  I  T  F  E  N  G  Y  C  Y
661  GAC GAC TGC CTG GCT ATC AAC TCT GGC ACC GGT ATC ACT TTC GAG AAC GGA TAC TGC TAC
221   G  S  H  G  L  S  I  G  S  V  G  G  R  S  D  N  T  V  Q  N
721  GGC TCC CAC GGC CTG TCC ATC GGT TCC GTT GGT GGA CGT AGC GAC AAC ACC GTC CAG AAC
241   V  I  I  R  D  S  T  I  E  K  A  D  N  G  I  R  I  K  T  I
781  GTC ATC ATC CGT GAC TCC ACC ATC GAG AAG GCC GAC AAC GGT ATC CGC ATC AAG ACC ATT
261   N  K  T  G  K  V  N  G  I  T  F  E  N  I  T  L  K  N  I  N
841  AAC AAG ACT GGC AAG GTC AAC GGT ATC ACC TTC GAG AAC ATC ACC CTC AAG AAC ATC AAC
281   A  K  K  G  I  V  I  Q  Q  D  Y  E  N  G  S  P  T  G  K  P
901  GCC AAG AAG GGC ATT GTC ATC CAG CAG GAT TAC GAG AAC GGC AGC CCT ACC GGC AAG CCC
301   T  A  G  I  P  I  T  D  V  T  V  K  N  V  K  G  T  V  A  A
961  ACC GCT GGT ATT CCC ATC ACC GAC GTC ACC GTC AAG AAC GTT AAG GGT ACC GTC GCC GCG
321   G  T  N  V  Y  I  L  C  A  K  G  A  C  S  N  W  K  W  S  G
1021 GGC ACC AAC GTG TAC ATC CTT TGC GCC AAG GGT GCC TGC TCC AAC TGG AAG TGG TCT GGC
341   K  V  S  V  T  G  G  K  S  S  S  E  C  S  G  I  P  S  G  S
1081 AAG GTC TCC GTT ACC GGT GGC AAG TCC TCT TCT GAG TGC TCT GGC ATC CCT TCC GGC TCC
361   G  A  K  C  *
1141 GGT GCC AAG TGC TAA
```

Fig. 3.23 Nucleotide and predicted amino acid sequence of the complete PG gene of *C. lupini* SHK 2148. The green block indicates the initiation codon while the asterisk indicates the termination codon. A putative signal peptide is indicated in red and the predicted intron is underlined.


PG SHK 2148 (1)
CmpgII (1)
ClpgII (1)
ClpgI (1)
BcPG1 (1)
PecB (1)
PecA (1)
BcPG2 (1)
PgaII (1)
BcPG5 (1)
BcPG4 (1)
BcPG6 (1)
FmPG (1)
BcPG3 (1) MRSAIILGGLASLALACDNPDHDSCANAFTVSSAAAGPFCATYTQAANSA STGLPAFATACASNPKKLSSACSCLQVATTLATVAKSTSAAAVSGVAASS
PG SHK 2148 (1)
CmpgII (1)
ClpgII (1)
ClpgI (1)
BcPG1 (1)
PecB (1)
PecA (1)
BcPG2 (1)
PgaII (1)
BcPG5 (1)
BcPG4 (1)
BcPG6 (1)
FmPG (1)
BcPG3 (101) AAAVPVASGSAAASSYVRSSYVKSVTASASVQAHKATTGVVASTSVPAVSSAAGASTAVITSA
PG SHK 2148 (40)
CmpgII (40)
ClpgII (40)
ClpgI (42)
BcPG1 (62)
PecB (47)
PecA (44)
BcPG2 (47)
PgaII (43)
BcPG5 (56)
BcPG4 (71)
BcPG6 (38)
FmPG (40)
BcPG3 (183)

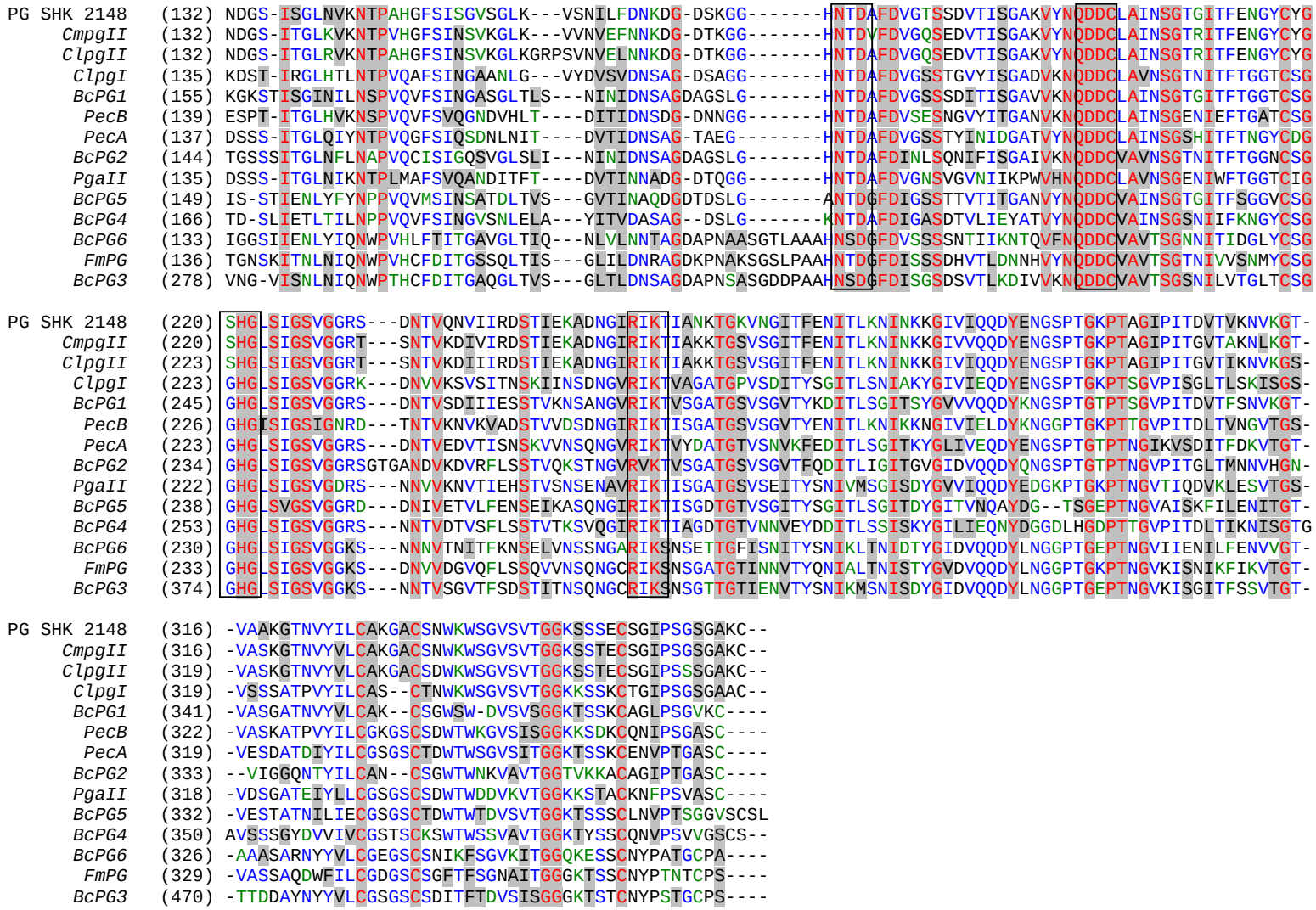


Fig. 3.24 Amino acid sequence alignment of the PG gene from *Colletotrichum* SHK 2148 with the amino acid sequences of various other fungal PG genes indicating the four conserved domains found in polygalacturonases. (The four most conserved domains, present in PGs, are indicated in blocks).

2.3.7 Northern blot analysis

Electrophoretic analyses of RNA on the gel that was used for Northern blotting showed that the RNA samples on the gel were not equally loaded, even though concentrations were determined with the spectrophotometer. Day 12 had the lowest RNA concentration and day 5 the highest according to gel analyses (Fig. 3.25). The RNA samples from days 6 and 12 seem to be slightly degraded (Fig. 3.25). Hybridization of the northern membrane with the full length cloned PG gene showed that the PG transcript was present on day 3, 4 and 7 after inoculation. The transcript was absent for days 5, 6 and 12 (Fig. 3.26).

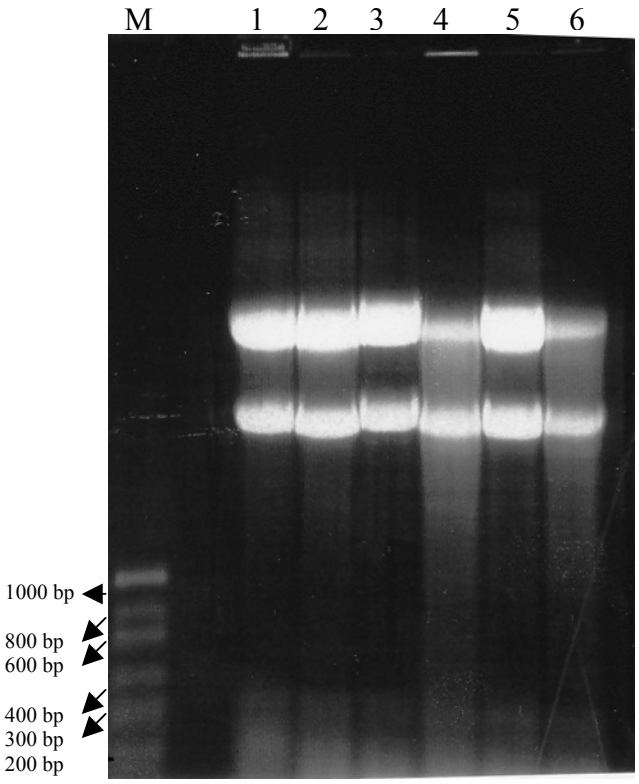


Fig. 3.25 RNA extractions of *C. lupini* SHK 2148 grown on pectin from different time periods. Total RNA extracted from mycelia of the fungus *C. lupini* SHK 2148 grown for 3, 4, 5, 6, 7 and 12 days (lanes 1, 2, 3, 4, 5 and 6 respectively) on pectin. M indicates the RNA molecular marker included on the gel.

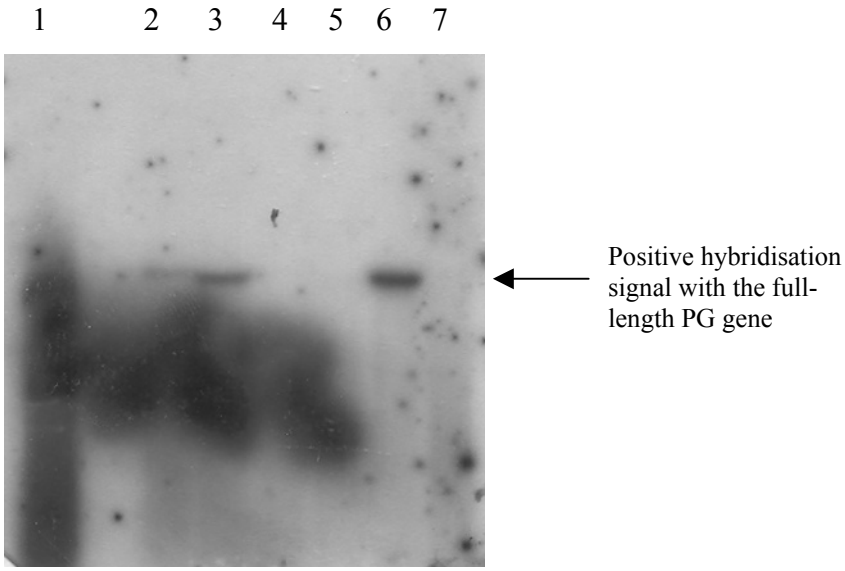


Fig. 3.26 Northern blot analysis of *C. lupini* SHK 2148 RNA samples, harvested from the fungus grown on pectin for different time periods, using the full-length PG gene as probe. Bands displayed of the full length PG gene hybridising to RNA samples obtained from mycelia of *C. lupini* SHK 2148 grown on pectin as sole carbon source for 3, 4, 5, 6, 7 and 12 days (lanes 2, 3, 4, 5, 6 and 7). A molecular marker was included in the analyses (lane1).

3.3.8 RT PCR amplification

RT-PCR analyses were done on RNA extracted from day 4 since this day displayed PG activity and expression according to the agarose diffusion assay and northern blot analyses respectively. The expected PCR product size of 1.1 kb was obtained with PCR amplification of cDNA from day 4 using primers C. lupini_Compl F I and C. lupini_Compl R I (Fig. 3.27 lane 4). A size difference of the cDNA copy vs. the genomic copy was evident on the gel (Fig. 3.27 lanes 2 and 3 vs. lane 4). The complete cDNA PG gene fragment was cloned into pGEM-T-Easy and transformed into *E. coli*. Five transformants, pPGcDNAcomplA- pPGcDNAcomplE, were subjected to colony PCR screening using both a complete PG gene primer set, C. lupini_Compl F I and C. lupini_Compl R, as well as an internal PG gene primer set, C. lupini_Int F I and C. lupini_Int R I. From the colony PCR results using both the complete primer set (Fig. 3.28) and internal primer set (Fig. 3.29), a slight size difference can be seen between the genomic and the cDNA fragments (Fig. 3.28 lane 2 vs. lanes 3 – 7 and Fig. 3.29 lane 7 vs. lanes 2- 6). One of the clones, pPGcDNAcomplA (Fig. 3.30), was subjected to restriction enzyme analysis with

EcoRI and compared to the *EcoRI* restricted genomic equavalent pPGcompl1 on a 2% TAE gel (Fig. 3.31). There is a slight size difference visible between the excised cDNA PG fragment (Fig. 3.31 lane 3) and the excised genomic PG fragment (Fig. 3.31 lane 5). Sequence analyses of pPGcDNAcomplA revealed that the size difference was due to the presence of a single intron of 59 bp, as predicted in the genomic copy that was spliced out in the cDNA copy (Fig. 3.32).

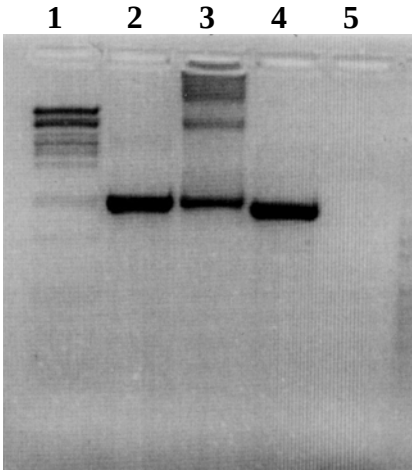
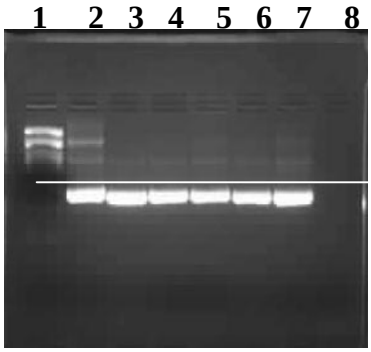


Fig. 3.27 RT-PCR analysis of *C. lupini* SHK 2148 cDNA sample grown on pectin for a time period of 4 days.
Lane 1: λPst molecular marker, genomic PG copy amplified from genomic DNA (lane 2), genomic DNA PG copy amplified from pPGcompl1 (lane 3), putative cDNA PG copy amplified from cDNA template of the Day 4 sample (lane 4) and water control (lane 5).



Line indicating size difference between the amplified genomic and cDNA PG fragments.

Fig. 3.28 Colony PCR of analyses of clones, pPGcDNAcomplA-pPGcDNAcomplE, containing the putative complete cDNA PG copy using a complete primer set.
The amplified genomic PG fragment from pPGcompl1 (lane2) was electrophoresed with the colony PCR products of clones pPGcDNAcomplA, pPGcDNAcomplB, pPGcDNAcomplC, pPGcDNAcomplD and pPGcDNAcomplE (lanes 3, 4, 5, 6 and 7 respectively). A water control (lane 8) and λPst molecular marker (lane1) were included in the electrophoresis.

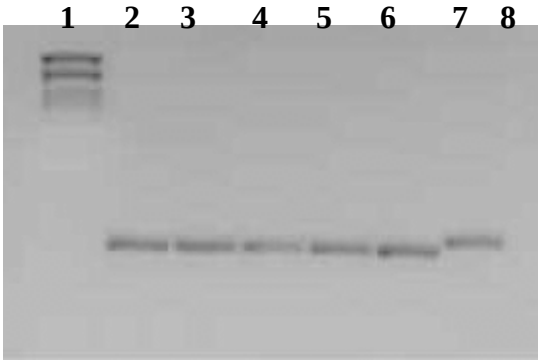


Fig. 3.29 Colony PCR of analyses of clones, pPGcDNAcomplA-pPGcDNAcomplE, containing the putative complete cDNA PG copy using an internal primer set.
The internal genomic PG fragment amplified from pPGcompl1 (lane7) were separated on an agarose gel with the colony PCR products of clones pPGcDNAcomplA, pPGcDNAcomplB, pPGcDNAcomplC, pPGcDNAcomplD and pPGcDNAcomplE (lanes 2, 3, 4, 5, and 6 respectively) as well as a water control (lane 8) and λPst molecular marker (lane1).

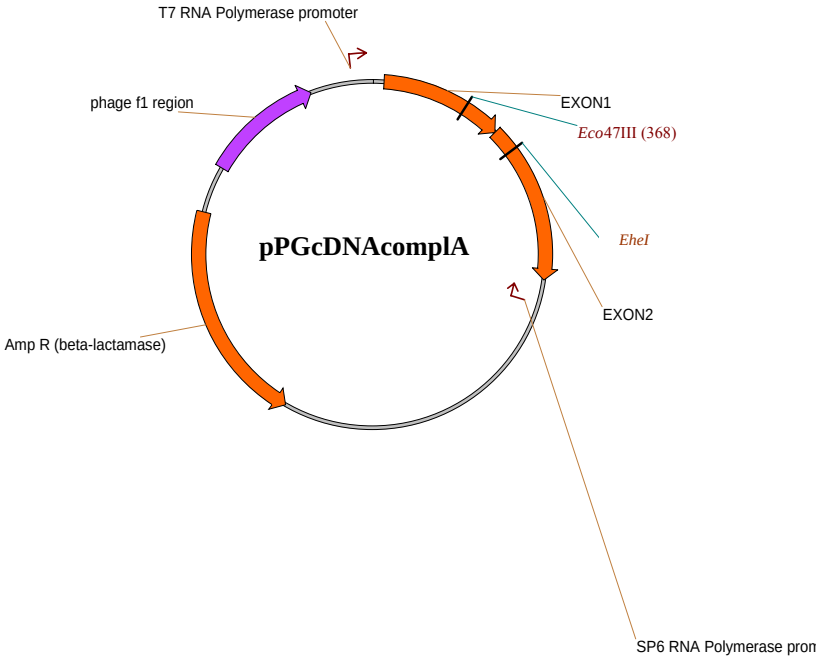


Fig. 3.30 Plasmid map of pPGcDNAcomplA, harbouring the full cDNA PG fragment, indicated by filled red arrows (Exon1 and Exon2).

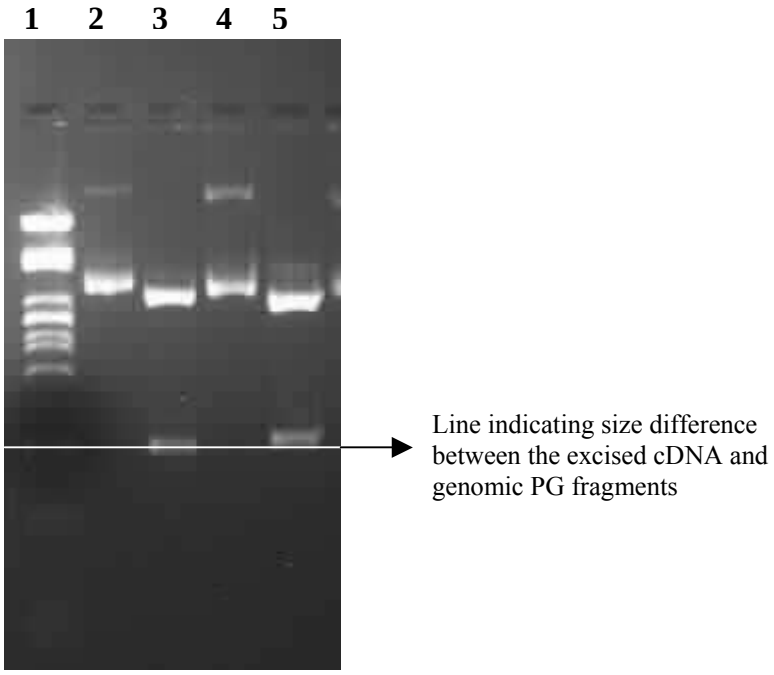


Fig. 3.31 *EcoRI* restriction analysis of pPGcDNAcomplA and pPGcompl1 on a 2% TAE agarose gel.
Unrestricted pPGcDNAcomplA and pPGcompl1 (lanes 2 and 4 respectively) were separated on a 2% agarose gel with *EcoRI* restricted pPGcDNAcomplA and pPGcompl1 (lanes 3 and 5 respectively). A λPst molecular marker was loaded in lane 1.

```
Genomic PG fragment     (451) ATCAGCGGCGTTTCTGGCCTCAAGGTGTAAGTCACAACTCAATGTTGGGT
cDNA PG fragment        (225) ATCAGCGGCGTTTCTGGCCTCAAGGT------------------------

Genomic PG fragment     (501) TTTTGGACAGTAATACTGACCCGACTATTCTCTAGCTCCAACATCCTTTT
cDNA PG fragment        (251) ----------------------------------CTCCAACATCCTTTT

Genomic PG fragment     (551) CGACAACAAGGATGGTGACTCCAAGGGTGGCCACAACACCGATGCCTTCG
cDNA PG fragment        (266) CGACAACAAGGATGGTGACTCCAAGGGTGGCCACAACACCGATGCCTTCG
```

Fig. 3.32 Nucleotide alignment of a section of the genomic and full cDNA PG gene confirming the predicted intron (indicated in red).

3.4 Discussion

A PG gene was cloned and characterised from a lupin anthracnose fungus *C. lupini* SHK 2148. Culture filtrates of this pathogenic fungus, grown on pectin medium, displayed PG activity in an agarose diffusion assay, suggesting the presence and expression of one or more PG genes. A full-length copy of an endoPG gene as well as 420 bp of the 5 ' UTR was cloned from *C. lupini* SHK 2148 using internal PCR primers and inverse PCR. Northern and RT-PCR analysis confirmed that the gene was expressed using pectin as sole carbon source. Furthermore, sequence analysis of the cDNA copy revealed that a single putative intron is spliced out post-transcriptionally (Fig. 3.30).

Colletotrichum had previously been reported to have the highest PG activity for days 4, 5, 6 and 7 with slightly less activity for day 3 after inoculation in the pectin containing media (Oelofse, 2003). In this study the PG activity from a crude extract of *C. lupini* SHK 2148 grown in pectin media for 12 days were evaluated on days 3, 4, 5, 6, 7 and 12. PG activity was stronger during the early growth period, day 3 and 4 after inoculation, and decreased towards the end of the growth period. This was similar to the PG activity observed for *C. gloeosporioides* f.sp. *malvae*, in which the authors observed the highest PG activity 3 days after inoculation, which declined towards day 9 (Li *et al*, 2002).

A full length PG gene was cloned and characterised from *C. lupini* isolate SHK 2148, that has been reported has highly virulent on lupins (Personal communication, Dr. S.H. Koch). The full-length PG gene was cloned by first cloning an internal PG fragment from three *C. lupini* isolates (SHK 788, SHK 1033 and SHK 2148) using degenerate primers. Sequence analysis revealed that the internal PG gene fragment in all three isolates was identical, suggesting that the PG genes in the isolates also will have very high sequence homology. The complete PG gene cloned from *C. lupini* SHK 2148 is 1153 bp and contains a single intron of 59 bp that displays the typical 5' GT and AT 3' border sequences (Fig. 3.23 and Fig. 3.30). The intron was similar in size and position compared to the introns of the PG genes of *C. gloeosporioides* f. sp. *malvae* and *C. lindemuthianum* (*CmpgII* and *ClpgII* respectively). Splicing of the intron predicted from sequence analyses was confirmed by cloning the cDNA copy of

the PG gene by RT-PCR. The cloned PG from *C. lupini* SHK 2148 has a potential signal peptide of 23 amino acids. This suggests that it will be exported, similar to other fungal endoPGs. The 5' untranscribed region of the PG gene displays several characteristics of filamentous fungal genes such as the conserved border sequences of the intron, a putative TATAA signal and a CAAT box (Chapter 1; Gurr *et al*, 1987).

The predicted protein product, after intron splicing, consists of 363 amino acids, which falls in the size range of other predicted endoPG proteins. Further comparison of the predicted amino acid sequence of the PG gene from *C. lupini* SHK 2148 with other fungal PG genes revealed that the gene had the highest similarity to the *CmpgII* gene of *C. gloeosporioides* f. sp. *malvae* and the *ClpgII* gene of *C. lindemuthianum*. The *C. lupini* SHK 2148 predicted PG protein furthermore displayed all four domains present in fungal PGs as described by Markovič *et al* (2001) as outlined in Fig. 3.3.20; the NTD and DD domain of the *C. lupini* SHK 2148 PG protein was identical to the majority of the fungal PGs compared with variations present in *BcPG6* and *BcPG3* of *B. cinerea*. The third domain of *C. lupini* SHK 2148 PG protein was similar to that of *cmpgII* (*C. gloeosporioides* f. sp. *malvae*) and *clpgII* (*C. lindemuthianum*), while the fourth domain, RIK, was identical to all the other fungal PGs with the exception of *BcPG2* of *B. cinerea.*

The Southern blot membrane was UV fixed before storage, this enhanced the background colour of the membrane leading to very faint bands. Only single bands were detected on the membrane, which could indicate that this particular PG gene is present as a single copy in the genome. However, due to the poor quality of the developed membrane and the fact that fungi usually produce more than one PG, it is difficult to support this observation.

The PG cloned from *C. lupini* SHK 2148 is very similar to *CmpgII* of *Colletotrichum* f.sp. *malvae*, which is present as a single copy in the genome, has a similar size and contains a single intron of 64 bp (Li *et al*, 2002). These two genes are also very similar to the *Clpg2* gene of *C. lindemuthianum*, which is expressed during the early stages of infection (Dumas *et al*, 1999).

Northern blot analysis evaluated the *in vitro* transcription of the fungus grown at pH 5, on pectin media as sole carbon source. Transcripts were only detected for days 3, 4, and 7. The slight degradation of the RNA from days 6 and 12 might explain why no transcript was present for those days, yet no transcript was obtained from the RNA from day 5, which were not degraded. It could be that the regulation of the PG is pH dependant as is the case for the PG gene from *C. gloeosporioides* f.sp. *malvae*, transcripts for the PG gene were only detected when the fungus was grown on mallow cell wall extracts at pH 3.5 and not at pH 7.5 or other carbon source like pectin (Li *et al*, 2002). Thus, it might be possible that the growth of the fungus alters the pH and the transcription of the gene. This would however not explain the presence of a transcript at day 7. Another explanation for the transcripts at day 7 could be that more than one PG are involved. The first PG is expressed during the early growth period at pH5, while the other PG(s) are only activated later on at another pH level. There are reports of differentially regulated PGs that are activated at different time periods such as the PGs from *C. lindemuthianum* (Centis *et al*, 1997; Dumas *et al*, 1999) where both genes are induced by pectin during the early growth phase, while only *Clpg1* is expressed later on. There have also been reports of pH dependant expression of other CDWE; transcripts of the *pel* (pectate lyase) gene of *C. gloeosporioides* on avocado fruits were only detected if the pH of the pectin containing media was pH 5.8 and higher (Yakoby *et al*, 1999).

Future research needs to be done address the expression and regulation of the PG gene(s) from *C. lupini* SHK 2148 on different carbon sources and under different pH conditions.

Chapter 4

Heterologous expression of a Polygalacturonase gene from *Colletotrichum lupini* in *Pichia pastoris*

Abstract

A cDNA copy of the PG gene of *Colletotrichum lupini* SHK 2148 was successfully constructed by switching an internal genomic fragment flanking the intron with the corresponding internal cDNA fragment. This cDNA copy was used as a template for designing several PG gene constructs for expression in *Pichia pastoris*. The mature PG protein with its own signal peptide as well as the mature protein with the α-MF signal peptide was expressed in *P. pastoris*. In addition to this, two hybrid PGs, the PG from *C. lupini* SHK 2148 containing the N-terminal part of the mature *Fusarium moniliforme* PG and the latter containing the N-terminal part of the *C. lupini* SHK 2148 PG were also transformed into *P. pastoris*. None of the transformants had PG activity. Western blot analysis with an antibody directed against the *F. moniliforme* PG gave no signal while hybridisation was observed with an antibody directed against the *Aspergillus niger* PG. However, the size of the hybridising bands was very large which might be due to extensive post translational modifications such as glycosylation. Conclusive evidence could not be provided for expression of the *C. lupini* SHK 2148 PG in *P. pastoris.*

4.1 Introduction

Several expression systems are currently available for heterologous protein expression. They vary from simple bacterial systems to more complex mammalian cells (Anderson *et al*, 2002; Rai *et al*, 2001). Although bacterial systems are very easy and inexpensive to use, they do lend themselves to some limitations especially in regard to translational and post translational modifications of eukaryotic proteins (Rai *et al*, 2001).

A favourable alternative to bacterial expression systems has been presented in the form of yeasts such as *Saccharomyces* strains. The latter presented itself as a system that could be grown with ease on simple media to high cell densities (Rai *et al*, 2001), and was supported by the complete genomic sequence of the yeast, which made it easy to manipulate on a molecular level. Although *Saccharomyces* were a good alternative to the bacterial systems, it had some disadvantages; it was a fermentative system in which metabolic by-products could build up to toxic levels, fermentative conditions, such as pH levels and carbon source feed rate could become expensive and demanding to control and the secretion level of the desired proteins were sometimes very low (Rai *et al*, 2001).

Pichia is one of the genera of the methylotrophic yeasts that is able to utilize methanol as sole carbon source (Houard *et al*, 2002). *Pichia pastoris*, isolated from an exudate of oak, has been developed during the past 15 years for heterologous protein expression and is a better alternative to *Saccharomyces* (Cereghino *et al*, 1999; Houard *et al,* 2002). This system is fairly easy to manipulate, expresses proteins at high levels both intra-and extra cellular and provides higher eukaryotic protein modification functions (Cereghino *et al*, 1999). Furthermore, the *Pichia* system is a respiratory system, which is not limited by the accumulation of toxic fermentative products as in the *Saccharomyces* system (Cereghino *et al*, 1999). Apart from this, genes carried on transformation vectors are stably integrated into the genome of *Pichia* (Houard *at al*, 2002). So far, more that 400 proteins have been expressed successfully in *P. pastoris* including two fungal pectinases; *Fusarium solani* pectate lyases (*pelC* and *pelD*) (Cereghino *et al*, 2000). Several vectors and

strains are available for the expression of foreign genes (Cereghino *et al,* 2000). The expression is mainly driven under the influence of the methanol induced AOX1 (alcohol oxidase gene 1) or constitutive induced GAP (glyceraldehyde 3-phosphate dehydrogenase) promoters (Cereghino *et al*, 2000; Houard *et al*, 2002). The use of different promoters allows the constitutive or induced expression of recombinant proteins. The GAP promoter provides a constitutive production of the recombinant protein to levels comparable to that obtained with the AOX1 promoter. With the GAP promoter it is unnecessary to transfer cultures from one growth media to another and it is not necessary to build up and maintain the methanol concentrations in the media (Cereghino *et al, 2000*).

Yeast signal sequences are often used for expression of foreign proteins in yeast systems. Several signal sequences are available to secrete foreign proteins in yeast; so far the α-MF factor (α-mating factor signal sequence) from *S. cerevisiae* has given the best results. In some cases protein production using the α-MF signal gave better success than the native protein signal sequence (Cereghino *et al, 2000*).

This chapter describes attempts to express a polygalacturonase (PG) gene cloned from *Colletotrichum* (chapter 3) in *Pichia pastoris*. The native genomic PG gene copy of *Colletotrichum* contains an intron (chapter 3). Therefore, it was necessary to obtain a cDNA copy without the intron for the heterologous expression in *P. pastoris*. Attempts to obtain a full length PG cDNA copy without the intron through RT-PCR failed, and only a partial internal fragment of the PG gene without the intron could be obtained. Therefore, a synthetic cDNA construct of the *Colletotrichum* PG gene was constructed by restriction digestion the genomic gene copy to remove the region containing the intron, followed by ligation of the partial cDNA fragment without the intron (obtained through RT-PCR), yielding a synthetic intronless cDNA copy of the full length PG gene. Several PG gene constructs for expression in *P. pastoris* were constructed from this cDNA construct, transformed into a *Pichia pastoris* and their PG activity evaluated. The work was performed in the laboratories of Prof. F. Cervone, La Sapienza University, Rome.

4.2 Materials and Methods

4.2.1 Ligation and cloning reactions

DNA and cDNA fragments were purified from agarose gels using Qiagen PCR purification kit (Qiagen, Germany), the Qiagen gel extraction kit (Qiagen) or Nucleospin PCR purification kit (Nucleospin, Macherey Nagel).

Ligation reactions were performed using 1U T4 ligase (Roche Diagnostics, Mannheim, Germany) and 50ng pGEM-T-Easy (Promega, Madison, USA) or 5ng pGAPZαA and pPICZA respectively. All ligation reactions were performed overnight at 5 °C. Ligation mixtures were mixed with 40µl of competent *E. coli* cells, placed on ice for 30 minutes, heat shocked for 45 sec. at 42 °C, transferred to 1ml LB medium and incubated for 1 hour at 37 °C. Transformants were plated onto LB agar plates containing 100mg/ml ampicillin, 40µl IPTG (100mM) and 40µl X-gal (100mM) for pGEM-T-Easy transformants or LSLB (low salt luria broth) agar plates containing 25µg/ml zeocin for pGAPZαA and pPICZA transformants. Alternatively ligation mixes were electroporated into cells using a Biorad electroporator at 1.8kV, 25m Faradys and 200Ω.

4.2.2 RNA extractions

C. lupini SHK 2148 was grown in Czapek Dox medium (Difco, Detroit, USA) and transferred to pectin containing media as described in chapter 3. Total RNA was isolated from fungal mycelia (collected at day 3 after inoculation) using the RNeasy kit from Qiagen (Hilden, Germany). A quick check for the presence of RNA was done by running RNA samples on a 1% TAE agarose gel. From the total RNA, mRNA was isolated using the Oligotex mRNA isolation kit (Qiagen). The concentration of RNA samples was determined with a photometer (Eppendorf Biophotometer, Brinkmann Instruments, Westbury, NY, USA).

4.2.3 RT-PCR amplification

A range of gene specific primers (Table 4.1) was used to amplify the PG cDNA copy from mRNA, using the Ready-To-Go RT-PCR bead kit (Amersham Biosciences, Buckinghamshire, UK). RT-PCRs were performed with four different PG primers sets: (1) a complete primer set, C. lupini_Compl F I and C. lupini_Compl R; (2) complete primer set C. lupini_Compl F II and C. lupini_Compl R II), (3) internal primer set PG-COLL-F2 and PG-COLL-R; and (4) internal primer set C. lupini_Int F I and C. lupini_Int R I. The RT-PCRs with the Ready-To-Go Beads were set up by first dissolving the dried pellet in the appropriate amount of RNAse free water, followed by addition of the various primer pairs (20µM each) and 230ng of mRNA as template per bead tube. The reagents added to each Ready-To-Go Bead tube were mixed thoroughly, where after the reverse transcription reaction was done by incubating the tubes at 42 °C for 30 min. Following reverse transcription, PCR cycles were conducted that consisted of an initial denaturation step at 96 °C for 2 min, followed by a ten-cycle repeat of a denaturation step at 94 °C for 20 sec., an annealing step at 55 °C for 45 sec. and an elongation step at 72 °C for 45 sec. This was followed with another thirty cycles that each consisted of a denaturation step at 94 °C for 30 sec.; an annealing step at 55 °C for 40 sec. and an elongation step at 72 °C for 45 sec. The final elongation step was carried out at 72 °C for 7 minutes in a GeneAmp 2700 thermal cycler (ABI Advanced Biotechnological Institute, Perkin-Elmer Corporation, Foster City, USA). The PCR cycles for the four different primers were identical except for the annealing temperatures: the internal primer sets and the complete primer set I was annealed at 55 °C, while the complete primer set II was annealed at 65 °C in a PCR cycle that consisted of an initial denaturing step of 5 minutes at 95 °C, a thirty-three cycle repeat of three steps of 94 °C for 1 minute, the relevant annealing temperature for 1 minute and 72 °C for 1 minute. A final elongation step of 72 °C for 5 minutes completed the PCR reaction.

Each RT-PCR reaction was accompanied by a positive control PCR that contained the full length cloned plasmid PG gene copy (pPGcompl1) as template. The PCR reaction consisted of 40ng of pPGcompl1 (Chapter 3), 0.5µl Biotaq (1.25U/µl, Bioline, Germany), 5mM $MgCl_2$, 200µM of each dNTP and 10µM of

C. lupini_Compl F I and C. lupini_Compl R. The PCR amplification cycles were the same as those used in the RT-PCR.

A control RT-PCR of the β-tubulin gene was also included in each RT-PCR with the PG gene primers to ensure that the mRNA was of good quality and that reverse transcription was taking place. The size product amplified from genomic DNA with the βt1 primers, are approximately 540 bp for ascomycetes (Glass *et al*, 1995). This region is interrupted with a 59 bp intron in *Neurospora crassa,* but the size of the intron might vary between the different ascomycetes (Glass *et al*, 1995). The RT-PCR of the β-tublin gene consisted of 2µl of each β-tublin primer (Table 4.1) βt1a and βt1b (10mM respectively) and 230ng m-RNA per bead reaction in a final volume of 50µl. A control β-tublin PCR reaction with genomic DNA as template was also included. The β-tublin PCR reactions consisted of 30ng of genomic DNA, 0.25µl Biotaq (1.25U/µl), 1.5mM $MgCl_2$ and dNTPs (200µM of each).

Table 4.1 Primers used for RT-PCRs, sequencing reactions and the construction of the PG constructs.

Primer	Sequence	Annealing Temp
βt1a βt1b	5' TTCCCCCGTCTCCACTTCTTCATG 3' 5' GACGAGATCGTTCATGTTGAACTC 3'	58 °C
C. lupini_Compl F C. lupini_Compl R	5' CCACGTGTTGATCACATACC 3' 5' CCCACCATCAAGCATTTAGC 3'	55 °C
C. lupini_Compl FII C. lupini_Compl RII	5' CGATGAAGTTCCTCTCCGTTGTC 3' 5' TTTAGCACTTGGCACCGGAGCCGGAAGG 3'	65 °C
PG-COLL-F2 PG-COLL-R	5' CGC GCC TCG TGC ACC TTC 3' 5' GCT GAC GCC GGA CCA CTT CCA 3'	55 °C
C. lupini_Int F I C. lupini_Int R I	5' CCTTCGGCTACAAGGAGT 3' 5' AACGGAACCGATGGACAG 3'	55 °C
T7 SP6	5' TAATACGACTCACTATAGGG 3' 5' ATTTAGGTGACACTATAG 3'	55 °C
FMPG FWEco_pichia	5-ATGC**GAATTC**GATCCCTGCTCCGTGACT-3	55 °C
FMPG RVXba_pichia	5-ATGC**AGATCT**CTAGCTGGGGCAAGTGTTG-3	55 °C
CollyPGEcoFWII	5' ACGTA**GAATTC**GCCCCCGCCGAGCTC 3'	58 °C
CollyPGEcoFWIII	5' ACGTA**GAATTC**GCCTCGTGCACCTTCAC 3'	58 °C
CollyPGXbaRV	5' ATCGA**TCTAGA**TTAGCACTTGGCACCGGA 3'	58 °C
SOE a.sense CLFMPG	5' CTGTTAGAACCTTTGCCATCCCACCAGCGCTCACCCT 3'	55 °C
SOE sense CLFMPG	5' AGGGTGAGCGCTGGTGGGATGGCAAAGGTTCTAACAG3'	55 °C
SOE Forward FMCLPG	5' GGTCAGGCGTACTGGGACGGCAAGGGAGGCAA 3'	55 °C
SOE Reverse FMCLPG	5' TTGCCTCCCTTGCCGTCCCAGTACGCCTGACC 3'	55 °C

4.2.4 Cloning and characterization of an internal cDNA PG gene fragment

40ng purified cDNA PG fragment was cloned into pGEM-T-Easy and transformed into *E.coli* JM 109 competent cells as described before (Section 4.2.2). Plasmid DNA was isolated from clones according to the method of Sambrook *et al* 1989. An aliquot of 5µl of each plasmid preparation was subjected to restriction analysis with 0.3µl

EcoRI (10U/µl, Roche) in a 10µl reaction volume for 1h at 37 °C. Restriction reactions were evaluated on an EtBr containing 1% (w/v) TAE agarose gel. Clones were subjected to sequence analysis with T7 and SP6 primers (Table 4.1). The sequence reaction was set up as described in Chapter 3 (3.2.4).

4.2.5 Construction of a full-length PG cDNA copy

pPGcompl1 (100ng) and pPGcDNAint4 (100ng) were transformed into *E.coli* JM110 (dam^- strain) via electroporation using a Biorad electroporator as described above. Clones were screened using colony PCR with the complete primer set C. lupini_Compl FII and C. lupini_Compl RII and the internal PCR primer set II (C. lupini_Int F I and C. lupini_Int R I) and previously described PCR parameters (Section 4.2.5). Plasmid DNA was isolated and subjected to *EcoRI* analyses as described previously (section 4.2.6). pPGcompl1 (≈1µg) and pPGcDNAint4 (≈1µg) were digested with 2.5µl *EheI* (10U/µl) and *Eco47111*(10U/µl) (Fermentas, Maryland, USA) respectively in a 50µl reaction volume. The reaction was evaluated on a 1.2% agarose gel. The *EheI* and *Eco47111* restricted internal cDNA PG fragment, was gel-purified and ligated to *EheI* and *Eco47111* digested pPGcompl1 (Fig. 4.1). The ligation reaction was set up as previously described and chemically transformed into JM109. Transformants were screened via colony PCR with the C. lupini_Int F I and C.lupini_Int R I primers. Plasmid constructs were isolated from clones, subjected to restriction enzyme analysis with *EheI* and *Eco47111* and sequenced with the universal primer sets SP6 and T7.

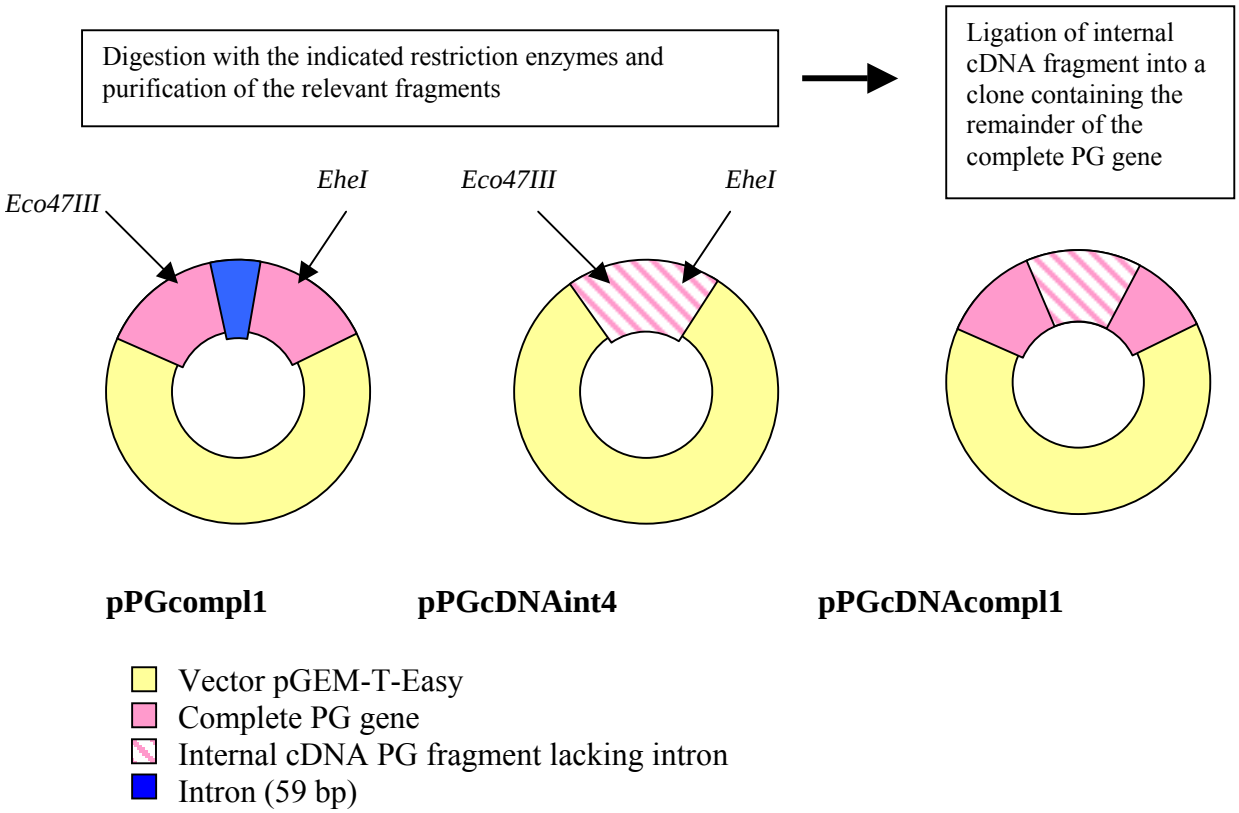


Fig. 4.1 Schematic presentation of the construction of a cDNA clone of the PG gene from *Colletotrichum lupini* SHK 2148.

4.2.6 Expression vectors

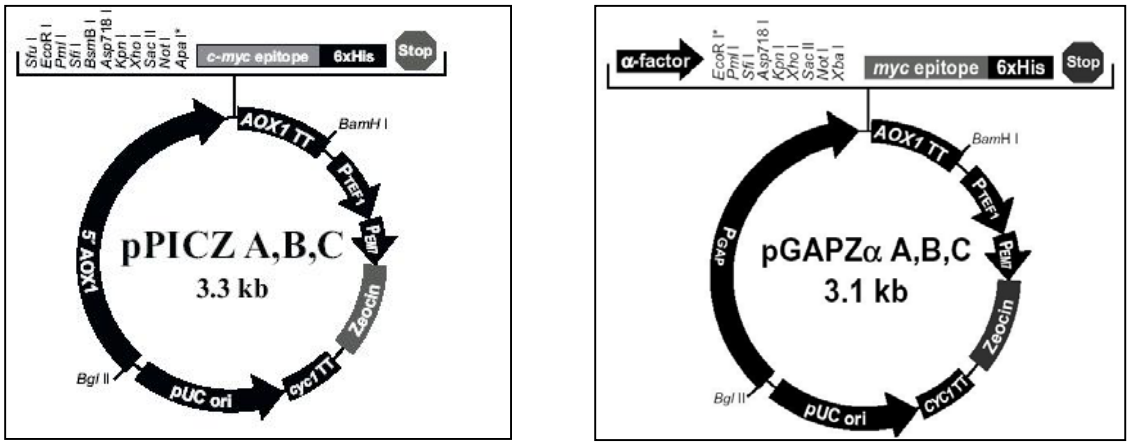


Fig. 4.2 *Pichia pastoris* expression vectors:
(www.invitrogen.com)

Heterologous expression of the PG gene was performed with the easy select *Pichia* expression kit from Invitrogen (Invitrogen Research Corporation Technologies, Inc. Tucson, Arizona, Cat. No. K174001).

Two vectors, pGAPZαA and pPICZA (Fig. 4.2) were used to design the PG gene constructs for expression in *P. pastoris*. The vectors are provided in three reading frames (A, B, and C), contains a MCS (multiple cloning site) as well as a C-terminal myc epitope (Glu-Gln-Lys-Leu-Ile-Ser-Glu-Glu-Asp-Leu) (Evans *et al*, 1985) and a C-terminal polyhistidine tag (encoding six histidine residues that form a metal-binding site) that facilitates the detection and affinity purification of the recombinant protein respectively (Fig. 4.3 and Fig. 4.4). The selectable marker for the vectors is derived from the zeocin resistance gene that is encoded by the *Sh ble* gene (*Streptoalloteichus hindustanus ble* gene). Two different promoters drive protein expression; in pGAPZαA, the GAP promoter allows constitutive expression of the foreign protein, while the expression in pPICZA is under the regulation of the methanol induced AOX1 promoter (Fig. 4.4 and Fig. 4.3 respectively). Another significant difference between the two vector systems is the α-Factor secretion signal that is present in pGAPZαA (Fig. 4.4). This is the native *Saccharomyces cerevisiae* α-factor, which has proven to be very successful for the secretion of recombinant proteins produced in *P. pastoris*. The AOX1 transcription termination (TT) region is the native transcription termination and polyadenylation region from the AOX1 gene, which ensures correct processing of the foreign gene to provide a more stable mRNA transcript (Fig. 4.3 and Fig. 4.4). Both vectors are shuttle vectors that are able to replicate in *E. coli* (with the pUC origin) and be integrated into the *P. pastoris* genome via a homologous recombination between the GAP and AOX1 promoter region and loci on the genome respectively.

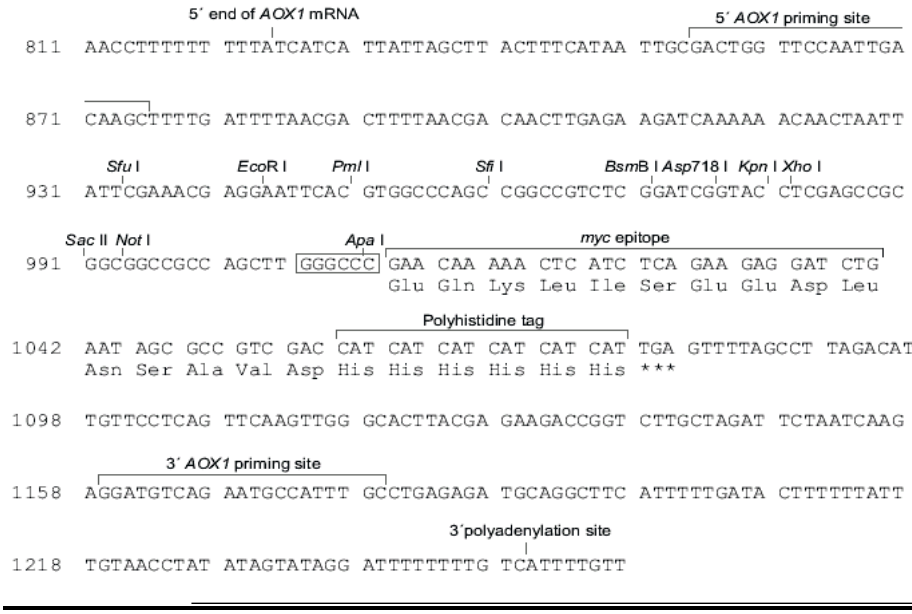


Fig. 4.3 pPICZA multiple cloning site.

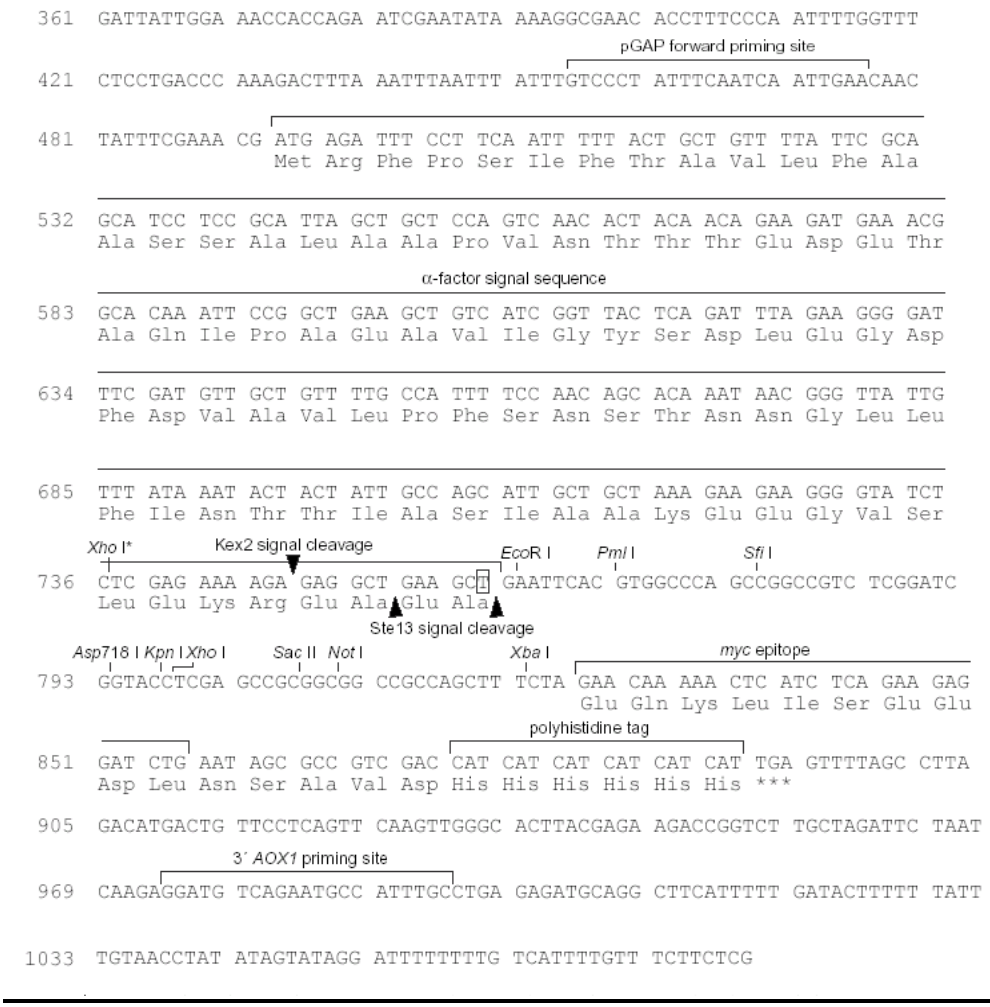


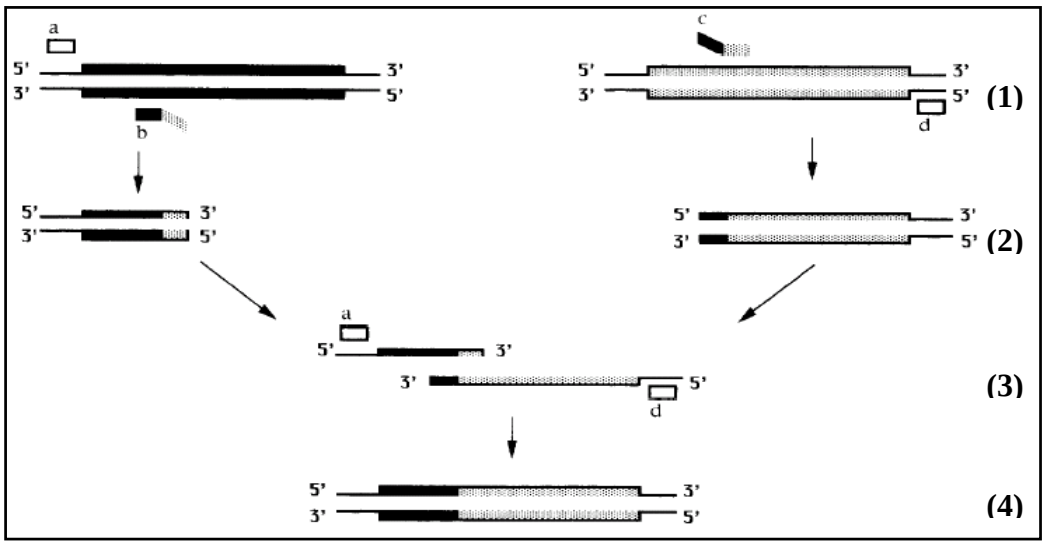
Fig. 4.4 pGAPZαA multiple cloning site.

4.2.7 PG gene constructs for expression in *P. pastoris*

4.2.7.1 PCR amplification

The PG fragment for PG constructIII was amplified from pPGcDNAcompl7 using primer pair CollyPGEcoFWIII (50µM) and CollyPGXbaRV (50µM) (Fig. 4.8).

The N- terminus sequence of the *Fusarium moniliforme* mature PG gene (Fig. 4.7) sequence was switched with the N- terminus sequence of the *C. lupini* SHK 2148 PG gene (Fig. 4.8) and vice versa via Splicing by Overlap Extension by PCR (SOE PCR) (Fig. 4.5 and Fig. 4.6). SOE PCR involves three separate PCR reactions. In the first two PCR reactions (Fig. 4.5 (1)) two hybrid primers (Fig. 4.5 b & c) are involved, which are designed from the known sequence and are tipped with overlapping sequence data. In this study SOE sense CLFMPG and SOE a.sense CLFMPG are the hybrid primers for the first hybrid construct while SOE Reverse FMCLPG and SOE Forward FMCLPG are for the second hybrid construct (Fig. 4.6). SOE sense CLFMPG and SOE Reverse FMCLPG are designed from *F. moniliforme* PG gene sequence and tipped with *C. lupini* SHK 2148 sequence, while SOE a.sense CLFMPG and SOE Forward FMCLPG are designed from *C. lupini* SHK 2148 gene sequence and tipped with sequence data from *F. moniliforme* PG gene (Fig. 4.6). These hybrid primers are used with two terminal primers (Fig. 4.5 a & d) to create PCR products, one which contains sequence data 5' to the splice point and one which contain sequence data 3' to the splice point (Fig. 4.5 (2)). In this case FMPG RVXba_pichia creates SW1, CollyPGEcoFWIII creates SW2III, FMPG FWEco_pichia delivers SW3 and CollyPGXbaRV produces SW4 (Fig. 4.6). These templates are denatured and will partly anneal at the overlapping sequence (Fig. 4.5 (3)) that was incorporated by the hybrid primers. The partly annealed DNA serves as template for the third PCR reaction in which the terminal primers are used to produce the final hybrid product ((Fig. 4.5 (4)). In this study SWAIII produces the *F.moniliforme* PG with the *Colletotrichum* N terminus using the SWIII and SW1 PCR products, while SWB produces the *Colletotrichum* PG with the *F. moniliforme* N terminus from the SW3 and SW4 products (Fig. 4.6).



a & d: Terminal primers

b & c: Hybrid primers

SW1 (Swop 1):

SOE sense CLFMPG

FMPG RVXba pichia

SW2 III (Swop 2- Mature PG *C. lupini*)

CollyPGEcoFWIII

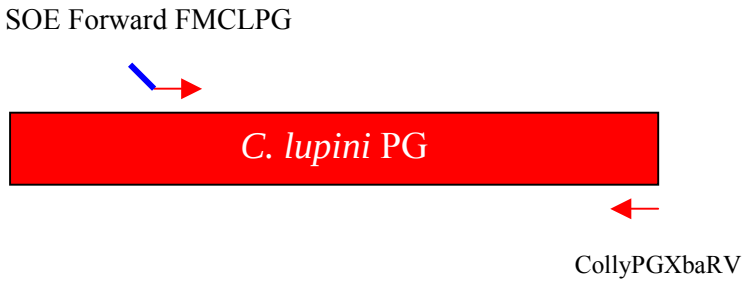
SOE a.sense CLFMPG

SW3(Swop 3):

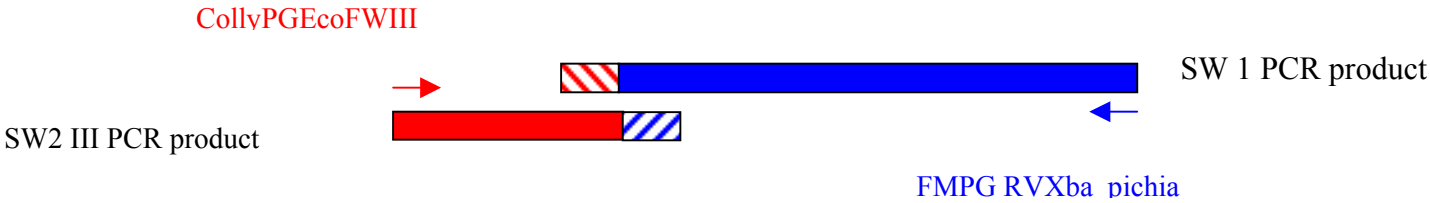
FMPG FWEco_pichia

SOE Reverse FMCLPG

SW4(Swop 4):



SW A III:



SW B:

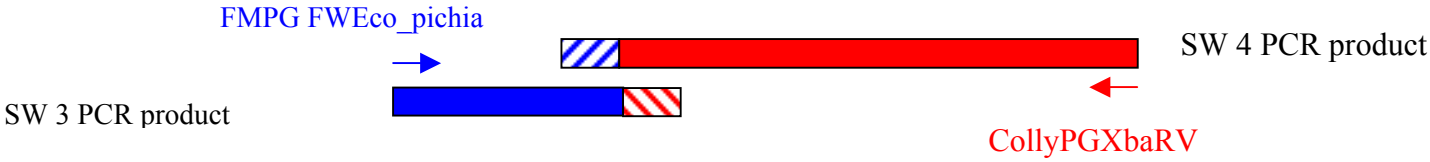


Fig. 4.6 SOE PCR amplification steps creating the hybrid PG gene construct.

```
   M   V   R   N   I   V   S  R   L   C   S   Q   L   F   A   L   P
5' ATG GTT CGA AAC ATC GTA TCG CGG CTT TGC TCC CAG CTG TTT GCT CTG CCC

 S   S   S   L   Q   E   R
TCA AGC TCT CTC CAG GAA CGA
```

FMPG FWEco_pichia

```
1    GAT CCC TGC TCC GTG ACT GAG TAC TCT GGC CTC GCC ACC GCT GTC TCA TCC
1    D   P   C   S   V   T   E   Y   S   G   L   A   T   A   V   S   S
                                                 1
52   TGC AAA AAC ATC GTG CTC AAC GGT TTC CAA GTC CCG ACA GGC AAG CAA CTC
18   C   K   N   I   V   L   N   G   F   Q   V   P   T   G   K   Q   L

103  GAC CTA TCC AGC CTC CAG AAT GAC TCG ACC GTT ACC TTC AAG GGC ACG ACC
35   D   L   S   S   L   Q   N   D   S   T   V   T   F   K   G   T   T

154  ACT TTT GCC ACC ACT GCT GAT AAC GAC TTT AAT CCT ATC GTC ATT AGT GGA
52   T   F   A   T   T   A   D   N   D   F   N   P   I   V   I   S   G

205  AGT AAC ATC ACT ATC ACT GGT GCA TCT GGC CAT GTC ATT GAT GGC AAC GGT
69   S   N   I   T   I   T   G   A   S   G   H   V   I   D   G   N   G
```

SOE PCR splice point

```
256  CAG GCG TAC TGG GAT GGC AAA GGT TCT AAC AGC AAT AGC AAC CAA AAG CCC
86   Q   A   Y   W   D   G   K   G   S   N   S   N   S   N   Q   K   P

307  GAT CAC TTC ATC GTT GTT CAG AAG ACC ACC GGC AAC TCA AAG ATC ACA AAC
103  D   H   F   I   V   V   Q   K   T   T   G   N   S   K   I   T   N

358  CTA AAT ATC CAG AAC TGG CCC GTT CAC TGC TTC GAC ATT ACA GGC AGC TCG
120  L   N   I   Q   N   W   P   V   H   C   F   D   I   T   G   S   S

409  CAA TTG ACC ATC TCA GGG CTT ATT CTT GAT AAC AGA GCT GGC GAC AAG CCT
137  Q   L   T   I   S   G   L   I   L   D   N   R   A   G   D   K   P

460  AAC GCC AAG AGC GGT AGC TTG CCC GCT GCG CAT AAC ACC GAC GGT TTC GAC
154  N   A   K   S   G   S   L   P   A   A   H   N   T   D   G   F   D

511  ATC TCG TCC AGT GAC CAC GTT ACG CTG GAT AAC AAT CAT GTT TAT AAC CAA
171  I   S   S   S   D   H   V   T   L   D   N   N   H   V   Y   N   Q

562  GAT GAT TGT GTT GCT GTT ACT TCC GGT ACA AAC ATC GTC GTT TCT AAC ATG
188  D   D   C   V   A   V   T   S   G   T   N   I   V   V   S   N   M

613  TAT TGC TCC GGC GGC CAT GGT CTT AGT ATC GGA TCT GTT GGT GGA AAG AGC
205  Y   C   S   G   G   H   G   L   S   I   G   S   V   G   G   K   S

664  GAC AAT GTC GTT GAT GGT GTT CAG TTC TTG AGC TCG CAG GTT GTG AAC AGT
222  D   N   V   V   D   G   V   Q   F   L   S   S   Q   V   V   N   S

715  CAG AAT GGA TGT CGC ATC AAG TCC AAC TCT GGC GCA ACT GGC ACG ATC AAC
239  Q   N   G   C   R   I   K   S   N   S   G   A   T   G   T   I   N

766  AAC GTC ACC TAC CAG AAC ATT GCT CTC ACC AAC ATC AGC ACG TAC GGT GTC
256  N   V   T   Y   Q   N   I   A   L   T   N   I   S   T   Y   G   V

817  GAT GTT CAG CAG GAC TAT CTC AAC GGC GGC CCT ACT GGA AAG CCG ACC AAC
273  D   V   Q   Q   D   Y   L   N   G   G   P   T   G   K   P   T   N

868  GGA GTC AAG ATC AGC AAC ATC AAG TTC ATC AAG GTC ACT GGC ACT GTG GCT
290  G   V   K   I   S   N   I   K   F   I   K   V   T   G   T   V   A

919  AGC TCT GCC CAG GAT TGG TTT ATT CTG TGT GGT GAT GGT AGC TGC TCT GGA
307  S   S   A   Q   D   W   F   I   L   C   G   D   G   S   C   S   G

970  TTT ACC TTC TCT GGA AAC GCT ATT ACT GGT GGT GGC AAG ACT AGC AGC TGC
324  F   T   F   S   G   N   A   I   T   G   G   G   K   T   S   S   C
```

FMPG RVXba_pichia

```
1020 AAC TAT CCT ACC AAC ACT TGC CCC AGC TAG  3'
341  N   Y   P   T   N   T   C   P   S   STOP
```

Fig. 4.7 *F. moniliforme* PG gene sequence (Dr. C. Caprari).

CollyPGEcoFWII

```
 1     M   K   F   L   S   V   V   A   G   L   S   A   L   A   A   A   A   P   A   E
 1 5'  ATG AAG TTC CTC TCC GTT GTC GCG GGC CTC AGC GCC CTG GCT GCC GCC GCC CCC GCC GAG
```

CollyPGEcoFWIII

```
21     L   D   T   R   A   S   C   T   F   T   D   A   K   T   A   M   S   K   K   T
61    CTC GAT ACC CGT GCC TCG TGC ACC TTC ACC GAC GCC AAG ACG GCC ATG TCC AAG AAG ACC
41     S   C   T   D   I   V   L   N   G   I   K   V   P   A   G   E   T   L   D   L
121   TCC TGC ACC GAC ATC GTC CTC AAC GGC ATC AAG GTT CCC GCC GGC GAG ACC CTC GAC CTC
61     T   G   L   K   D   G   T   K   V   T   F   K   G   T   T   T   F   G   Y   K
181   ACC GGC CTC AAG GAC GGA ACC AAG GTT ACC TTC AAG GGC ACC ACC ACC TTC GGC TAC AAG
81     E   W   E   G   P   L   I   A   I   G   G   K   K   V   S   V   V   G   A   S
241   GAG TGG GAG GGC CCT CTC ATC GCC ATC GGC GGT AAG AAG GTC AGC GTT GTC GGT GCT TCC
101    G   N   S   I   S   C   Q   G   E   R   W   W   D   G   K   G   G   N   G   G
301   GGC AAC TCC ATC TCC TGC CAG GGT GAG CGC TGG TGG GAC GGC AAG GGA GGC AAC GGT GGC
```

SOE PCR splice point

```
121    K   K   K   P   K   F   F   K   V   K   I   N   D   G   S   I   S   G   L   N
361   AAG AAG AAG CCC AAG TTC TTC AAG GTC AAG ATC AAC GAC GGT TCC ATC TCT GGC CTG AAC
141    V   K   N   T   P   A   H   G   F   S   I   S   G   V   S   G   L   K   V
421   GTC AAG AAC ACC CCG GCC CAC GGA TTC TCC ATC AGC GGC GTT TCT GGC CTC AAG GT
481   GTA AGT CAC AAC TCA ATG TTG GGT TTT TGG ACA GTA ATA CTG ACC CGA CTA TTC TCT AG
161    V   S   N   I   L   F   D   N   K   D   G   D   S   K   G   G   H   N   T   D
541    C TCC AAC ATC CTT TTC GAC AAC AAG GAT GGT GAC TCC AAG GGT GGC CAC AAC ACC GAT
181    A   F   D   V   G   T   S   S   D   V   T   I   S   G   A   K   V   Y   N   Q
601   GCC TTC GAT GTC GGT ACG TCT TCT GAC GTG ACC ATC TCC GGC GCC AAG GTC TAC AAC CAG
201    D   D   C   L   A   I   N   S   G   T   G   I   T   F   E   N   G   Y   C   Y
661   GAC GAC TGC CTG GCT ATC AAC TCT GGC ACC GGT ATC ACT TTC GAG AAC GGA TAC TGC TAC
221    G   S   H   G   L   S   I   G   S   V   G   G   R   S   D   N   T   V   Q   N
721   GGC TCC CAC GGC CTG TCC ATC GGT TCC GTT GGT GGA CGT AGC GAC AAC ACC GTC CAG AAC
241    V   I   I   R   D   S   T   I   E   K   A   D   N   G   I   R   I   K   T   I
781   GTC ATC ATC CGT GAC TCC ACC ATC GAG AAG GCC GAC AAC GGT ATC CGC ATC AAG ACC ATT
261    N   K   T   G   K   V   N   G   I   T   F   E   N   I   T   L   K   N   I   N
841   AAC AAG ACT GGC AAG GTC AAC GGT ATC ACC TTC GAG AAC ATC ACC CTC AAG AAC ATC AAC
281    A   K   K   G   I   V   I   Q   Q   D   Y   E   N   G   S   P   T   G   K   P
901   GCC AAG AAG GGC ATT GTC ATC CAG CAG GAT TAC GAG AAC GGC AGC CCT ACC GGC AAG CCC
301    T   A   G   I   P   I   T   D   V   T   V   K   N   V   K   G   T   V   A   A
961   ACC GCT GGT ATT CCC ATC ACC GAC GTC ACC GTC AAG AAC GTT AAG GGT ACC GTC GCC GCG
321    G   T   N   V   Y   I   L   C   A   K   G   A   C   S   N   W   K   W   S   G
1021  GGC ACC AAC GTG TAC ATC CTT TGC GCC AAG GGT GCC TGC TCC AAC TGG AAG TGG TCT GGC
341    K   V   S   V   T   G   G   K   S   S   S   E   C   S   G   I   P   S   G   S
1081  AAG GTC TCC GTT ACC GGT GGC AAG TCC TCT TCT GAG TGC TCT GGC ATC CCT TCC GGC TCC
361    G   A   K   C  STOP
1141  GGT GCC AAG TGC TAA  3'
```

CollyPGXbaRV

Fig. 4.8 Complete PG gene sequence from *C. lupini* SHK 2148.

Italics Signal peptide

Regions from which primers were designed

Splice point region for the SOE PCR

---- Intron

4.2.7.2 PCR parameters and cycle

Each PCR reaction was carried out in a final volume of 50µl, using 10ng of pPGcDNAcpmpl7 template (or less then 10ng for the second set of PCR reactions of the SOE PCR method), dNTPs (final concentration 0.2mM), 1.25 units of high fidelity PWO taq (Roche, Mannheim, Germany) and the relevant primer pairs (final concentration 1µM). As a positive control, CollyPGEcoFWII (50µM) and CollyPGXbaRV (50µM) primer pair was used to amplify a PG fragment from the pPGcDNAcompl7.

The PCR cycle, performed in a Gene pulser x-cell (Biorad) consisted of a denaturing cycle of 4 minutes at 94°C, followed by a three cycle repeat of 94°C for 1:30 minutes; the relevant annealing temperature of the primers (table 4.1) for 1:30 minutes, an extension temperature of 72 °C for 1:30 minutes. The cycle was finished by a final elongation step of 72°C for 7 minutes.

A tenth of the PCR reactions were evaluated on a 1% TAE agarose gel containing EtBr. The PCR products were excised, purified from the gel and electrophoresed with a dilution series of λ DNA to determine their concentration.

4.2.7.3 Construction of the PG gene constructs

pPGcDNAcompl7 was digested with *EcoRI*, while amplified PG fragments were restricted with *EcoRI* and *XbaI* Digested PG fragments were gel-purified and subjected to concentration determination on a 1% TAE agarose gel with a dilution series of λ DNA.

Pichia expression vectors, approximately 200ng of each, were digested with the 10U of the relative restriction enzymes; pPICZA were restricted with *EcoRI* while pGAPZαA were restricted *EcoRI* and *XbaI* for 1h at 37 °C. The restriction reactions were evaluated on a 1% TAE agarose gel, purified and subjected to concentration determination with a dilution series of λ DNA on a 1% TAE agarose gel. Purified vector and PG fragments were ligated and transfored into into *E. coli* top 10 F'as previously described (section 4.2).

4.2.7.4 Screening of transformants

Colony PCR was used to screen several transformants (table 4.2)

Table 4.2 PG clones screened with colony PCR with different primer pairs and annealing conditions.

Construct	Number of colonies screened	Primer pair	Annealing temperature
PG construct I	60	CollyPGEcoFWIII (50µM)	58 °C
PG construct III	10	CollyPGXbaRV (50µM)	
SWAIII	5	CollyPGEcoFWIII (50µM)	55 °C
		FMPGRVXba_pichia (50µM)	
SWB	5	FMPG FWEco_pichia (50µM)	55 °C
		CollyPGXbaRV (50µM)	

Positive clones were inoculated into 5ml LSLB media with 25µg/ml zeocin incubated overnight at 37 °C whereafter plasmid isolations were performed with the Nucleospin plasmid extraction kit (Nucleospin plasmid DNA purification kit, Macherey Nagel).

Plasmid DNA was subjected to restriction enzyme analysis; PG construct I clones were digested with *EcoRI*, *KpnI* or *XhoI* respectively, PG construct III clones were digested with *EcoRI* and *XbaI* respectively as well as with *EcoRI* and *KpnI* in double digest restriction reactions and SWAIII and SWB clones were digested with *EcoRI* and *XbaI* in a double digestion reaction. Restriction reactions were performed in a 10 µl reaction volume for 1h at 37 °C.

4.2.8 Transformation of *P. pastoris*

P. pastoris strain X-33, with the wild-type genotype and methanol utilization (mut+) phenotype were prepared for electroporation according to the suppliers instructions (Invitrogen) :

The *P. pastoris* strain was shake-incubated overnight in 5ml YPD at 28 °C. The 5ml culture was used to inoculate 500ml fresh YPD medium. The culture was grown overnight until it reached an OD_{600} between 1.3 – 1.5. *Pichia* cells were collected by centrifugation at 1500 x g for 5 minutes at 4°C. The pellet was resuspended in 500ml ice-cold sterile water. This step was repeated and cells were resuspended in 250ml ice-cold sterile water. Cells were centrifuged again and resuspended in 20ml ice-cold sorbitol (1M). After a final centrifugation step, cells were resuspended in 1 ml ice-cold sorbitol (1M) and kept on ice for the electroporation procedure.

Clones were prepared for *Pichia* transformation by linearising pGAPZαA constructs with 5 U *AvrII* or pPICZA constructs with 5U *BstXI*. Linearised DNA were precipitated for 30 minutes at –80 °C with 2 volumes isopropanol and ammonium acetate (final concentration of 2M). The precipitated DNA were centrifuged for 30 minutes at 4 °C at 18 000 rpm and vacuum dried for 5 minutes. The concentrations of the linearised product were determined before electroporation of *P. pastoris*.

Between 50ng/µl and 200ng/µl of DNA were added to 80µl of *Pichia* cells, cells were pulsed under the predetermined Gene Pulser Xcell Biorad electroprator conditions: C = 25 uF; PC = 200ohm; V = 2.0kV, (Biorad, Life Science Research Group 2000 Alfred Nobel Drive Hercules, CA) and immediately transferred to 1 ml ice-cold sorbitol (1M). The transformed cells were incubated for 3-4 h at 28 °C without shaking, where after 20 – 100µl were plated onto LSLB plates with zeocin (100µg/ml). Plates were incubates for 2- 3 days at 28 °C.

Colonies transformed with pPICzA were inoculated into 3ml BMGY containing zeocin (100µg/ml) and incubated for 24-36h before they were transferred to BMMY light containing methanol (0.5 –3%) (Appendix A), while colonies harbouring pGAPzAα constructs were inoculated into 3ml BMMY light (Appendix A), with zeocin (100µg/ml). Clones in the BMMY light medium were grown for approximately four days

4.2.9 PG activity assay

The cup plate method was adapted from the agarose diffusion assay of Taylor and Secor (1987). Four *P. pastoris* clones of each construct were analysed on 90mm diameter cup plates containing 30 ml of a solution of polygalacturonic acid from citrus spp. (0.5%), agarose (0.8%) (Invitrogen) both dissolved in Sodium acetate (100mM, pH 4.6).

Wells were pressed into the cup plate and filled with 60µl crude culture extract from each *P. pistoris* clone. The crude culture extract was obtained from an aliquot of the culture that was grown BMMY light media with methanol (for the pPICzA clones) or BMMY light media (for the pGAPzAα clones). Purified *Fusarium moniliforme* PG (0.5ng) were included as a positive control on each plate. Plates were incubated overnight at 28 ° C and stained with HCl (6M).

4.2.10 SDS PAGE analysis

P. pistoris clones were grown for 4 days in BMMY light media with methanol (for the pPICzA clones) and BMMY light media (for the pGAPzAα clones) at 28 °C with shaking at 280 rpm. Supernatant were obtained from these cultures by spinning 1ml of culture at 5 000 rpm for 1 min. Proteins were precipitated from 500µl of the supernatant using TCA (trichloroacetic acid). TCA was added to the supernatant (0.11v/v) and mixed well. The samples were incubated at 4 °C for 30 min and centrifuged at maximum speed (12 000 rpm) for 30 min. TCA was discarded and the pellets were washed three times with 70% ice-cold ethanol. Pellets were air dried and resuspended in 10µl SDS loading buffer (3×). Approximately a third of the samples were electrophoresed on the SDS PAGE gel (See Appendix for preparation). A purified PG sample from *Fusarium moniliforme* (1ng/µl) and the LMW ladder from the LMW calibration kit from Amersham were included on the gels.

Electrophoresis proceeded for approximately 1h at 170V and 300mA in the Biorad Mini Trans-Blot system. The SDS-PAGE gel was silver stained by first fixating the gel for 30 minutes in a fixation buffer (50%) mercaptoetanol, 12% Acetic acid and 0.0185% of a37 % formamide stock solution. Subsequently, the gel was washed

twice for 10 minutes in a 50% ethanol solution, pre-treated for 1 minute in a 200mg/10ml sodium tiosulphate ($Na_2S_2O_3 \times 5\ H_2O$) solution, and then briefly washed three times in ultra pure water where after it was incubated for 10 minutes in the silver nitrate solution (0.2% $AgNO_3$, 0.0280% of the 37% Formamide stock solution). After the incubation period, two brief wash steps in ultra pure water removed the excess silver nitrate. The gel was developed in a developing solution (6% Na2CO3, 0.0185% of the 37% formamide stock solution and 200mg/10 ml sodium tiosulphate) until clear bands were visible on the gel. The gel was briefly washed in ultra pure water and the development reaction terminated by a solution containing 50% mercaptoetanol and 12% acetic acid.

4.2.11 Western blot analysis

One clone for each construct expressed in *P. pastoris* was chosen for a Western blot analysis. The analysis was performed on the supernatant of each chosen clone as well as the boiled cells themselves. Two antibodies were used in the study, the first set of blots were performed with the antibody directed towards the *F. moniliforme* PG while the another set of blots were probed with an antibody directed towards the *A. niger* PG (both antibodies were provided by Dr. Caprari, University or Rome). The positive controls of each blot included purified PG from *F. moniliforme* and *A. niger* respectively (1ng/µl each). 3µl of each sample was boiled for 5 minutes and loaded onto a SDS-PAGE gel. This was performed in duplicate. The electrophoresis proceeded as described before, where after one gel was subjected to silver staining while the other gel was used for the western blot.

The blot was set up as follows:
A sponge, soaked in the transfer buffer, were placed in the transfer "cassette" of the TRANS-BLOT system (Biorad), followed by two soaked filter papers, the SDS-PAGE gel a Nitrocellulose membrane and finally another soaked filter paper and sponge. The cassette was closed, placed into the TRANS_BLOT system, filled with transfer buffer and transfer of the proteins to the membrane was allowed to proceed at 100V (100-200mA) for 3h.

After the transfer procedure was completed, the membrane was incubated in blocking solution (Appendix A) for 10 minutes and then twice for 5 minutes. The membrane was incubated for 12h in a solution containing 0.5% BSA with the *FmPG* antibody or A. *niger* antibody (1:1000 dilution).

The membrane was washed with washing buffer (Appendix A) for one 10 minute and two 5 minute intervals. It was then incubated with the second antibody (anti-rabbit, Amersham, 1:5000 dilution) and 0.5% BSA for 2h. The membrane was washed as described before. The ECL advanced Western blotting detection kit (Amersham) was used to develop the western blots. The membrane was briefly dried between two filter papers and then completely air dried before adding solution 2 (a substrate buffer containing oxidant) and then solution 1 (luminol, enhancher, substrate buffer) from the kit. The membrane was incubated for 1 minute in these solutions and developed with Hyperfilm in a cassette for approximately 1, 5 and 15 minutes.

4.3 Results

4.3.1 RNA extraction and RT PCR amplification

Total RNA was isolated from *C. lupini* SHK2148 mycelia collected after three days of growth in pectin containing media (Fig. 4.9). The concentration of the RNA samples, as determined with a photometer, was 206ng/µl (sample1), 92ng/µl (sample 2), 98ng/µl (sample 3), and 159ng/µl (sample 5). The total RNA samples were all pooled to isolate mRNA and conduct RT-PCR. RT-PCR of the β-tubulin gene was successful, revealing a RT-PCR band of approximately 450 bp (Fig. 4.10). This falls in the expected size range as reported by Glass *et al* (1995). This showed that the m-RNA was of good quality, and could be used for PG gene RT-PCRs.

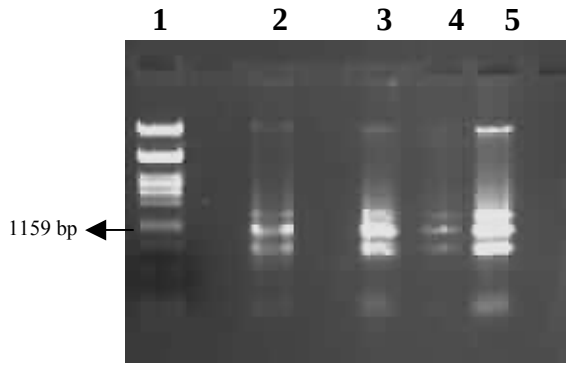


Fig. 4.9 RNA extracted from *C. lupini* SHK2148 mycelia collected on day 3 after inoculation in pectin media.
Approximately 1µg of total RNA isolated from samples 1, 2, 3 and 4 (lanes 2, 3 and 4 respectively) collected from mycelia harvested on day 3 after inoculation in pectin media. λ PstI molecular marker (lane 1, Appendix A)

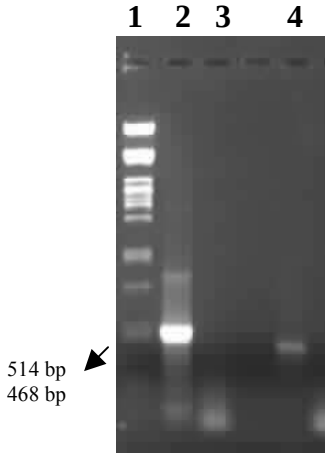


Fig. 4.10 PCR amplification of the β- tubulin gene from DNA and mRNA template of *C. lupini* SHK 2148.
The β- tubulin genomic copy (lane 2) and cDNA copy (lane 4) amplified via PCR and RT-PCR from genomic and mRNA template of *Colletotrichum lupini* respectively. A water control (lane 3 and) and a λ*PstI* molecular marker (lane 1) were included on the TAE gel.

RT-PCR using four PG gene specific primer sets, were only successful with one of the internal primer sets. A PCR product of approximately 500 bp was obtained only from primer set C. lupini_Int F I and C. lupini_Int R I. No other products were obtained for the other primer pairs (Fig. 4.11). The internal cDNA PG fragment was cloned into pGEM-T-Easy, and plasmids successfully isolated from 9 clones pPGcDNAint1 – 9 (Fig. 4.12). Restriction digestion of two selected clones (pPGcDNAint1 and pPGcDNAint4) with *EcoRI* showed that only pPGcDNAint4 (Fig. 4. 13) harboured the expected insert size of 500 bp, whereas the insert size of pPGcDNAint1 was too small (≈ 339 bp) (Fig. 4.14). The larger bands present above these excised fragments represent the partly or undigested plasmid from which these fragments were excised (Fig. 4.14 lane 3 and lane 4). Sequence analyses of pPGcDNAint4 with SP6 and T7 showed that the internal PG fragment formed part of the previously characterised PG gene and that the predicted intron (chapter 3) was spliced out.

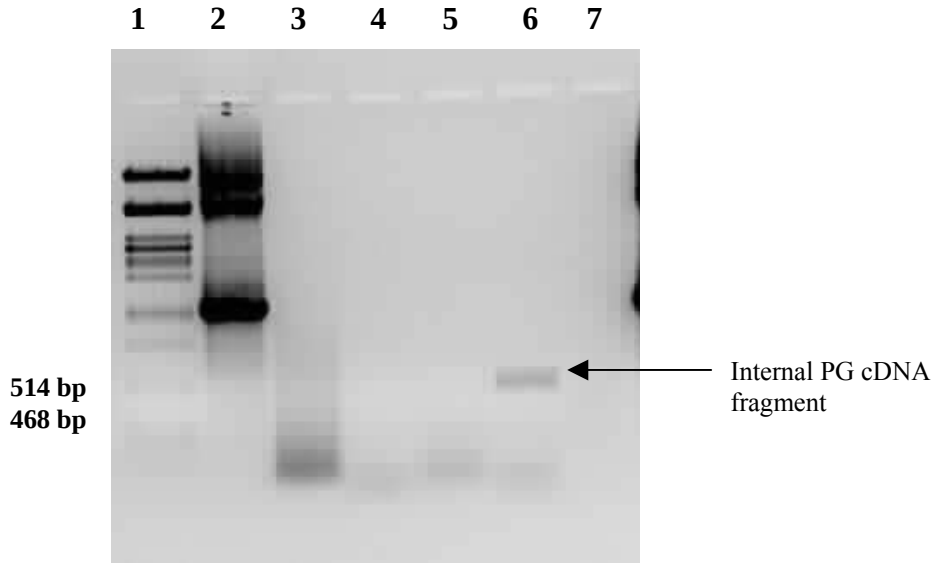


Fig. 4.11 RT-PCR amplification of an internal PG cDNA fragment with different PG gene specific primers.
PCR amplification of the complete genomic copy from plasmid pPGcompl1 using primer pair C. lupini_Compl F I and C. lupini_Compl RI (lane 1). Unsuccessful RT-PCR amplification of a full cDNA PG copy using PCR primer pairs C. lupini_Compl F I and C. lupini_Compl RI (lane 4) as well as a failed amplification of an internal PG cDNA fragment using PG-COLL-F2 and PG-COLL-R (lane 5). Successful amplification of the internal cDNA PG fragment (approximately 500 bp) using RT-PCR with primer pair C. lupini_Int F I and C. lupini_Int R I (lane 6). A λ PstI molecular marker (lane1) as well as PCR negative water control (lane 3) were electrophoresed with the PCR products**.**

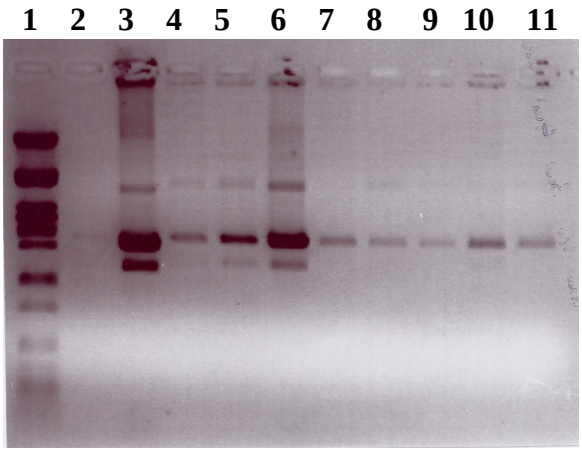


Fig. 4.12 Evaluation of plasmids pPGcDNAint1- pPGcDNAint9, isolated from clones, harbouring the internal cDNA PG fragment cloned into pPGEM-T-Easy.
Plasmid DNA isolations from nine clones desinated as pPGcDNAint1 –9 (lane 3-11), transformed with the putative internal cDNA PG fragment, were isolated and electrophoresed together with a λ PstI molecular marker (lane 1).

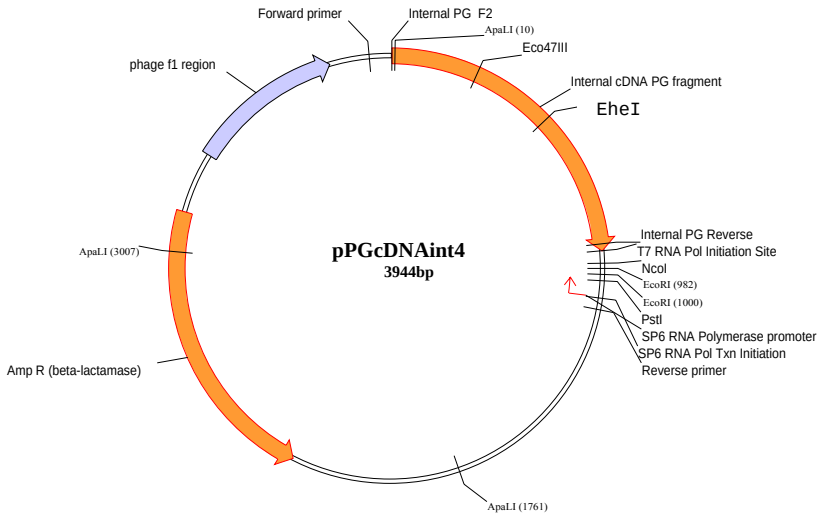


Fig. 4.13 Plasmid map of pPGcDNAint4 harbouring the internal cDNA PG fragment.

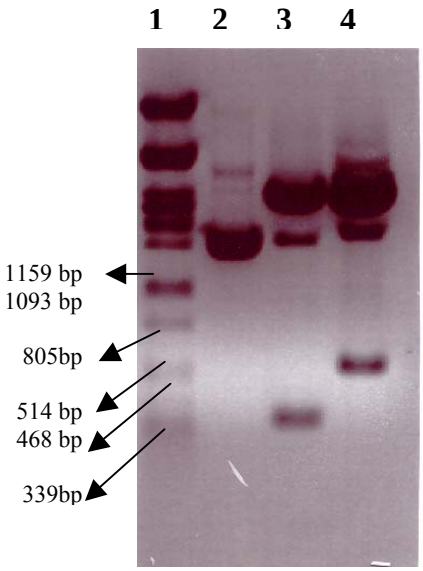


Fig. 4.14 Restriction analysis of pPGcDNAint1 and pPGcDNAint4 with *EcoRI*. Undigested pPGcDNAint1 (lane 2), *EcoRI* digested pPGcDNAint1 (lane 3), *EcoRI* digested pPGcDNAint4 (lane 4) and λ PstI molecular marker (lane 1).

4.3.2 Construction of a full-length cDNA PG clone

The internal fragment of the PG gene without the intron, obtained through RT-PCR, was used as basis to construct a synthetic full length PG gene copy without an intron. Unfortunately no full-length cDNA gene copy could be obtained with RT-PCR,

requiring the construction of a synthetic full length PG gene without the intron using constructs pPGcDNAint4 and pPGcompl1 as basis (section 4.2.5). pPGcDNAint4 and pPGcompl1 was first transformed into dam$^-$ *E. coli* strains (does not methylate DNA), since the cloning strategy required the use of the methylation sensitive enzymes *EheI* and *Eco47111*(section 4.2.5). Colony PCR analyses showed that transformation of pPGcDNAint4 and pPGcompl1 into dam- *E. coli* strains yielded two positive *E. coli* JM110 dam$^-$ clones containing pPGcompl1-1 and one containing pPGcDNAint4-1 (Fig. 4.15). Restriction analyses of pPGcompl1-1 and pPGcDNA4-1 with *EcoRI* confirmed that the clones contained the correct inserts (Fig. 4.15, lane8 and lane 9).

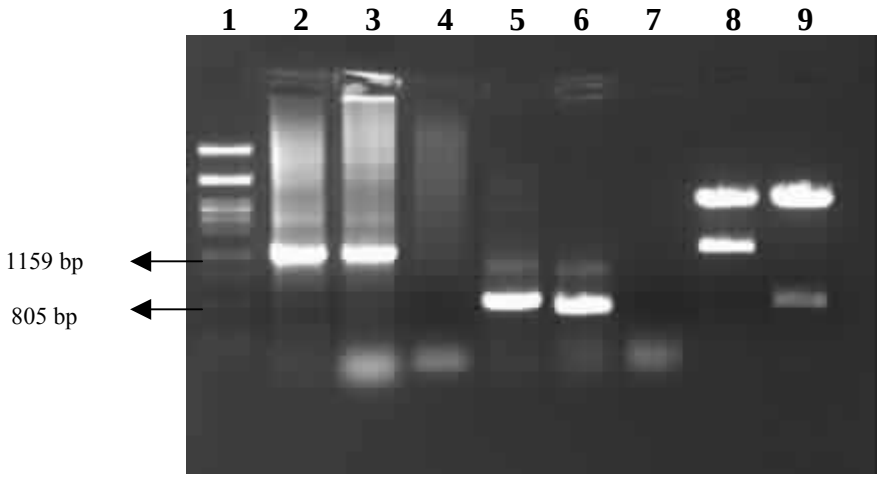


Fig. 4.15 Colony PCR and *EcoRI* restriction analysis of pPGcompl1-1 and pPGcDNAint4-1 isolated from *E.coli* dam$^-$ strains.
PCR amplification from plasmid pPGcompl1 with complete primer set C. lupini_Compl FII and C. lupini_Compl RII (lane2) as well as internal primer set C. lupini_Int F I and C. lupini_Int R I (lane 5) were included as positive controls to compare with the colony PCR amplification of clone pPGcompl1-1 with primers C. lupini_Compl FII and C. lupini_Compl RII (lane 3) and clone pPGcDNAint4-1 with primers C. lupini_Int F I and C. lupini_Int R I (lane6). Water controls are included (lane 4 and 7). The *EcoRI* restriction analysis of pPGcDNAint4-1 (lane 8) and pPGcDNAint4 (lane 9) was electrophoretically separated with a λ PstI molecular marker (lane 1).

Restriction digestion of pPGcDNAint4-1 and pPGcompl1-1 (obtained from the dam- *E. coli* strains) with the methylation sensitive enzymes *EheI* and *Eco47111* yielded the expected band sizes (data not shown). The cDNA internal PG fragment band from pPGcDNAint4, and the band from pPGcompl1 from which the genomic internal

fragment was excised, were successfully gel purified (data not shown) and used in a ligation reaction to construct pPGcDNAcompl. Transformation of *E. coli* JM 109 cells with the ligation mix yielded 14 clones designated as pPGcDNAcompl1 – pPGcDNAcompl14. Colony PCR showed that clones 1,2, 4, 7 and 8 contained inserts of the expected size range of approximately 458 bp (59 base pairs smaller than the genomic control of 517 bp) (Fig. 4.16, lanes 4, 5, 7, 10 and 12). Sequence analyses showed that only one, pPGcDNAcompl7 (Fig. 4.17) of the five clones had the insert in the correct orientation (data not shown). Restriction digestion of pPGcDNAcompl7 with *EheI* and *Eco47111* confirmed that the intron was spliced out, seen by the smaller fragment excised from pPGcDNAcompl7 than from pPGcompl1, and that no mutations occurred at the ligation points in pPGcDNAcompl7 (Fig. 4.18).

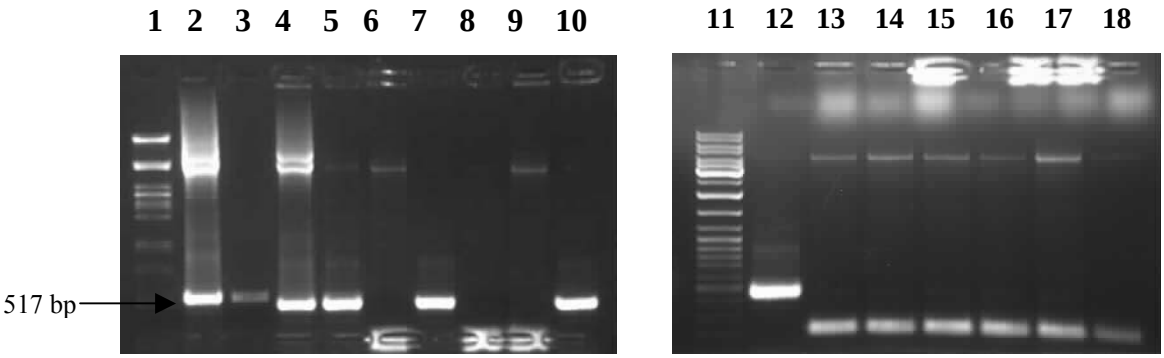


Fig. 4.16 Colony PCR amplification of clones transformed with the pPGcDNAcompl construct.
PCR amplification of pPGcompl1 using the internal primer set C. lupini_Int F I and C. lupini_Int R I (lane 2). Colony PCR of clones pPGcDNAcompl1 – pPGcDNAcompl14 (lanes 4, 5, 6, 7, 8, 9, 10, 12, 13, 14, 15, 16 and 17) using the primer set C. lupini_Int F I and C. lupini_Int R I. Lane 18 represents the water control and a λ PstI molecular marker (lane 1) as well as a100 bp molecular marker (lane 11, Appendix A) were included during the electrophoresis.

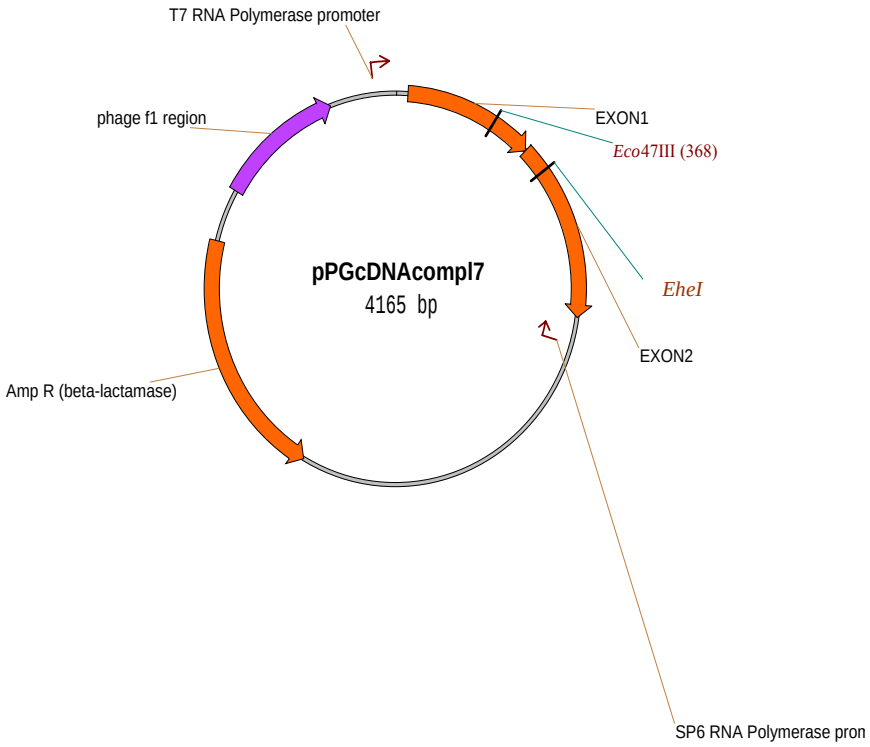


Fig. 4. 17 Plasmid map of pPGcDNAcompl7 harbouring the constructed cDNA PG copy.

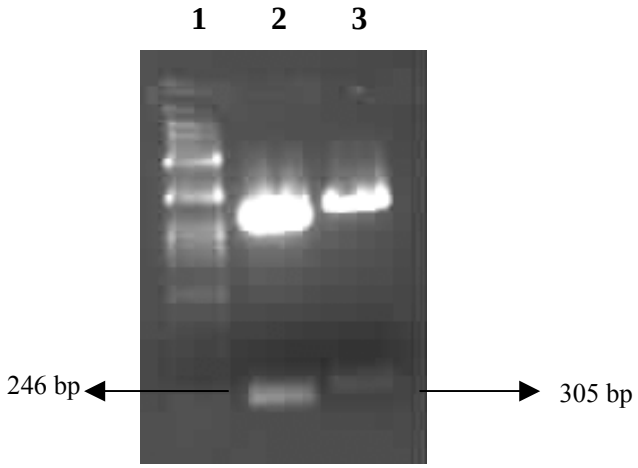


Fig. 4.18 Restriction digestion of pPGcDNAcompl7 and pPGcompl1 with *EheI* and *Eco47111*.
pPGcDNAcompl7 and pPGcompl1 digested with *EheI* and *Eco47111* (lane 2 and lane 3 respectively) electrophoresed with a λ *PstI* molecular marker (lane 1).

4.3.3 PG gene constructs used for expression of the gene in *P. pastoris*

PG construct I was created by excising the cDNA fragment of the PG gene from pPGcDNAcompl7 through *EcoRI* restriction digestion, and subcloning the fragment into pPICZA. Restriction digestion of pPGcDNAcompl7 with *EcoRI* yielded an approximately 1.2 Kb and 3 Kb band (Fig. 4.19, lane 2), whereas pPICzA was only linearized by *EcoRI* (Fig. 4.19, lane 3).

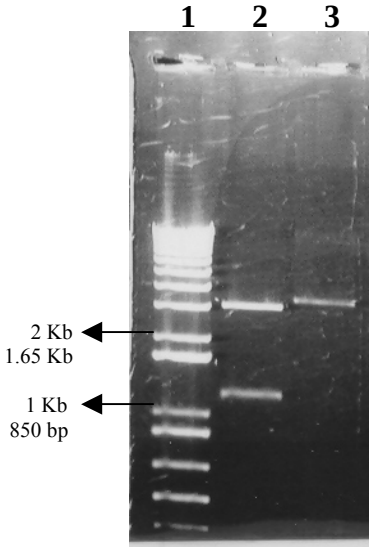


Fig. 4.19 *EcoRI* restriction of pPGcDNAcompl7 and pPICzA.
pPGcDNAcompl7 digested with *EcoRI* releasing the cDNA PG fragment (lane 2), pPICzA linearised with *EcoRI* (lane 3) and a 1 Kb plus molecular marker (lane 1, Appendix A) were electrophored on a 1% TAE gel.

Transformation of *E. coli* cells with a ligation mixture of the 1.2 Kb band of pPGcDNAcoml7 and linearized pPICzA yielded many transformed clones, from which 60 were selected for colony PCR. Colony PCR of the *E. coli* clones revealed several positive clones of which six clones designated as PG construct I- 15, PG construct I- 19, PG construct I- 20, PG construct I- 23, PG construct I- 24 and PG construct I- 28, were digested with *XhoI* and *KpnI*. *XhoI* was used to show successful linearization of the plasmid (Fig. 4.20 lanes 4, 7, 10, 13, 16 and 20) whereas *KpnI* was used to determine the orientation of the ligated fragment by evaluating the size of the band excised by *KpnI* (Fig. 4.20, lanes 3, 6, 9, 12, 15 and 18). PG construct I- 19, PG construct I- 20 and PG construct I- 28 contained the fragment in the correct orientation; a fragment of approximately 200 bp was excised from the clones with *KpnI* (The small fragment excised was visible on the Agarose gel, but not in the photo) (Fig. 4.20, lanes 12, 15 and 18). PG construct I- 23 and PG construct I- 24

contained the cloned fragment in the incorrect orientation, since an approximately 1Kb band was excised from the clones by *KpnI* digestion (Fig. 4.20, lanes 3 and 6). Restriction of PG construct I- 15 was unsuccessful (Fig. 4.20 lanes 9 and 10).

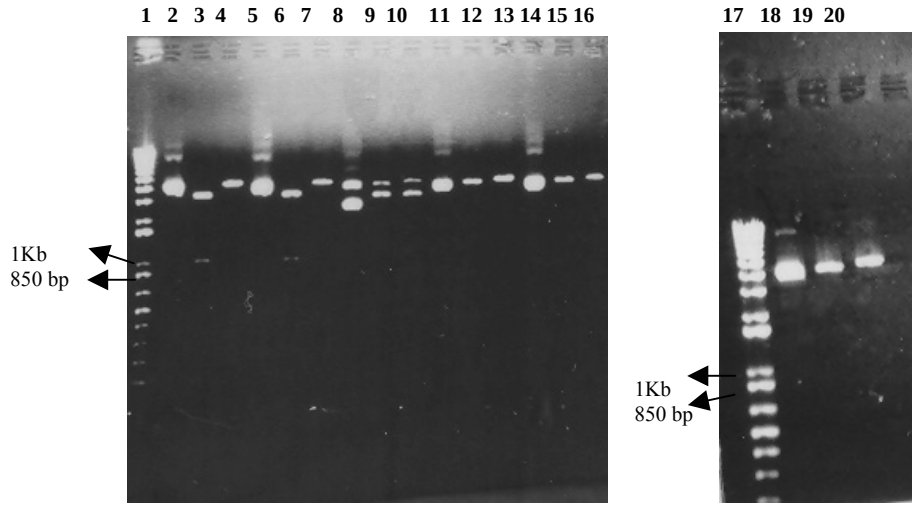


Fig. 4.20 Restriction analysis of PG construct I clones with *KpnI* and *XhoI*.
Uncut PG constructs I – 23, PG constructs I – 24, PG construct I – 15, PG construct I – 19, PG construct I – 20 and PG construct I – 28 (lanes 2, 5, 8, 11, 14 and 18), *KpnI* restricted PG constructs I-23, PG constructs I-24, PG constructs I-15, PG constructs I-19, PG constructs I-20 and PG constructs I-28 (lanes 3, 6, 9, 12, 15 and 19). *XhoI* restricted PG constructs I-23, PG constructs I-24, PG constructs I-15, PG constructs I-19, PG constructs I-20 and PG constructs I-28 (lanes 4, 7, 10, 13, 16 and 20). 1Kb plus molecular marker were electrophoresed in lanes 1 and 17.

PG construct III was constructed by cloning the PCR fragment, amplified from pPGcDNAcompl7 (Fig. 4.21, lane 3) into pPGAPzAα. Transformation of the ligation mixture into *E. coli* Top 10 yielded numerous transformants, however colony PCR was only performed on 10 clones (Fig. 4.22). Expected PCR amplification products in the size range of a 1000 bp are evident. The PG fragments were cloned in frame with the α- signal factor.

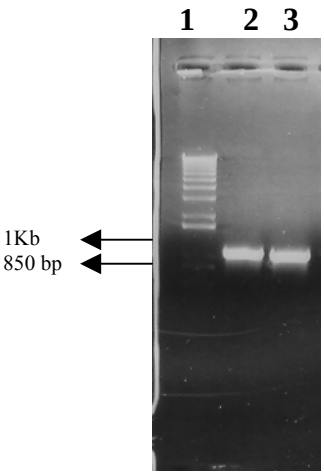


Fig. 4.21 PCR amplification of the PG fragment from pPGcDNAcompl7 for the construction of the PG construct III.
PCR amplification of a PG fragments that will represent PG construct III (lane 3) were elctrophoresed with1 Kb plus molecular marker (lane 1). A positive PCR amplification with primer pairs CollyPGEcoFWII and CollyPGXbaRV using pPGcDNAcompl7 as template as were included during gel electrophoresis (lane2)

E. coli clones potentially harbouring PG construct III were subjected to restriction enzyme analysis. Three clones of PG construct III, designated as PG construct III –4, PG construct III –5 and PG construct III –6, were restriction digested with *EcoRI and KpnI* and yielded a fragment of approximately 850 bp (Fig. 4.22), confirming cloning of the correct fragment. Similarly, restriction digestion of the three clones with *EcoRI and XbaI* revealed the expected excised PG fragment of approximately 1Kb (Fig. 4.22).

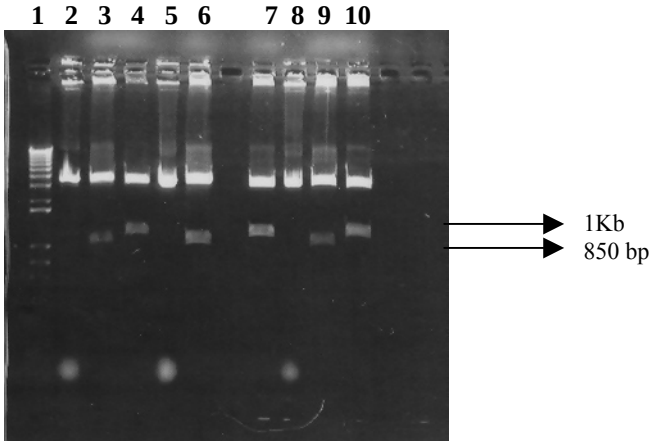


Fig. 4.22 *EcoRI –KpnI* and *EcoRI –XbaI* digestion of PG construct III clones.Uncut plasmid DNA from PG construct III –4, PG construct III –5 and PG construct III –6 uncut (lanes 2, 5 and 8 respectively), *EcoRI –KpnI* restricted PG construct III –4, PG construct III –5 and PG construct III –6 (lanes 3, 6 and 9 respectively) and *EcoRI –XbaI* restricted PG construct III –4, PG construct III –5 and PG construct III –6 (lanes 4, 7 and 10 respectively). A 1Kb plus molecular marker were loaded in lane1.

4.3.4 PG hybrid constructs

PG hybrid constructs were constructed via SOE PCR (Section 4.2.7). The results of the first set of PCRs SW1, SW2III, SW3 and SW4 are displayed in Fig. 4.23 SW1 (lane 2) represents the PG sequence of *F. moniliforme* excluding the front region from the swap point. SW2III (lane 4) represents the front part of the *C. lupini* SHK 2148 PG to the swap point. SW3 (lane 5) represents the front part of the *F. moniliforme* PG gene to the splice point and SW4 (lane 6) depicts the end part of the *C. lupini* SHK 2148 PG gene to the swap point.

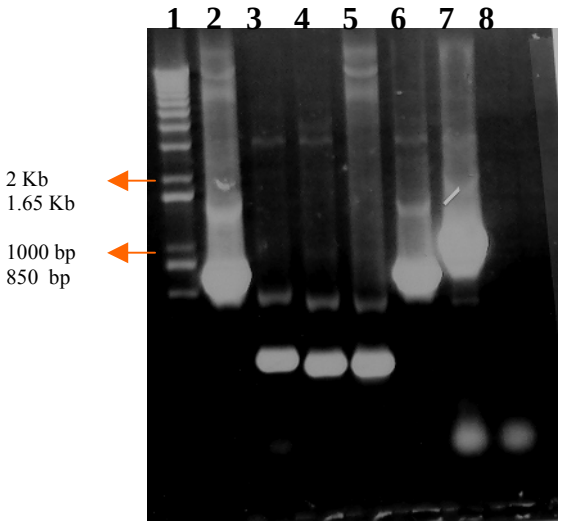


Fig. 4.23 PCR amplifications for the first set of the splicing overlap by extension PCR (SOE PCR).
PCR products obtained for SW1, SW2III, SW3 and SW4 SOE PCR reactions (lanes 2, 4, 5 and 6). PCR amplification of pPGcDNAcompl7 using primer pairs CollyPGEcoFWII and CollyPGXbaRV (lane 7). A water control (lane 8) and 1 Kb plus molecular marker (lane 1) were also included. (Non-relevant PCR reaction, lane3).

The second SOE PCR set used the diluted products of the first set as templates (Fig. 4.24). In the PCR reaction of SWAIII (Fig. 4.24 lane 3) SW1 and SW2III were used as template to create a hybrid PG gene with the front part originating from the *C. lupini* SHK 2148 PG gene and the end part from the *F. moniforme* PG. SWB (Fig. 4.24 lane 4) used SW3 and SW 4 as template to create a hybrid PG with the front part from *F. moniforme* PG and the end part from the *C. lupini* SHK 2148 PG.

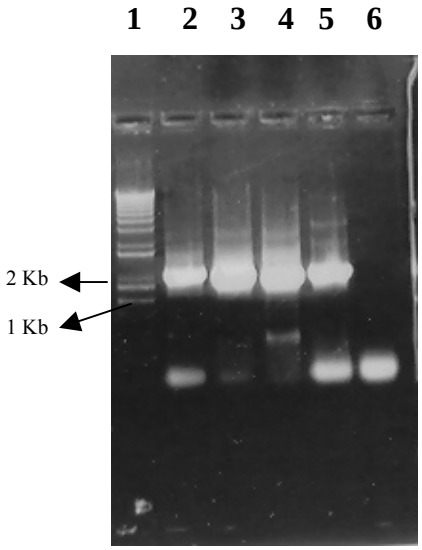


Fig. 4.24 PCR amplifications for the second set of the splicing overlap by extension PCR (SOE PCR) resulting in hybrid PG fragments.
PCR amplification of SWAIII (lane 3) and SWB (lane 4) were electrophoresed with the PCR product of pPGcDNAcompl7 using primer set CollyPGEcoFWII and CollyPGXbaRV (lane 5), a water control (lane 6) and a 1 Kb plus molecular marker (lane 1). Lane 2 contains a PCR reaction not relevant to this study.

Following transformation, 5 *E. coli* clones of each hybrid construct were subjected to colony PCR (results not shown). PG hybrid clones SWAIII-4 and SWB-1 were selected and digested with *EcoRI* (Fig. 4.25 lanes 8 and 13), *XbaI* (Fig. 4.25 lanes 9 and 14) and an *EcoRI-XbaI* combination (lanes 11 and 16). The constructs harboured an insert of approximately 1000bp as revealed by the *EcoRI-XbaI* double digestion. The plasmids were linearised with *AvrII* (Fig. 4.25 lanes 10 and 15).

Table 4.3 illustrates the different constructs and their key properties that were prepared for transformation into *P. pastoris*.

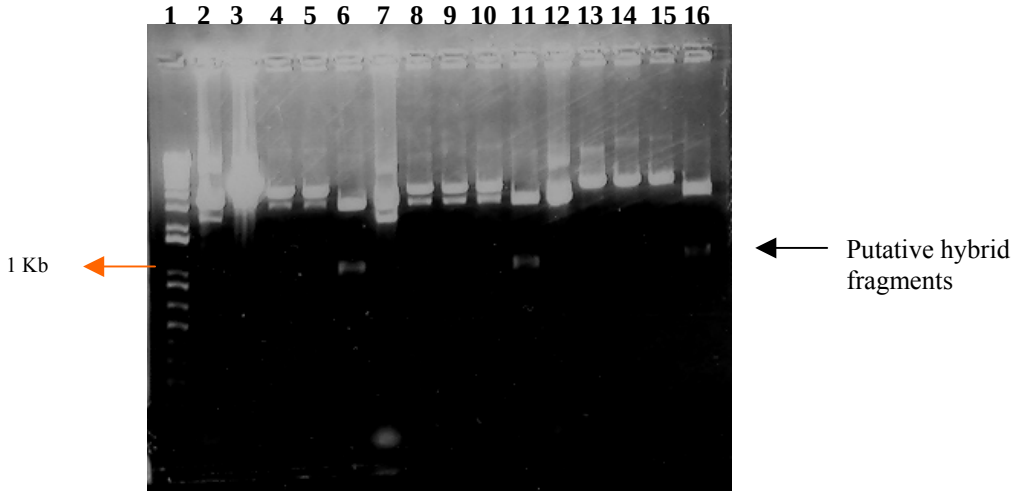


Fig. 4.25 Restriction analysis of hybrid PG clones.

Plasmid DNA of SWAIII-4 and SWB-1which were undigested (lanes 7 and 12 respectively), subjected to *EcoRI* restriction (lanes 8 and 13 respectively), digested with *XbaI* (lanes 9 and 14 respectively), linearised with *AvrII* (lanes 10 and 15 respectively) and double digested with *EcoRI* and *XbaI* (lanes 11 and 16 respectively). Compared to the 1Kb molecular marker (lane 1), the clones contained a fragment of approximately 1000 bp as revealed by the double digestion with *EcoRI-XbaI*. Lanes 2-6 represents work that is not discussed in this study.

Table 4.3 Key features of PG constructs analysed prior to transformation of *P. pastoris.*

PG construct	Clones analysed	Key feature
PG construct I	PG construct I-23, PG construct I-24, PG construct I-15, PG construct I-19, PG construct I-20 and PG construct I-28.	Mature PG protein with native peptide signal.
PG construct III	PG construct III-4, PG construct III-5 and PG construct III-6.	Mature PG protein with α-MF signal peptide.
SWAIII	SWAIII-4	PG protein from *C. lupini* SHK 2148 containing the N-terminal part of the mature PG protein from *F. moniliforme* with the α-MF signal peptide.
SWB	SWB-1	PG protein from *F. moniliforme* containing the N-terminal part of the mature PG protein from *C. lupini* SHK 2148 with the α-MF signal peptide.

4.3.5 Transformation of *P. pastoris* and PG activity analysis

Electroporation of the various PG constructs into *P. pastoris* electrocompetent cells, yielded time constants between 5.2 and 5.5, indicating that the electroporation was successful. Small colonies only appeared after 4 days of incubation. Several colonies were inoculated into the appropriate media and incubated for 6 days at 28 °C. No growth however was observed. Since the colonies were very small, the selection was repeated, however this time they were plated onto a master plate, allowed to grow to an appropriate size before inoculation in the media. Colonies grew well after 6 days at 28 °C. Cup plate analysis were performed on each clone, a positive control of purified *F. moniliforme* PG was included on each cup plate (Fig. 4.26). No zones were observed for any of the clones harbouring the PG constructs; the only zone observed was for the positive control. The lack of PG activity for each clone selected for further analysis was confirmed by a repeat of the cup plate analysis (results not shown).

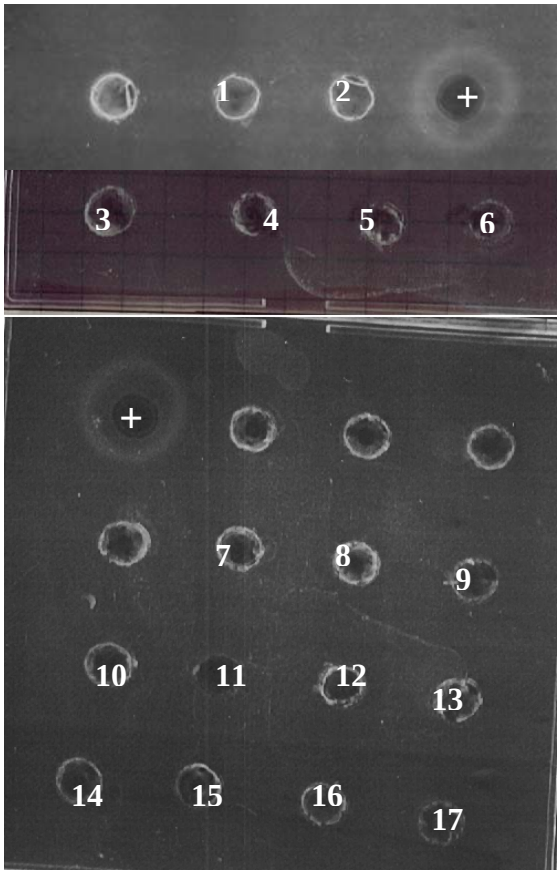


Fig. 4.26 PG activity analysed with an agarose diffusion assay, of *Pichia* cultures transformed with the PG constructs and PG hybrid constructs.
Crude culture extract of *Pichia* clones harbouring several PG gene constructs were evaluated for PG activity using the agarose diffusion assay. Clones evaluated for their PG activity included PG constructI-28.1, PG constructI-28.2, SWAIII-2.1, SWAIII-2.2, SWAIII-2.3, SWAIII-2.4, SWB-1.1, SWB-1.2, SWB-1.3, SWB-1.4, PG construct III- 5.1, PG construct III- 4.2 and PG construct III- 4.1. (Numbers 1, 2, 3, 4, 5, 6,7, 8, 9, 10, 11, 12, 13, 14, 15, 16 and 17 respectively). Purified *F. moniliforme* PG (0.5 ng) were included as a positive control.

4.3.6 SDS-PAGE analysis of *Pichia* transformants

Clones selected for SDS-PAGE and western blot analysis included two clones from PG construct I-28, designated PG construct I-28.1 and PG construct I-28.2; three clones from PG construct III, designated PG construct III-4.1, PG construct III-4.2 and PG construct III-5.1 and four clones from each hybrid construct SWAIII-2 and SWB-1, designated as SWAIII-2.1, SWAIII-2.2, SWAIII-2.3, SWAIII-2.4, SWB-1.1, SWB-1.2, SWB-1.3 and SWB-1.4.

Total protein from the supernatant of several clones, as outlined above, grown for four days were isolated and concentrated. An aliquot of 3 µl of each sample were evaluated with SDS-PAGE analyses (Fig. 4.27). There is a high concentration of proteins present in all the clones investigated in the size range of reported PGs (30- 40 kDa). The size of the *F. moniliforme* PG is approximately 40kDa. The SDS PAGE gel for the PG construct III clones are not presented here, however during Western blot analysis the proteins of these clones were visualised on a separate SDS PAGE gel. The profiles for the different clones appeared to be similar; unfortunately it is difficult to compare these profiles with the protein profile of the untransformed *Pichia* clone (Fig. 4.27, lane 5), since the latter was present as darker smear from which distinct bands could not be identified.

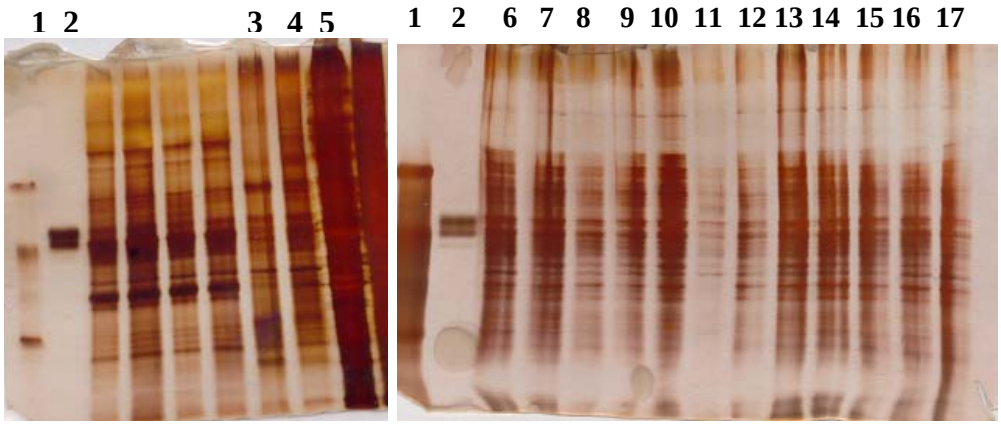


Fig. 4.27 SDS PAGE analysis for *Pichia pastoris* clones transformed with different PG constructs.
Purified PG protein from *Fusarium moniliforme* (lane 2) were separated on the SDS-PAGE gel with total protein isolations from clones transformed with PG constructI-28.1, PG constructI-28.2, SWAIII-2.1, SWAIII-2.2, SWAIII-2.3, SWAIII-2.4, SWB-1.1, SWB-1.2, SWB-1.3 and SWB-1.4 (lanes3, 4, 10, 11, 12, 13, 14, 15, 16 and 17). A protein molecular marker (lane 1, Appendix A) as well as an untransformed *pichia* clone (lane 5) was included in the analyses. (Lanes 6, 7, 8 and 9 contain clones that are not relevant to this study).

4.3.7 Western Blot analysis of *Pichia* transformants

From the SDS PAGE analysis, one clone for each construct, which displayed a high protein concentration in the desired size range, were chosen for Western blot analysis. To ensure that proteins were present on the SDS page gel used in Western analysis,

samples were separated on two SDS PAGE gels; one was used for silver staining to confirm the presence of proteins and the other for the Western blot (SDS-PAGE gels not presented). An untransformed *Pichia* clone was included in the analysis to determine any false positive hybridisation to other proteins present in *P. pastoris*. Membranes containing proteins from the supernatant of *P. pastoris* transformants harbouring the PG construct I and hybrid constructs, did not reveal any positive hybridising signal with the antibody directed against the *F. moniliforme* PG (results not shown). The only positive signal obtained was with the purified *F. moniliforme* PG protein that was included as a positive control in the blot (results not shown). Further analysis of the selected clones included a Western blot analysis of the boiled transformed *Pichia* cells, this blot revealed non specific hybridisation to all the clones including the untransformed *Pichia* clones (results not shown). Since the PG proteins of *F. moniliforme* and *Colletotrichum lupini* were only 42 % identical a Western blot analysis with an antibody directed towards the *A. niger* PG, which is more similar to the PG of *Colletotrichum lupini* (50%), was performed on both the supernatant of the selected clones of PG construct I and the PG hybrid constructs (Fig. 4.28) as well as the boiled cells (Fig. 4.29). The Western blot analysis of the supernatant of the clones revealed the presence of a high molecular weight band in all the clones except for the untransformed control of wild type *P. pastoris* (Fig. 4.28, lane 12). *Pichia* cells were boiled on order to release any protein (PG) that is not secreted. Analysis of the boiled *Pichia* cells resulted in non-specific hybridisation (Fig. 4.29). In both analyses the positive control (of *A. niger*) gave a signal (Fig. 4.28 & 4.29 lanes 2).

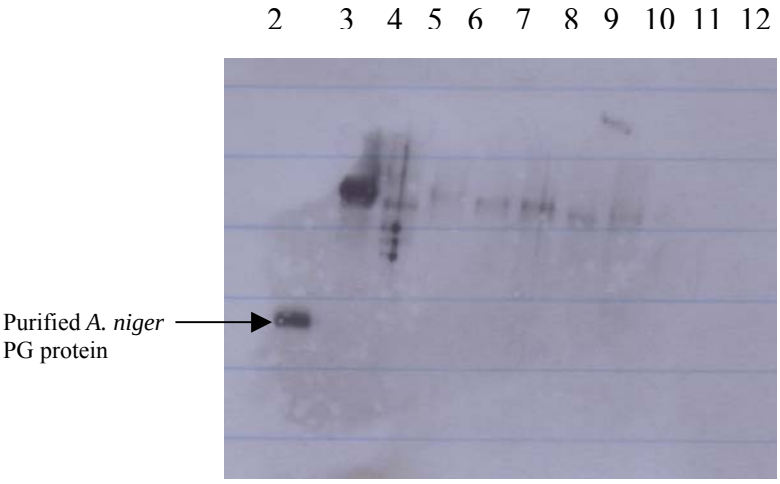


Fig. 4.28 Western blot analysis of the supernatant of *P. pastoris* clones containing different PG constructs. The blot was detected with an antibody directed against the PG of *A. niger*.
The western blot contained purified PG protein from *A. niger* (lane 2), proteins isolated from *Pichia* cells that were transformed with PG construct I-28.1, PG construct III-4.1, SWAIII-2.1 and SWB-1.1 (lanes 4, 6, 8 and 9 respectively) as well as an untransformed control of wild type *P. pastoris* (lane 12). A protein molecular marker (lane 1) was included in the analyses. Lanes 3, 5, 7,10 and 11 represent additional clones not applicable to this study.

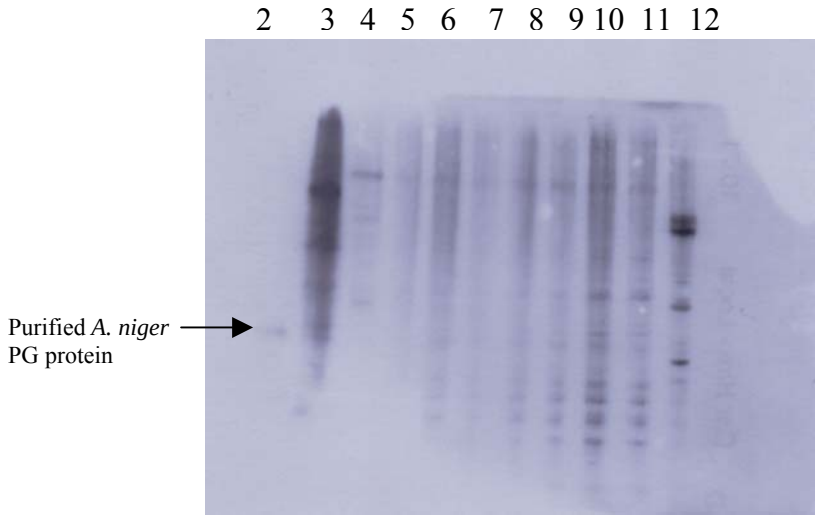


Fig. 4.29 Western blot analysis of boiled *P. pastoris* cells containing different PG constructs. Blots were detected with an antibody directed against a PG of *A. niger*.
The blot contained proteins isolated from *Pichia* cells that was transformed with PG construct I-28.1, PG construct III-4.1, SWAIII-2.1 and SWB-1.1 (lane 4, 6, 8 and 9 respectively). Purified A. niger PG protein (lane 2), an untransformed wild type *P. pastoris* (lane 12) as well as a molecular marker (lane 1) were included on the blot. Lanes 3, 5, 7, 10 and 11 represents additional clones not applicable to this study.

4.4 Discussion

A full-length cDNA PG copy, without an intron, was constructed by restriction digestion and ligation reactions. This construct was used to construct several PG gene constructs suitable for expression in *P. pastoris*. One construct (PG construct I) included the complete gene sequence to allow potential expression under the native signal peptide. Another construct (PG construct III) with the mature protein sequence was constructed by comparing previously reported sequence data for the *A. niger* PG and the *PG* genes from *C. lindemuthianum* with the PG sequence data of *Colletotrichum lupini*. This was used to determine the putative signal sequence and to construct a vector containing only the mature PG protein sequence or one where the N-terminal part of the mature *C. lupini* PG protein sequence was exchanged with that of the *F. moniliforme* PG. In addition to this, several other constructs were made, which were not discussed in this chapter. These included a construct which contained the mature protein sequence with additional upstream propeptide sequence, since it was reported that a propeptide sequence from a plant PG might play a role in expression and secretion (Dal Degan *et al*, 2001). Two tagged PG constructs, one containing the mature protein sequence and the other containing the additional propeptide sequence, were also included in the expression studies but not discussed in this study.

Even though clones selected for transformation of *P. pastoris* appeared to harbour the desired fragments, no PG activity was observed for any of the constructs expressed in *P. pastoris*. SDS-PAGE analysis revealed high protein concentrations in the reported size range for PGs, it was however difficult to determine if these proteins were only present in the transformed *Pichia* clones, since the presence or absence of these bands could not be clearly distinguished in the untransformed *Pichia* control. Western blot analysis was performed to determine if any protein, active or inactive was produced, intracellular or extra cellular. No clear positive signal was obtained for any of the clones and analysis of the boiled cells resulted in non-specific hybridisation. Since the PG of *F. moniliforme* is only 42 % identical to that of *Colletotrichum lupini*, it was decided to do a Western blot analysis of the clones with the antibody directed against *A. niger* which had a higher identity (50 %) to the PG of *Colletotrichum lupini*. With this antibody a putative positive signal was observed for the supernatant

of the clones, the size of the band was however very large. Glycosylation was considered as a reason for the increase in the protein size. Several post translational modifications including glycosylation, might increase the size of a protein. However since only a single N-glycosylation site was predicted for the PG protein of *C. lupini* SHK 2148 (http://www.cbs.dtu.dk/services/NetNGlyc/), it is unlikely that the size of the protein will increase dramatically to yield such a large band. Four potential glycosylation sites were predicted for the *F. moniliforme* PG (Caprari *et al*, 1993) and one for the *A. niger* PG (van Santen *et al*, 1999). Non-specific hybridisation was again observed when the boiled cells were analysed. Although the PG of *Colletotrichum lupini* could not be expressed in *P. pastoris* several other cell wall degrading enzymes (CWDE) of fungi have been expressed in *P. pastoris* or other yeast systems. Previous CWDE that were successfully expressed in *P. pastoris* included an endoPG gene of *Sclerotinia sclerotiorum* that has been successfully expressed in *Kluyveromyces lactis* under the control of the constitutive *pgk* promoter. The expressed protein was 37.5 kDa and not heavily glycosylated (Cotton *et al*, 2002). The PG gene of *F. moniliforme* was expressed in *S. cerevisiae* under the control of a galactose inducible promoter and the signal peptide sequence of the *Kluyveromyces lactis* killer toxin gene (Caprari *et al*, 1996). The *F. moniliforme* PG was in the size range 40-50 kDa (Caprari *et al*, 1996). *Fusarium solani* pectate lyase (*pelC*), *Fusarium solani* pectate lyase (*pelD*) were expressed and in *Pichia pastoris, pel*C and *PelD* were both secreted out of the cells and expressed under the *P. pastoris* acid phosphatase signal peptide (PHO) and native signal peptide respectively (Gonzalez-Candelas Guo *et al*, 1995 and Gonzalez-Candelas Guo *et al*, 1996).

Although results from this study suggest that the *Colletotrichum* PG is not expressed in *P. pastoris,* several additional controls need to be included in future studies to confirm these results that are only tentative; a *P. pastoris* isolate that has previously been shown to express the *F. moniliforme* PG should be included in the transformation of both the *E. coli* and *P. pastoris* system. Unfortunately this control was not included in the present study since the *P. pastoris* expression system, of the lab of Prof. Cervone in Rome, has been established and has always been very effective. However, recent communications with the lab suggest that they might currently be experiencing problems with the *P. pastoris* expression system, emphasizing the need to always include a *P. pastoris* control that expresses a known

protein should this be available. This would serve as a positive control in the PG activity assay in addition with purified PG protein of *F. moniliforme.* Apart from this a clear untransformed *Picha* clone as well as PG(s) extracted from *Colletotrichum* SHK 2148 should be included in both the SDS-PAGE and Western blot analysis to determine what proteins are unique to the transformed clones and similar to the extracted PG(s). Should these positive controls have been included it could have revealed whether there is a problem with the expression constructs or the *P. pastoris* expression system.

Furthermore, due to time constraints, none of the PG constructs were sequenced to confirm that no mutations occurred during their construction. PG construct I was subcloned while the others were constructed using PCR, which renders them more likely to mutations than PG construct I. Future studies should include a full sequence analysis of the plasmid DNA constructs that were isolated from the *E. coli*, prior to the transformation of *P. pastoris*. However, since the cDNA PG fragment was sequenced and had no mutations and the recently isolated complete cDNA PG displayed a 100% homology to the constructed cDNA PG gene (pPGcDNAcomplA, chapter 3) it was unlikely that mutations could have been introduced in all the constructs that were expressed in *P. pastoris*.

Chapter 5

Concluding discussion

Lupin anthracnose is a serious disease of lupin industries worldwide. This disease has also been reported in South Africa. The causal agent of lupin anthracnose in South Africa was previously identified as *Colletotrichum tortuosum* (Koch *et al*, 1996). Since this identification was based only on morphological properties, the aim was to re-evaluate the taxonomic placing of the lupin anthracnose isolates of South Africa. *Colletotrichum* SHK 2148 was compared with two type cultures of *C. lupini*, which represented the two recently described variants *C. lupini* var. *setosum*, and *C. lupini* var. lupini (Nirenberg *et al*, 2002). The culture morphology of *Colletotrichum* SHK 2148 was similar to that of *C. lupini* var. *setosum*, it furthermore produced setae, which were also reported for *C. lupini* var. *setosum*. The conidial shape and size of the three different isolates were alike under all the growth conditions. ITS and β-tubulin sequence data were collected for all three isolates and compared with previously submitted sequence data of *C. lupini*, *C. acutatum*, *C. gloeosporioides* and other *Colletotrichum* isolates. Phylogenetic analyses of the sequence data revealed that *Colletotrichum* SHK 2148 grouped closely with the *C. lupini* species especially the *C. lupini* var. *setosum*. Thus, morphological as well as molecular data supported the grouping of *Colletotrichum* SHK 2148 with the newly described *C. lupini* species. Two other isolates from South Africa, *Colletotrichum* SHK 1033 and *Colletotrichum* SHK 788, resembled *Colletotrichum* SHK 2148 in both morphology and ITS sequence data. Therefore, they all could be from the same original isolate that was introduced into South Africa and thus be grouped as *C. lupini* var. *setosum*.

Several plant pathogenic fungi produced endopolygalacturonase enzymes (endoPGs), which might be critical for their pathogenicity. EndoPG activity has been reported previously for *Colletotrichum lupini* SHK isolates. Chapter 3 of this study confirmed that activity for *C. lupini* SHK 2148, which reached its highest level after the fungus was grown for three days on the pectin media at pH 5. The aim of chapter 3 was to identify and characterise the gene(s) responsible for the endoPG activity and to determine the *in vitro* expression of this gene(s) when the fungus is grown on pectin as sole carbon source at pH 5. Conserved regions of previously identified endoPG genes from *C. gloeosporioides* f.sp. *malvae* (*cmpgII*) and *C. lindemuthianum* (*clpg2*), were used to design primers for the amplification of an internal fragment of a PG gene from *C. lupini* SHK 2148. The remaining sequence data of this PG gene was resolved

with inverse PCR. A complete PG gene was subsequently isolated from the genome of *C. lupini* SHK 2148. This gene was approximately 1Kb and very similar to the *C. gloeosporioides* f.sp. *malvae cmpgII* and *C. lindemuthianum clpg2* genes. A single 59 bp intron interrupted the gene sequence. The intron displayed the typical border sequences of filamentous fungal gene introns and the position of the intron appeared to be similar to that of *cmpgII* and *clpg2* genes. A putative TATAA signal and a CAAT box were detected in the 5' untranslated region of the gene. The deduced amino acid sequence of the endoPG gene was compared with other fungal endoPGs; this comparison revealed that the four conserved motifs described for endoPGs, were present in the *C. lupini* SHK 2148 endoPG protein. Northern blot analyses showed that the gene was expressed at the same time of maximum PG activity. No transcript was observed when the fungus was grown for 5, 6 or 12 days on the pectin. It might be possible that the RNA isolated on days 6 and 12 were slightly degraded or that more than one PG is responsible for PG activity and that the others are too different to be detected with the same probe. Another explanation for the absence of the transcript might be that the expression of the gene is pH dependant and that pH levels for that time periods did not support the expression. A full cDNA copy of the PG gene was obtained via RT-PCR amplification using mRNA isolated from the fungus after 4 days of growth on the pectin media. The cDNA copy was identical to the genomic copy except for the presence of the predicted intron.

A cDNA copy of the PG gene was constructed, since a full-length cDNA copy was not available at the time for expression studies, which were conducted in the Laboratories of Prof. F. Cervone in Rome. An internal genomic fragment, flanking the intron, was exchanged with the corresponding cDNA fragment. (A full cDNA copy, which was isolated via RT-PCR only after returning from Prof. Cervone's laboratory, were cloned, sequenced and compared to the sequence of the constructed cDNA copy and confirmed to be identical). The constructed cDNA copy was used to make PG constructs for the expression in *P. pastoris*. The mature PG gene with its own signal peptide, the PG gene with the α-MF signal peptide as well as a PG gene with the N-terminal sequence of the *F. moniliforme* PG gene were transformed into *P. pastoris*. None of the resulting clones showed any PG activity. SDS-PAGE gels were performed to evaluate the total protein profiles of the clones. The boiled cells and

supernatant of the clones were subjected to Western blot analyses with an *A. niger* and *F. moniliforme* PG antibody. Positive hybridisation was only observed with the supernatant and *A. niger* antibody. The size of the band displayed in the Western blot was higher than expected, and although glycosylation for PG genes has been reported and a single glycosylation site for the *Colletotrichum* SHK2148 protein has been predicted, it is unlikely to increase the size of the protein to such an extent. Personal communication with the laboratories of Prof. F. Cervone revealed that they might be experiencing problems with the *P. pastoris* expression system, thus results from the expression studies are not conclusive.

Regarding future work, it might be of interest to determine the expression of the PG gene under different pH and substrate conditions and further to express the complete cDNA copy, isolated from the mRNA, in *P. pastoris* with a suitable positive control.

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Appendix A

TE- buffer

10 mM Tris- HCl
1 mM EDTA
pH 8.0

Genomic DNA extraction buffer

200mM	Tris HCl pH8.5
250 mM	NaCl
25mM	EDTA
0.5 %	SDS

2 × Wash buffer

2 × SSC
0.1% SDS

0.5 × Wash buffer

0.5 × SSC
0.1% SDS

Maleic acid buffer (Southern blot)

0.1 M	Maleic acid
0.15 M	NaCl

Adjust pH to 7.5 with NaOH (solid)

Washing Buffer (Southern blot)

0.1 M Maleic acid
0.15 M NaCl
Adjust pH to 7.5 with NaOH (solid)
0.3 % (v/v) Tween 20

Detection buffer (Southern blot)

0.1 M Tris-HCl
0.15 M NaCl
pH 9.5

Formamide hybridisation buffer (Southern blot)

5 × SSC
50 % formamide, deionised
0.1 % sodium-lauroylsarcosine
0.02 % SDS
2% Blocking agent (fat-free milk powder)

Pectin medium

NH_4NO_3	1g / 500ml
KH_2PO_4	0.5g / 500ml
$MgSO_4$	0.05g / 500ml
Yeast extract	0.25g / 500ml
NaOH	0.5g / 500ml
D-maleic acid	1.5g / 500ml

0.25 g/ 25 ml washed pectin

BMMY (Buffered methanol-complex medium)

1% yeast extract
2% peptone
100mM potassium phosphate, pH 6.0
1.34 % YNB
4 × 10 –5 % biotin
1% glycerol or 0.5% methanol

LSLB (Low Salt Luria Broth) Medium

10 g Tryptone
5 g NaCl
5 g Yeast Extract

Combine the dry reagents above and add deionized, distilled water to 950 ml.
Adjust pH to 7.5 with 1 N NaOH. Bring the volume up to 1 liter. For plates, add 15 g/L agar before autoclaving.
Autoclave on liquid cycle at 15 lbs/sq. in. and 121°C for 20 minutes.
Allow the medium to cool to at least 55°C before adding the Zeocin to 25 μg/ml final concentration.
Store plates at 4°C in the dark. Plates containing Zeocin. are stable for 1-2 weeks.

5× RNA Loading buffer

Saturated bromophenol blue	16µl
500 mM EDTA, pH 8.0	80µl
37% (12.3 M) formaldehyde	700µl
100% glycerol	2ml
Formamide	3.084ml
10×FA gel buffer	4ml
RNase-free water to 10 ml	

10× FA gel buffer

200mM 3-[Morpholino]propanesulfonic acid(MOPS) (free acid)
50mM Sodium acetate
10 mM EDTA
pH to 7.0 with NaOH

1× FA gel buffer

10 × FA gel buffer	100 ml
37% (12.3 M)	20 ml
RNase-free water to 1L	

SDS-PAGE separating gel (10 %)

Acrilamide stock (30%)	1650 µl
Tris- HCl (1.5 M) pH 6.8	620 µl
SDS (10%)	50 µl
APS	25 µl
Temed	8 µl
dH_2O	2020 µl

SDS-PAGE stacking gel

Tris-HCl (0.5M) pH 6.8	620 µl
SDS (10%)	25 µl
Acrilamide stock (30%)	330 µl
APS (10%)	15 µl
Temed	8 µl
dH_2O	1520 µl

Loading Buffer (SDS-PAGE) 3 ×

Tris-HCl pH 6.8 (50mM)	3 ml
Glicerol (8%)	2.4 ml
SDS (20%)	2,4 ml
2-β-mercaptoetanol (4%)	1.2 ml
dH_2O to an end volume of 10 ml	
Bromophenolblue to the desired colour intensity	

Transfer stock solution 10 × (Western Blot)

Tris (base)	96.8 g/l
Glycine	9.74 g/l
pH 9.2	

Transfer Buffer (Western Blot)

Methanol	200 ml
Transfer stock solution 10×	100 ml
dH_2O	700 ml

PBS 10 × Stock

NaCl	1.3 M
Na_2HPO_4 .$12H_2O$	70 mM
NaH_2PO_4. $1H_2O$	30 mM
pH 7.3	

Blocking buffer (Western blot)

1× PBS	
Tween	(0.2%)
Fat free milk powder	(5%)
or BSA	(3%)

Washing buffer (Western blot)

1× PBS	
Tween	(0.2%)
H_2O (ultra pure)	

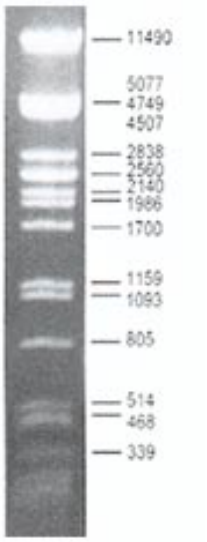
Incubation buffer (Western blot)

1× PBS	
Tween	(0.2%)
BSA	(0.5%)
H_2O (ultra pure)	

1 × TNE Buffer

10 mM Tris-HCl pH 8.0
1 mM EDTA pH 8.0
0.2 M NaCl
pH 7.4

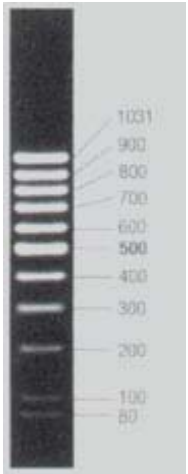
Appendix B



λ *PstI* generated DNA molecular marker

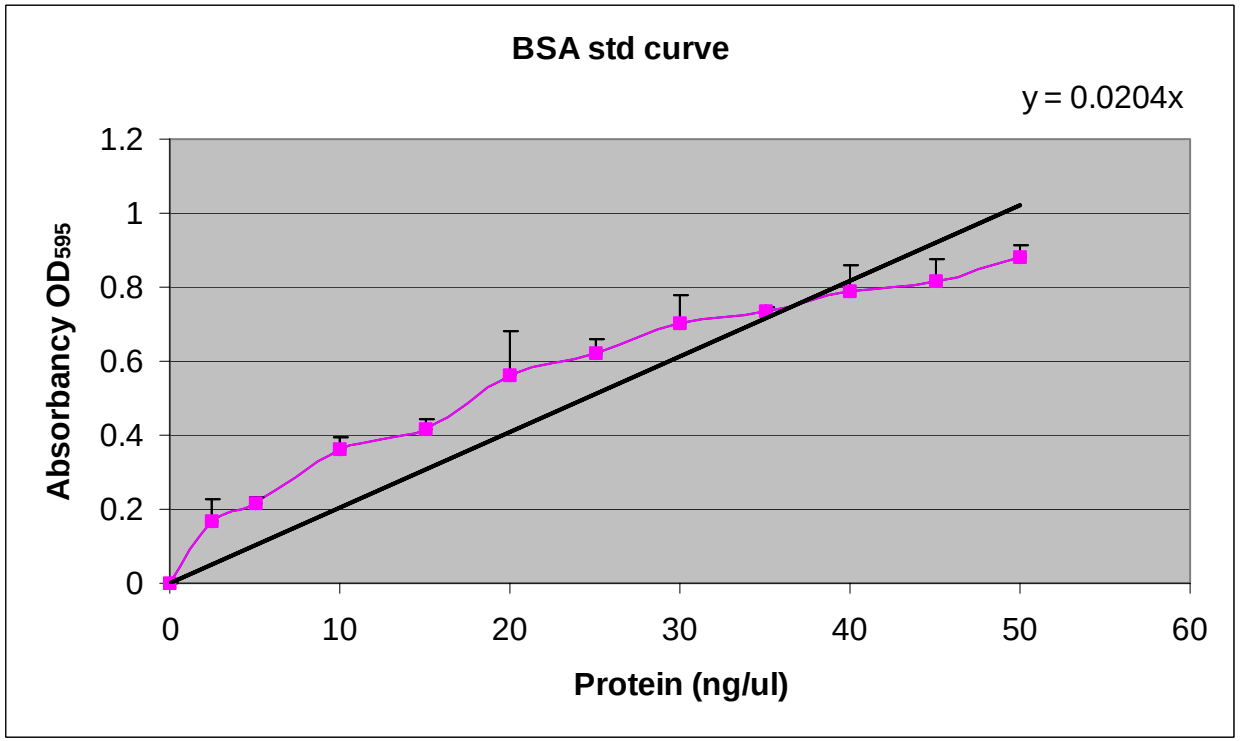
1KB plus DNA molecular marker

SDS protein markers



100 bp molecular marker

Appendix C



BSA standard curve for the Biorad microassay procedure performed in triplicate.

Appendix D

Complete aligned nucleotide sequences of the ITS1, 5.8 S rDNA and ITS2 region for 31 different taxa including *Colletotrichum* SHK 2148

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                                           111111111122222222223333333333444444444455555555556
Taxon/Node                        123456789012345678901234567890123456789012345678901234567890
-----------------------------------------------------------------------------------------------
C. gloeosporioides (AF090855)     ---------------TAGGGTCTCCGCGACCCTCCCGGCCTCCC-GCCTCCGGGCGGGTC   [44]
C. gloeosporioides (HO19)         ---------------...................-.C.G.CT..C...............   [44]
C. gloeosporioides (CR45)         -----TAGGGTCTCCGC.A-----.C.TC..GG..TCCG.CT..-.G.---...TC..--   [44]
C. gloeosporioides (CR21)         TAGGG-----TCTCCGC.A-----.C.TC..GG..TC--.CG..-T..GGGC..GTC.--   [45]
C. coccodes (BBA71527)            ---GGACAACCCCCC.C...GGG.G.TCC.....-.C.G.CG.G-C....AC..GC.T--   [53]
C. cf. truncatum (BBA71528)       CGGGCAGAGGTTCCC.C.C.GAA..C.TC..GGTGAC.-.C.T.-A.-----..GC.T--   [51]
C. acutatum (AF081292)            CAGGGGAAGCCTCTCGC...C....C.TC..GG.G.C.G.C...CA..A.G...AC..--   [58]
C. acutatum (AF090853)            CAGGGGAAGCCTCTCGC...C....C.TC..GG.G.C.G.C...-A..A.G...AC..--   [57]
C. acutatum (BBA71292)            CAGGGGAAGCCTCTCGC...C....C.TC..GG.G.C.G.C...-A..A.G...AC..--   [57]
C. acutatum (C2897)               CAGGGGAAGCCTCTCGC...C....C.TC..GG.G.C.G.C...-A..A.G...AC..--   [57]
C. acutatum (CMG12)               CAGGGGAAGCCTCTCGC...C....C.TC..GG.G.C.G.C...-A..A.G...AC..--   [57]
C. lupini var. setosum (BBA70073) CAGGGGAAGCCTCTCGC...C....C.TC..GG.G.C.G.C...-A..A.G...AC..--   [57]
Colletotrichum SHK2148            CAGGGGAAGCCTCTCGC...C....C.TC..GG.G.C.G.C...-A..A.G...AC..--   [57]
C. lupini var. setosum (BBA70352) CAGGGGAAGCCTCTCGC...C....C.TC..GG.G.C.G.C...-A..A.G...AC..--   [57]
C. lupini (BBA71330)              CAGGGGAAGCCTCTCGC...C....C.TC..GG.G.C.G.C...-A..A.G...AC..--   [57]
C. lupini var. setosum (BBA71310) CAGGGGAAGCCTCTCGC...C....C.TC..GG.G.C.G.C...-A..A.G...AC..--   [57]
C. lupini var. lupini (BBA63879)  CAGGGGAAGCCTCTCGC...C....C.TC..GG.G.C.G.C...-A..A.G...AC..--   [57]
C. lupini var. lupini (BBA70884)  CAGGGGAAGCCTCTCGC...C....C.TC..GG.G.C.G.C...-A..A.G...AC..--   [57]
C. lupini var. lupini (BBA71249)  CAGGGGAAGCCTCTCGC...C....C.TC..GG.G.C.G.C...-A..A.G...AC..--   [57]
C. acutatum (HY09)                CAGGGGAAGCCTCTCGC...C....C.TC..GG.G.C.G.C...-A..A.G...AC..--   [57]
C. acutatum (BBA71370)            CAGGGGAAGCCTCTCGC...C....C.TC..GG.G.C.G.C..--A..A.G...AC..--   [56]
C. acutatum (BBA71371)            CAGGGGAAGCCTCTCGC...C....C.TC..GG.G.C.G.C..--A..A.G...AC..--   [56]
C. acutatum (JG05)                CAGGGGAAGCCTCTCGT...CGGA.C.TC..GG.G.C.G.C..--.T.A.G...GC..--   [56]
C. acutatum (TN47)                CAGGGGAAGCCTCTCGT...CGGA.C.TC..GG.G.C.G.C..--.T.A.G...GC..--   [56]
C. acutatum (CR46)                CAGGGGAAGCCTCTCGC...C....C.TC..GG.G.C.G.C...-A..A.G...AC..--   [57]
Neurospora crassa                 CCGGAAAGGCCTT---C...C....CG..T.--.T...GTCT..-----.GCTCGC..C-   [49]


                                                                         111111111111111111111
                                  666666666777777777788888888889999999999000000000011111111112
Taxon/Node                        123456789012345678901234567890123456789012345678901234567890
-----------------------------------------------------------------------------------------------
C. gloeosporioides (AF090855)     GGCGCCCGCCGGAGGAT---AACCAAACTCTGATTTAACGACGTTTCTTCTGAGTGGTAC   [101]
C. gloeosporioides (HO19)         .................---........................................   [101]
C. gloeosporioides (CR45)         --...............---........................................   [99]
C. gloeosporioides (CR21)         .-.......G.AG.AT----........................................   [100]
C. coccodes (BBA71527)            ................----T..........AT........................C..   [109]
C. cf. truncatum (BBA71528)       C...............----T..........AT........................C..   [107]
C. acutatum (AF081292)            ................----...........AT..AC.......C............C..   [114]
C. acutatum (AF090853)            ................----...........AT..AC.......C............C..   [113]
C. acutatum (BBA71292)            ................----...........AT..AC.......C............C..   [113]
C. acutatum (C2897)               ................----...........AT..AC.......C............C..   [113]
C. acutatum (CMG12)               ................----...........AT..AC.......C............C..   [113]
C. lupini var. setosum (BBA70073) ................----...........AT..AC.......C............C..   [113]
Colletotrichum SHK2148            ................----...........AT..AC.......C............C..   [113]
C. lupini var. setosum (BBA70352) ................----...........AT..AC.......C............C..   [113]
C. lupini (BBA71330)              ................----...........AT..AC.......C............C..   [113]
C. lupini var. setosum (BBA71310) ................----...........AT..AC.......C............C..   [113]
C. lupini var. lupini (BBA63879)  ................----...........AT..AC.......C............C..   [113]
C. lupini var. lupini (BBA70884)  ................----...........AT..AC.......C............C..   [113]
C. lupini var. lupini (BBA71249)  ................----...........AT..AC.......C............C..   [113]
C. acutatum (HY09)                ................----...........AT..AC.......C............C..   [113]
C. acutatum (BBA71370)            ................----...........AT..AC.......C............C..   [112]
C. acutatum (BBA71371)            ................----...........AT..AC.......C............C..   [112]
C. acutatum (JG05)                A...............----...........AT..AC.......C............C..   [112]
C. acutatum (TN47)                A...............----...........AT..AC.......C............C..   [112]
C. acutatum (CR46)                ................----...........AT..AC.......C............C..   [113]
Neurospora crassa                 --T...........TGCCGA...T.......TGA..TTT-.T..C...-......AA-..   [104]
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                                 1111111111111111111111111111111111111111111111111111111111111111
                                 2222222223333333333444444444455555555556666666666777777777778
Taxon/Node                       1234567890123456789012345678901234567890123456789012345678901234567890
-------------------------------------------------------------------------------------------------------------
C. gloeosporioides (AF090855)    AAGCAAATAATCAAAACTTTT-AACAACCGGATCTCTTGGTTCTGGCATCGATGAAGAAC   [160]
C. gloeosporioides (HO19)        ....................-......G.-............................   [159]
C. gloeosporioides (CR45)        ....................-......G.-............................   [157]
C. gloeosporioides (CR21)        ..........C.........-......G.-............................   [157]
C. coccodes (BBA71527)           ...........T.........-......G.-............................   [167]
C. cf. truncatum (BBA71528)      ...........T.........-......G.-............................   [165]
C. acutatum (AF081292)           ...........T.........T......G.............................   [174]
C. acutatum (AF090853)           ...........T.........-......G.-............................   [171]
C. acutatum (BBA71292)           ...........T.........-......G.-............................   [171]
C. acutatum (C2897)              ...........T.........-......G.-............................   [171]
C. acutatum (CMG12)              ...........T.........-......G.-............................   [171]
C. lupini var. setosum (BBA70073) ...........T.........-......G.-............................   [171]
Colletotrichum SHK2148           ...........T.........-......G.-............................   [171]
C. lupini var. setosum (BBA70352) ...........T.........-......G.-............................   [171]
C. lupini (BBA71330)             ...........T.........-......G.-............................   [171]
C. lupini var. setosum (BBA71310) ...........T.........-......G.-............................   [171]
C. lupini var. lupini (BBA63879) ...........T.........-......G.-............................   [171]
C. lupini var. lupini (BBA70884) ...........T.........-......G.-............................   [171]
C. lupini var. lupini (BBA71249) ...........T.........-......G.-............................   [171]
C. acutatum (HY09)               ...........T.........-......G.-............................   [171]
C. acutatum (BBA71370)           ...........T.........-......G.-............................   [170]
C. acutatum (BBA71371)           ...........T.........-......G.-............................   [170]
C. acutatum (JG05)               ...........T.........-......G.-............................   [170]
C. acutatum (TN47)               ...........T.........-......G.-............................   [170]
C. acutatum (CR46)               ...........T.........-......G.-............................   [171]
Neurospora crassa                TTTT......GTC...AC...C......G.-............................   [163]


                                 1111111111111111111222222222222222222222222222222222222222222222
                                 8888888889999999999000000000011111111112222222222333333333334
Taxon/Node                       1234567890123456789012345678901234567890123456789012345678901234567890
-------------------------------------------------------------------------------------------------------------
C. gloeosporioides (AF090855)    GCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAA   [220]
C. gloeosporioides (HO19)        ..................................................................   [219]
C. gloeosporioides (CR45)        ..................................................................   [217]
C. gloeosporioides (CR21)        ..................................................................   [217]
C. coccodes (BBA71527)           ..................................................................   [227]
C. cf. truncatum (BBA71528)      ..................................................................   [225]
C. acutatum (AF081292)           ..................................................................   [234]
C. acutatum (AF090853)           ..................................................................   [231]
C. acutatum (BBA71292)           ..................................................................   [231]
C. acutatum (C2897)              ..................................................................   [231]
C. acutatum (CMG12)              ..................................................................   [231]
C. lupini var. setosum (BBA70073) ..................................................................   [231]
Colletotrichum SHK2148           ..................................................................   [231]
C. lupini var. setosum (BBA70352) ..................................................................   [231]
C. lupini (BBA71330)             ..................................................................   [231]
C. lupini var. setosum (BBA71310) ..................................................................   [231]
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C. lupini var. lupini (BBA70884) ..................................................................   [231]
C. lupini var. lupini (BBA71249) ..................................................................   [231]
C. acutatum (HY09)               ..................................................................   [231]
C. acutatum (BBA71370)           ..................................................................   [230]
C. acutatum (BBA71371)           ..................................................................   [230]
C. acutatum (JG05)               ..................................................................   [230]
C. acutatum (TN47)               ..................................................................   [230]
C. acutatum (CR46)               ..................................................................   [231]
Neurospora crassa                ................G.................................................   [223]
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                                 2222222222222222222222222222222222222222222222222222222222223
                                 4444444445555555556666666666777777777788888888899999999990
Taxon/Node                       1234567890123456789012345678901234567890123456789012345678901234567890
-------------------------------------------------------------------------------------------------------
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C. gloeosporioides (HO19)        ..........................................................   [279]
C. gloeosporioides (CR45)        ..........................................................   [277]
C. gloeosporioides (CR21)        ..........................................-...............   [276]
C. coccodes (BBA71527)           ..........................................................   [287]
C. cf. truncatum (BBA71528)      ..........................................................   [285]
C. acutatum (AF081292)           ...........T................A..............................   [294]
C. acutatum (AF090853)           ...........T................A.................A............   [291]
C. acutatum (BBA71292)           ...........T................A..............................   [291]
C. acutatum (C2897)              ...........T................A..............................   [291]
C. acutatum (CMG12)              ...........T................A..............................   [291]
C. lupini var. setosum (BBA70073) ...........T................A..............................   [291]
Colletotrichum SHK2148           ...........T................A..............................   [291]
C. lupini var. setosum (BBA70352) ...........T................A..............................   [291]
C. lupini (BBA71330)             ...........T................A..............................   [291]
C. lupini var. setosum (BBA71310) ...........T................A..............................   [291]
C. lupini var. lupini (BBA63879) ...........T................A..............................   [291]
C. lupini var. lupini (BBA70884) ...........T................A..............................   [291]
C. lupini var. lupini (BBA71249) ...........T................A..............................   [291]
C. acutatum (HY09)               ...........T................A..............................   [291]
C. acutatum (BBA71370)           ...........T................A..............................   [290]
C. acutatum (BBA71371)           ...........T................A..............................   [290]
C. acutatum (JG05)               ...........T................A..............................   [290]
C. acutatum (TN47)               ...........T................A..............................   [290]
C. acutatum (CR46)               ...........T................A..............................   [291]
Neurospora crassa                ...........T......T.........A............................A.   [283]


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                                 0000000001111111111222222222233333333334444444445555555556
Taxon/Node                       1234567890123456789012345678901234567890123456789012345678901234567890
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C. gloeosporioides (AF090855)    CAAGCTCTGCTTGGTGTTGGGGCCCTACAGC-CGATGTAGGCCCTCAAAGGTAGTGGCGG   [339]
C. gloeosporioides (HO19)        ..............................-............................   [338]
C. gloeosporioides (CR45)        ..............................-............................   [336]
C. gloeosporioides (CR21)        ..............................-............................   [335]
C. coccodes (BBA71527)           .....C.....................G.T-...C.........T..............   [346]
C. cf. truncatum (BBA71528)      ............................G.TTAA.C.........T..............   [345]
C. acutatum (AF081292)           .....A.C.......T.........C..G..-AC.C..G......T..............   [353]
C. acutatum (AF090853)           .....A.C.......T.........C..G..-AC.C..G......T..............   [350]
C. acutatum (BBA71292)           .....A.C.......T.........C..G..-AC.C..G......T..............   [350]
C. acutatum (C2897)              .....A.C.......T.........C..G..-AC.C..G......T..............   [350]
C. acutatum (CMG12)              .....A.C.......T.........C..G..-AC.C..G......TG.............   [350]
C. lupini var. setosum (BBA70073) .....A.C.......T.........C..G..-AC.C..G......TG.............   [350]
Colletotrichum SHK2148           .....A.C.......T.........C..G..-AC.C..G......TG.............   [350]
C. lupini var. setosum (BBA70352) .....A.C.......T.........C..G..-AC.C..G......TG.............   [350]
C. lupini (BBA71330)             .....A.C.......T.........C..G..-AC.C..G......TG.............   [350]
C. lupini var. setosum (BBA71310) .....A.C.......T.........C..G..-AC.C..G......TG.............   [350]
C. lupini var. lupini (BBA63879) .....A.C.......T.........C..G..-AC.C..G......TG.............   [350]
C. lupini var. lupini (BBA70884) .....A.C.......T.........C..G..-AC.C..G......TG.............   [350]
C. lupini var. lupini (BBA71249) .....A.C.......T.........C..G..-AC.C..G......TG.............   [350]
C. acutatum (HY09)               .....A.C.......T.........C..G..-AC.C..G......TG.............   [350]
C. acutatum (BBA71370)           .....A.C.......T.........C..G..-AC.C..G......T..............   [349]
C. acutatum (BBA71371)           .....A.C.......T.........C..G..-AC.C..G......T..............   [349]
C. acutatum (JG05)               .....A.C.......T.........C..G..-...C..G......T..............   [349]
C. acutatum (TN47)               .....A.C.......T.........C..G..-...C..G......T..............   [349]
C. acutatum (CR46)               .....A.C.......T.........C..G..-...C..G......T..............   [350]
Neurospora crassa                ............-C.......AT.CG.G..-------.GTC.G......ATC........   [336]
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                                 6666666667777777777888888888899999999990000000000011111111112
Taxon/Node                       1234567890123456789012345678901234567890123456789012345678901234567890
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C. gloeosporioides (AF090855)    ACCCTCCCGGAGCCTCCTTTGCGTAGTAACTTTACGTCTCGCACTGGGATCCGGAGGGAC   [399]
C. gloeosporioides (HO19)        ........................................................      [398]
C. gloeosporioides (CR45)        ........................................................      [396]
C. gloeosporioides (CR21)        ........................................................      [395]
C. coccodes (BBA71527)           ..............................A.........................      [406]
C. cf. truncatum (BBA71528)      ......T.......................A-..................T........T   [404]
C. acutatum (AF081292)           ..............................A-..................T.........   [412]
C. acutatum (AF090853)           ...............................A..................T.........   [409]
C. acutatum (BBA71292)           ..............................A-..................T.........   [409]
C. acutatum (C2897)              ..............................A-..................T.........   [409]
C. acutatum (CMG12)              ..............................A-..........................   [409]
C. lupini var. setosum (BBA70073) ..............................A-........................-..   [408]
Colletotrichum SHK2148           ..............................A-..........................   [409]
C. lupini var. setosum (BBA70352) ..............................A-..........................   [409]
C. lupini (BBA71330)             ..............................A-..........................   [409]
C. lupini var. setosum (BBA71310) ..............................A-..........................   [409]
C. lupini var. lupini (BBA63879) ...............................-A...........C..............   [409]
C. lupini var. lupini (BBA70884) ..............................A-...........C..............   [409]
C. lupini var. lupini (BBA71249) ..............................A-...........C..............   [409]
C. acutatum (HY09)               ..............................A-...........C..............   [409]
C. acutatum (BBA71370)           ..............................A-..........................   [408]
C. acutatum (BBA71371)           ..............................A-..........................   [408]
C. acutatum (JG05)               ..............................A-..........................   [408]
C. acutatum (TN47)               ..............................A-..........................   [408]
C. acutatum (CR46)               ..............................A-..........................   [409]
Neurospora crassa                G.T.GT.A.TCA.AC.GA--....G..G...C...A..G.-T.TG.TCG.G...C...-T   [392]
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                                 4444444444444444444
                                 2222222223333333333
Taxon/Node                       1234567890123456789
-----------------------------------------------------------
C. gloeosporioides (AF090855)    TCTTGCCGTAAAACCCCCC   [418]
C. gloeosporioides (HO19)        ...................   [417]
C. gloeosporioides (CR45)        ...................   [415]
C. gloeosporioides (CR21)        ...................   [414]
C. coccodes (BBA71527)           ..................A   [425]
C. cf. truncatum (BBA71528)      ...A..............A   [423]
C. acutatum (AF081292)           ..................A   [431]
C. acutatum (AF090853)           ..................A   [428]
C. acutatum (BBA71292)           ..................A   [428]
C. acutatum (C2897)              ..................A   [428]
C. acutatum (CMG12)              ...................   [428]
C. lupini var. setosum (BBA70073) ...................   [427]
Colletotrichum SHK2148           ...................   [428]
C. lupini var. setosum (BBA70352) ...................   [428]
C. lupini (BBA71330)             ...................   [428]
C. lupini var. setosum (BBA71310) ...................   [428]
C. lupini var. lupini (BBA63879) ...................   [428]
C. lupini var. lupini (BBA70884) ...................   [428]
C. lupini var. lupini (BBA71249) ...................   [428]
C. acutatum (HY09)               ...................   [428]
C. acutatum (BBA71370)           ...................   [427]
C. acutatum (BBA71371)           ...................   [427]
C. acutatum (JG05)               .........T........A   [427]
C. acutatum (TN47)               .........T........A   [427]
C. acutatum (CR46)               .........T........A   [428]
Neurospora crassa                ...................   [411]
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Appendix E

Complete nucleotide alignment of the β-tubulin region 1 and β-tubulin region2 from several *Colletotrichum* isolates and one *Neurospora crassa* outgroup.

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                                           111111111122222222223333333333444444444455555555556
Taxon/Node                        123456789012345678901234567890123456789012345678901234567890
----------------------------------------------------------------------------------------------------
C. acutatum(PT30)                 ????????????????????????????????CCTTGATACAGGCAGAACATCTCTGGCG   [60]
C. acutatum(PT29)                 ATTTCGATATTTGACACGATCTC--GAACTGA............................   [58]
C. acutatum(JR15)                 ATTTCGATATTTGACACGATCTC--GAACTGA............................   [58]
C. acutatum(CR02)                 ATTTCGATATTTGACACGATCTC--GAACTGA............................   [58]
C. acutatum(96A4)                 ATTTCGATATTTGACACGATCTC--GAACTGA............................   [58]
C.lupini var setosum(CW52)        ATTTCGATATTTGACACGATCTC--GAACTGA............................   [58]
C.lupini var lupini(CW51)         ATTTCGATATTTGACACGATCTC--GAACTGA.....GC.....................   [58]
Colletotrichum SHK2148            ATTTCGATATTTGACACGATCTC--GAACTGA............................   [58]
C. acutatum(JR03)                 ATTCCGACATTTGACACGATCTC--GTACTGA.....G........A.............   [58]
C. acutatum(HO01)                 ATTCCGACATTTGACACGATCTC--GTACTGA.....G........A.............   [58]
C. acutatum(CR20)                 ATTCCGACATTTGACACGATCTC--GTACTGA.....G........A.............   [58]
C. acutatum(C2897)                ATTCCGACATTTGACACGATCTC--GTACTGA.....G........A.............   [58]
C. acutatum(JG05)                 ATTTCGACATTTGACACGATCTC--ATACTGA...C..C.......A.............   [58]
C. acutatum(CR46)                 ATTTCGACATTTGATACGATTTC--GTACTGA.....G........A.............   [58]
C. gloeosporioides(CR45)          ATATC-ATACTTGCGAGGACGGCAGATGTTGA.GA..GA.T.....A.....T.......   [59]
C. gloeosporioides(CR21)          ATATC-ATACTTGCGAGGACGGCAGATGTTGA.GA..GA.T.....A.....T.......   [59]
C. gloeosporioides(315)           ATAT--ATACTTGCGAGGACGGCAGATGTTGA.GA.AGAGT.....A.....T.......   [58]
C. gloeosporioides (HO19)         AATATCATACTTGCGAGGACGGCAGATGTTGA.GA..GA.T.....A.....T.......   [60]
C. falcatum(CFA12)                AGATCGGTCTCGGAAAGACTAGAT-ATATTGATACGAGC.......A.............   [59]
Neurospora crassa(M13906)         ????????????????????????????????????????????????????????????   [60]

                                                                         111111111111111111111
                                  666666666777777777788888888889999999999000000000011111111112
Taxon/Node                        123456789012345678901234567890123456789012345678901234567890
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C. acutatum(PT30)                 AGCACGGTCTCGACAGCAATGGCGTGTATGTCACTT--GTCCTCCAGTGCGGCATCCTCA   [118]
C. acutatum(PT29)                 ....................................--......................   [116]
C. acutatum(JR15)                 ....................................--......................   [116]
C. acutatum(CR02)                 ....................................--......................   [116]
C. acutatum(96A4)                 ....................................--......................   [116]
C.lupini var setosum(CW52)        ....................................--......................   [116]
C.lupini var lupini(CW51)         ....................................--......................   [116]
Colletotrichum SHK2148            ....................................--......................   [116]
C. acutatum(JR03)                 ...................................C--...........T...T...C.G   [116]
C. acutatum(HO01)                 ...................................C--...........T...T...C.G   [116]
C. acutatum(CR20)                 ...................................C--...........T...T...C.G   [116]
C. acutatum(C2897)                ...................................C--...........T...T...C.G   [116]
C. acutatum(JG05)                 ................................T...--............A..TA....G   [116]
C. acutatum(CR46)                 ....................................T-...............TG..C..   [117]
C. gloeosporioides(CR45)          .......C..A...........A..........TG-----..C.TTA.CT.T.CA.A.TG   [114]
C. gloeosporioides(CR21)          .......C..A...........A..........TG-----..C.TTA.CT.T.CA.A.TG   [114]
C. gloeosporioides(315)           .......C..............A..........TG----C..CTT.TCTG.C..CAT.G.   [114]
C. gloeosporioides (HO19)         .......C..A...........A..........T----.C..CTT.TCTGTC..CAT.GG   [116]
C. falcatum(CFA12)                .......C.......................AG.C---.A..C.AGA.CT..TCA.T.AC   [116]
Neurospora crassa(M13906)         ????????????????????????????????????????????????????????????   [120]

                                  111111111111111111111111111111111111111111111111111111111111
                                  222222222233333333334444444444555555555566666666667777777778
Taxon/Node                        123456789012345678901234567890123456789012345678901234567890
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C. acutatum(PT30)                 TGGACCCAG-CAGCTAATCACACCACAGGTACAACGGCACTTCCGAGCTCCAGCTCGAGC   [177]
C. acutatum(PT29)                 .........-..................................................   [175]
C. acutatum(JR15)                 .........-..................................................   [175]
C. acutatum(CR02)                 .........-..................................................   [175]
C. acutatum(96A4)                 .........-..................................................   [175]
C.lupini var setosum(CW52)        .........-..................................................   [175]
C.lupini var lupini(CW51)         .........-..........T.......................................   [175]
Colletotrichum SHK2148            .........-..................................................   [175]
C. acutatum(JR03)                 .........-..........T....T..................................   [175]
C. acutatum(HO01)                 .........-..........T....T..................................   [175]
C. acutatum(CR20)                 .........-..........T....T..................................   [175]
C. acutatum(C2897)                .........-..........T....T..................................   [175]
C. acutatum(JG05)                 .........-..........T.............T.........................   [175]
C. acutatum(CR46)                 ......T..-T.......T.T.......................................   [176]
C. gloeosporioides(CR45)          GT.GTTG.--.C.....A.T.G-A....C...........C..T................   [171]
C. gloeosporioides(CR21)          GT.GTTG.--.C.....A.T.G-A....C...........C..T................   [171]
C. gloeosporioides(315)           ...--TTGA-.C.....A.T.G-A....C...........C..T................   [170]
C. gloeosporioides (HO19)         ...--TTGA-.C.....A.T.GA-....C...........C..T................   [172]
C. falcatum(CFA12)                .CA.GTTTA-.C......TT.T.A....T...........C..T................   [175]
Neurospora crassa(M13906)         ???????????G...TTCGG.C.GGGGTCGG.....A.CACC.AAG..CGG.AAG.T.T.   [180]
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Taxon/Node                               123456789012345678901234567890123456789012345678901234567890
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C. acutatum(PT30)                        GCATGAGCGTCTATTTCAACGAAGTTT--GTTATCCTAGT-CCCCCAGTGTGCAGGC-AA   [233]
C. acutatum(PT29)                        ...........................--...........-................-..   [231]
C. acutatum(JR15)                        ...........................--...........-................-..   [231]
C. acutatum(CR02)                        ...........................--...........-................-..   [231]
C. acutatum(96A4)                        ...........................--...........-................-..   [231]
C.lupini var setosum(CW52)               ...........................--...........-................-..   [231]
C.lupini var lupini(CW51)                ...........................--...........-................-..   [231]
Colletotrichum SHK2148                   ...........................--...........-................-..   [231]
C. acutatum(JR03)                        .............C.............--...........-................-..   [231]
C. acutatum(HO01)                        .............C.............--...........-................-..   [231]
C. acutatum(CR20)                        .............C.............--...........-................-..   [231]
C. acutatum(C2897)                       .............C.............--...........-................-..   [231]
C. acutatum(JG05)                        .............C.............--...........-..T.............-..   [231]
C. acutatum(CR46)                        .T...........C.............--...........-................-..   [232]
C. gloeosporioides(CR45)                 .............C.............--....C.T..TAG....A..A.....A.AT..   [229]
C. gloeosporioides(CR21)                 ............C.............--....C.T..TAG....A..A.....A.AT..    [229]
C. gloeosporioides(315)                  .............C.............--....C.T..TAG.......A.....A.AT..   [228]
C. gloeosporioides (HO19)                .............C.............--....C.T..TAG....A..A.....A.AT..   [230]
C. falcatum(CFA12)                       .............C.............--...........-......TG.C......-..   [231]
Neurospora crassa(M13906)                CA.ACTCG...-....AG.G.....AAAA..CG.AAC.AGGT....GT..GTG.AC.--.   [237]

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Taxon/Node                               123456789012345678901234567890123456789012345678901234567890
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C. acutatum(PT30)                        TCCTATTGACGAATGCTGACC-TTCTCACCCAACCAGGCCTCCGGCAACAAGTACGTTCC   [292]
C. acutatum(PT29)                        .....................-......................................   [290]
C. acutatum(JR15)                        .....................-......................................   [290]
C. acutatum(CR02)                        .....................-......................................   [290]
C. acutatum(96A4)                        .....................-......................................   [290]
C.lupini var setosum(CW52)               .....................-......................................   [290]
C.lupini var lupini(CW51)                .....................-......................................   [290]
Colletotrichum SHK2148                   .....................-......................................   [290]
C. acutatum(JR03)                        .....................-......................................   [290]
C. acutatum(HO01)                        .....................-......................................   [290]
C. acutatum(CR20)                        .....................-......................................   [290]
C. acutatum(C2897)                       .....................-......................................   [290]
C. acutatum(JG05)                        .............A.......-......................................   [290]
C. acutatum(CR46)                        .....................-......................................   [291]
C. gloeosporioides(CR45)                 A.A.........G.A......-...G.T..T.C......T.................G..   [288]
C. gloeosporioides(CR21)                 A.A.........G.A......-...G.T..T.C......T.................G..   [288]
C. gloeosporioides(315)                  A.A.........G.A......-...G.T..T.C......T.................G..   [287]
C. gloeosporioides (HO19)                A.A.........G.A......-...G.T..T.C......T.................G..   [289]
C. falcatum(CFA12)                       C..C.....T....A......C..T....G..T......T..............T..C..   [291]
Neurospora crassa(M13906)                G.GG.GG..TC.T.A.A..G--.--.--G.A..A.TCC.ACAAAC..T.GC.A.TC..A.   [291]

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Taxon/Node                               123456789012345678901234567890123456789012345678901234567890
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C. acutatum(PT30)                        TCGCGCCGT--CCTCGTCGACTTGGAGCCCGGTACCATGGACGCCGTCCGTGCCGGTCCC   [350]
C. acutatum(PT29)                        .........--.................................................   [348]
C. acutatum(JR15)                        .........--.................................................   [348]
C. acutatum(CR02)                        .........--.................................................   [348]
C. acutatum(96A4)                        .........--.................................................   [348]
C.lupini var setosum(CW52)               .........--.................................................   [348]
C.lupini var lupini(CW51)                .........--.................................................   [348]
Colletotrichum SHK2148                   .........--.................................................   [348]
C. acutatum(JR03)                        C........--.................................................   [348]
C. acutatum(HO01)                        C........--.................................................   [348]
C. acutatum(CR20)                        C........--.................................................   [348]
C. acutatum(C2897)                       C........--.................................................   [348]
C. acutatum(JG05)                        C........--..........................................T......   [348]
C. acutatum(CR46)                        C........--.................................................   [349]
C. gloeosporioides(CR45)                 C..T.....--.........T......................................T   [346]
C. gloeosporioides(CR21)                 C..T.....--.........T......................................T   [346]
C. gloeosporioides(315)                  C..T.....--.........T......................................T   [345]
C. gloeosporioides (HO19)                C..T.....--.........T......................................T   [347]
C. falcatum(CFA12)                       C........--..............................T..T.....C.........   [349]
Neurospora crassa(M13906)                C..TA.G..TG.....G..-..G.CG.T....A.A---..C.TT..GG.C.C....AT..   [347]
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                                6666666667777777777888888888899999999990000000000011111111112
Taxon/Node                      1234567890123456789012345678901234567890123456789012345678901234567890
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C. acutatum(PT30)               TTTGGCCAGCTTTTCCGCCCCGACAACTTCGTCTTTGGCCAGTCCGGTGCCGGCAACAAC   [410]
C. acutatum(PT29)               ..............................................................   [408]
C. acutatum(JR15)               ..............................................................   [408]
C. acutatum(CR02)               ..............................................................   [408]
C. acutatum(96A4)               ..............................................................   [408]
C.lupini var setosum(CW52)      ..............................................................   [408]
C.lupini var lupini(CW51)       ..............................................................   [408]
Colletotrichum SHK2148          ..............................G..----------------------C.   [386]
C. acutatum(JR03)               ..............................................................   [408]
C. acutatum(HO01)               ..............................................................   [408]
C. acutatum(CR20)               ..............................................................   [408]
C. acutatum(C2897)              ..............................................................   [408]
C. acutatum(JG05)               ..............................G...............................   [408]
C. acutatum(CR46)               ..............................................................   [409]
C. gloeosporioides(CR45)        ..C..T.....C.....................C........T.....................   [406]
C. gloeosporioides(CR21)        ..C..T.....C.....................C........T.....................   [406]
C. gloeosporioides(315)         ..C........C.....................C........T.....................   [405]
C. gloeosporioides (HO19)       ..C..T.....C.....................C........T.....................   [407]
C. falcatum(CFA12)              ..C.....A..C.....................T..............................   [409]
Neurospora crassa(M13906)       .CG..T.TC.CGC..GCGG.T.C.CG.--..GAG.-.C.G.AA.TA--------...TCT   [396]

                                4444444444444444444444444444444444444444444444444444444444444444
                                2222222223333333333444444444455555555556666666666777777777778
Taxon/Node                      1234567890123456789012345678901234567890123456789012345678901234567890
-------------------------------------------------------------------------------------------------------
C. acutatum(PT30)               TG-----------------------------------ACCTGCTCTGCCATCTTGT-   [431]
C. acutatum(PT29)               ..-----------------------------------.................-   [429]
C. acutatum(JR15)               ..-----------------------------------.................-   [429]
C. acutatum(CR02)               ..-----------------------------------.................-   [429]
C. acutatum(96A4)               ..-----------------------------------.................-   [429]
C.lupini var setosum(CW52)      ..-----------------------------------.................-   [429]
C.lupini var lupini(CW51)       ..-----------------------------------.................-   [429]
Colletotrichum SHK2148          ..-----------------------------------.................-   [407]
C. acutatum(JR03)               ..-----------------------------------.................-   [429]
C. acutatum(HO01)               ..-----------------------------------.................-   [429]
C. acutatum(CR20)               ..-----------------------------------.................-   [429]
C. acutatum(C2897)              ..-----------------------------------.................-   [429]
C. acutatum(JG05)               ..-----------------------------------........C........-   [429]
C. acutatum(CR46)               ..-----------------------------------.................-   [430]
C. gloeosporioides(CR45)        ..-----------------------------------.................-   [427]
C. gloeosporioides(CR21)        ..-----------------------------------.................-   [427]
C. gloeosporioides(315)         ..-----------------------------------.................-   [426]
C. gloeosporioides (HO19)       ..GGCCAAGGGTCACTACACCGAGGGTGCCGAGCTCGTCG...A.G.TCT.G..G....C   [467]
C. falcatum(CFA12)              ..-----------------------------------.................-   [430]
Neurospora crassa(M13906)       ..-----------------------------------.T.GAA...TTG.A.--.CA   [416]

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                                8888888889999999990000000000111111111122222222233333333334
Taxon/Node                      1234567890123456789012345678901234567890123456789012345678901234567890
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C. acutatum(PT30)               ------------AAGTGCCACA----CGACGA-----TGACCCTAG-AATATAA------   [463]
C. acutatum(PT29)               ------------.....-.....-.....-.....-.....------   [461]
C. acutatum(JR15)               ------------.....-.....-.....-.....-.....------   [461]
C. acutatum(CR02)               ------------.....-.....-.....-.....-.....------   [461]
C. acutatum(96A4)               ------------.....-.....-.....-.....-.....------   [461]
C.lupini var setosum(CW52)      ------------.....-.....-.....-.....-.....------   [461]
C.lupini var lupini(CW51)       ------------.....-.....-.....-.....-.....------   [461]
Colletotrichum SHK2148          ------------.....-.....-.....-.....-.....------   [439]
C. acutatum(JR03)               ------------.............T.....-.....-.....G.------   [461]
C. acutatum(HO01)               ------------.............T.....-.....-.....G.------   [461]
C. acutatum(CR20)               ------------.............T.....-.....-.....G.------   [461]
C. acutatum(C2897)              ------------.............T.....-.....-.....G.------   [461]
C. acutatum(JG05)               ------------.....-.....-----ATT..-------....TT.T.G...GCG.------   [459]
C. acutatum(CR46)               ------------....T---G.-----.T..A.-------.C.......-.G.GCG.------   [459]
C. gloeosporioides(CR45)        ------------G...T---G.-----.ACGA.--------...TT.CTT--T.CC.T------   [455]
C. gloeosporioides(CR21)        ------------G...T---G.-----.ACGA.--------...TT.CTT--T.CC.T------   [455]
C. gloeosporioides(315)         ------------G...T----GACCCGA.T..TT-------..T.--TTCCG.G.------   [456]
C. gloeosporioides (HO19)       CGCCGCGAGGCTG..G..TG.GACTG.AG.AGATGTTC.....C.AG..C..G.TGGCTG   [527]
C. falcatum(CFA12)              ------------.....T.--.TTTC.AG.C.----------.....T-...GACT------   [461]
Neurospora crassa(M13906)       ------------C.T..----------..CTCG----------..-..T.T.C.GG------   [439]
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Taxon/Node                       1234567890123456789012345678901234567890123456789012345678901234567890
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C. acutatum(PT30)                ----------------------------------------------------------GCAA   [467]
C. acutatum(PT29)                ----------------------------------------------------------....   [465]
C. acutatum(JR15)                ----------------------------------------------------------....   [465]
C. acutatum(CR02)                ----------------------------------------------------------....   [465]
C. acutatum(96A4)                ----------------------------------------------------------....   [465]
C.lupini var setosum(CW52)       ----------------------------------------------------------....   [465]
C.lupini var lupini(CW51)        ----------------------------------------------------------....   [465]
Colletotrichum SHK2148           ----------------------------------------------------------....   [443]
C. acutatum(JR03)                ----------------------------------------------------------....   [465]
C. acutatum(HO01)                ----------------------------------------------------------....   [465]
C. acutatum(CR20)                ----------------------------------------------------------....   [465]
C. acutatum(C2897)               ----------------------------------------------------------....   [465]
C. acutatum(JG05)                ----------------------------------------------------------A..C   [463]
C. acutatum(CR46)                ----------------------------------------------------------ATG.   [463]
C. gloeosporioides(CR45)         ----------------------------------------------------------.ATT   [459]
C. gloeosporioides(CR21)         ----------------------------------------------------------.ATT   [459]
C. gloeosporioides(315)          ----------------------------------------------------------TT   [458]
C. gloeosporioides (HO19)        CCTCTGACTTCCGCAACGGTCGCTACTTGACCTGCTCTGCCATCTTGTGAGTTGACA.G.   [587]
C. falcatum(CFA12)               ----------------------------------------------------------AT.T   [465]
Neurospora crassa(M13906)        ----------------------------------------------------------C.AGC   [444]

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                                 0000000001111111111222222222233333333334444444445555555556
Taxon/Node                       1234567890123456789012345678901234567890123456789012345678901234567890
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C. acutatum(PT30)                ATGCTAACTTTGTCGT----------------TCCCAGCCGTGGTAAGGTCGCCATGAA   [510]
C. acutatum(PT29)                ................----------.....................................   [508]
C. acutatum(JR15)                ................----------.....................................   [508]
C. acutatum(CR02)                ................----------.....................................   [508]
C. acutatum(96A4)                ................----------.....................................   [508]
C.lupini var setosum(CW52)       ................----------.....................................   [508]
C.lupini var lupini(CW51)        ................----------.....................................   [508]
Colletotrichum SHK2148           ................----------.....................................   [486]
C. acutatum(JR03)                .........G.A....----------..T.....................................   [508]
C. acutatum(HO01)                .........G.A....----------..T.....................................   [508]
C. acutatum(CR20)                .........G.A....----------..T.....................................   [508]
C. acutatum(C2897)               .........G.A....----------..T.....................................   [508]
C. acutatum(JG05)                ..A.......GT.T..----------.T.T....................................   [506]
C. acutatum(CR46)                ............T..----------.....................................   [506]
C. gloeosporioides(CR45)         T........CC---CC----------.T.T........C.............................   [499]
C. gloeosporioides(CR21)         T........CC---CC----------.T.T........C.............................   [499]
C. gloeosporioides(315)          T........CA---T.----------.T..........C........T.....   [498]
C. gloeosporioides (HO19)        ...A.TC....TC.A.GATTTTGCTAACTCCCC.T.T........C.............   [647]
C. falcatum(CFA12)               T.A.....AGC-.GCC----------..T.....................................   [507]
Neurospora crassa(M13906)        ....CTGT.CGAG..----------CA.TT..A..A.--C...C..TG.T...--   [485]

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                                 6666666667777777777888888888899999999900000000011111111112
Taxon/Node                       1234567890123456789012345678901234567890123456789012345678901234567890
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C. acutatum(PT30)                GGATGTTGAGGACCAGATGCGCAACGTTCAGAACAAGAACTCGTCCTACTTCGTCGAGTG   [570]
C. acutatum(PT29)                ..........................................................   [568]
C. acutatum(JR15)                ..........................................................   [568]
C. acutatum(CR02)                ..........................................................   [568]
C. acutatum(96A4)                ..........................................................   [568]
C.lupini var setosum(CW52)       ..........................................................   [568]
C.lupini var lupini(CW51)        ..........................................................   [568]
Colletotrichum SHK2148           ..........................................................   [546]
C. acutatum(JR03)                ......C...................................................   [568]
C. acutatum(HO01)                ......C...................................................   [568]
C. acutatum(CR20)                ......C...................................................   [568]
C. acutatum(C2897)               ......C...................................................   [568]
C. acutatum(JG05)                ......C.....................C.............................   [566]
C. acutatum(CR46)                ......C.................T..C.............................   [566]
C. gloeosporioides(CR45)         ......C.....................C.............C...............   [559]
C. gloeosporioides(CR21)         ......C.....................C.............C...............   [559]
C. gloeosporioides(315)          ......C.....................C.............C..........T.....   [558]
C. gloeosporioides (HO19)        ......C.....................C.............C...............   [707]
C. falcatum(CFA12)               ...C......................................................   [567]
Neurospora crassa(M13906)        ---C....G...T.C.CG..TGTC..C...A..TC..TGGCG.G.-----.....-----   [532]
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Taxon/Node                       1234567890123456789012345678901234567890123456789012345678901234567890
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C. acutatum(PT30)                GATTCCCAACAA-CGTCCAGACCGCCCTTTGCTCCATTCCTCCCCGCGGCCTCAAGATGT   [629]
C. acutatum(PT29)                ...........-.....................................................   [627]
C. acutatum(JR15)                ...........-.....................................................   [627]
C. acutatum(CR02)                ...........-.....................................................   [627]
C. acutatum(96A4)                ...........-.....................................................   [627]
C.lupini var setosum(CW52)       ...........-.....................................................   [627]
C.lupini var lupini(CW51)        ...........-.....................................................   [627]
Colletotrichum SHK2148           ...........-.....................................................   [605]
C. acutatum(JR03)                ...........-.....................................................   [627]
C. acutatum(HO01)                ...........-.....................................................   [627]
C. acutatum(CR20)                ...........-.....................................................   [627]
C. acutatum(C2897)               ...........-.....................................................   [627]
C. acutatum(JG05)                ...........-.....................................................   [625]
C. acutatum(CR46)                ...........-.....................C..C............................   [625]
C. gloeosporioides(CR45)         ...C........-.............C.....T..............................   [618]
C. gloeosporioides(CR21)         ...C........-.............C.....T..............................   [618]
C. gloeosporioides(315)          ...C........-.............C......................................   [617]
C. gloeosporioides (HO19)        ...C........-.............C.....T..............................   [766]
C. falcatum(CFA12)               ...C........-.............C.....T..............................   [626]
Neurospora crassa(M13906)        -.G..A..C.G.G...GGT...TCTA.A.C...ATGG..G.G.G-....GT..TT.CC..   [590]

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                                 8888888889999999999000000000011111111112222222222333333333334
Taxon/Node                       1234567890123456789012345678901234567890123456789012345678901234567890
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C. acutatum(PT30)                CTTCCACCTTTGTCGGTAACTCTACCGCCATCCAGG-AGCTCTTCAAGCGTGTCGGTGAG   [688]
C. acutatum(PT29)                ..........................................-....................   [686]
C. acutatum(JR15)                ..........................................-....................   [686]
C. acutatum(CR02)                ..........................................-....................   [686]
C. acutatum(96A4)                ..........................................-....................   [686]
C.lupini var setosum(CW52)       ..........................................-....................   [686]
C.lupini var lupini(CW51)        ..........................................-....................   [686]
Colletotrichum SHK2148           ..........................................-....................   [664]
C. acutatum(JR03)                ......................C...........-....................   [686]
C. acutatum(HO01)                ......................C...........-....................   [686]
C. acutatum(CR20)                ......................C...........-....................   [686]
C. acutatum(C2897)               ......................C...........-....................   [686]
C. acutatum(JG05)                .C..........................................-....................   [684]
C. acutatum(CR46)                .C..................C.......................-....................   [684]
C. gloeosporioides(CR45)         .C........C...........C.........T....-....G..................   [677]
C. gloeosporioides(CR21)         .C........C...........C.........T....-....G..................   [677]
C. gloeosporioides(315)          .C........C...........C.............-....G..................   [676]
C. gloeosporioides (HO19)        .C........C...........C.........T....-....G..................   [825]
C. falcatum(CFA12)               .C..T.....................................-..................C...   [685]
Neurospora crassa(M13906)        AAAA.C..CCAT.TCTA.GG.TG...T.GGAT....T..GAA.A.CC..-..AACT.A..   [649]

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                                 4444444445555555555666666666677777777778888888888999999999990
Taxon/Node                       1234567890123456789012345678901234567890123456789012345678901234567890
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C. acutatum(PT30)                CAGTTCACT--GCC-ATGTTCCGTCGCAAGGCTTTCTTGCATTGGTACACTGGTGAGGGT   [745]
C. acutatum(PT29)                .........--...-.....................................................   [743]
C. acutatum(JR15)                .........--...-.....................................................   [743]
C. acutatum(CR02)                .........--...-.....................................................   [743]
C. acutatum(96A4)                .........--...-.....................................................   [743]
C.lupini var setosum(CW52)       .........--...-.....................................................   [743]
C.lupini var lupini(CW51)        .........--...-.....................................................   [743]
Colletotrichum SHK2148           .........--...-.....................................................   [721]
C. acutatum(JR03)                .........--...-.....................................................   [743]
C. acutatum(HO01)                .........--...-.....................................................   [743]
C. acutatum(CR20)                .........--...-.....................................................   [743]
C. acutatum(C2897)               .........--...-.....................................................   [743]
C. acutatum(JG05)                .........--...-.....................................................   [741]
C. acutatum(CR46)                ..........--..T-..................................C.........   [741]
C. gloeosporioides(CR45)         .........--...-.....................................................   [734]
C. gloeosporioides(CR21)         .........--...-.....................................................   [734]
C. gloeosporioides(315)          .........--...-.....................................................   [733]
C. gloeosporioides (HO19)        .........--...-.....................................................   [882]
C. falcatum(CFA12)               .........--..T-...........T.......................C......   [742]
Neurospora crassa(M13906)        ..TA...A.AA..GG.G.AAAA.AAA.C.ACAGGGA....CC.A...-..-..C...T.A   [707]
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Taxon/Node                       123456789012345678901234567890123456789012345678901234567890
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C. acutatum(PT30)                ATGGACGAGATGGAGTTCACTGAGGCTGAGTCCAACATGAACGATCTCGTCTCCGAGTAC   [805]
C. acutatum(PT29)                ............................................................   [803]
C. acutatum(JR15)                ............................................................   [803]
C. acutatum(CR02)                ............................................................   [803]
C. acutatum(96A4)                ............................................................   [803]
C.lupini var setosum(CW52)       ..................??????????????????????????????????????????   [803]
C.lupini var lupini(CW51)        ........................C.T......A...C........A????????   [803]
Colletotrichum SHK2148           ..................??????????????????????????????????????????   [781]
C. acutatum(JR03)                ............................................................   [803]
C. acutatum(HO01)                ............................................................   [803]
C. acutatum(CR20)                ............................................................   [803]
C. acutatum(C2897)               ............................................................   [803]
C. acutatum(JG05)                .....T......................................................   [801]
C. acutatum(CR46)                ...........................................................A...   [801]
C. gloeosporioides(CR45)         ................T.......................CT.G..T.........   [794]
C. gloeosporioides(CR21)         ................T.......................CT.G..T.........   [794]
C. gloeosporioides(315)          ..........................................T.G............   [793]
C. gloeosporioides (HO19)        ................T.......................CT.G..T.........   [942]
C. falcatum(CFA12)               .....T............C..........T..........CT.G.....T......   [802]
Neurospora crassa(M13906)        .GC.G.A.C.GCTCAAATTTGA.AT...GC.T.GG.CC..G??????????????????   [767]

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                                 66666666677777777788888888889999
Taxon/Node                       12345678901234567901234567890123
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C. acutatum(PT30)                CAGCAATACCAGGACGCTGGTGTTGACGAGGAG   [838]
C. acutatum(PT29)                ................................   [836]
C. acutatum(JR15)                ................................   [836]
C. acutatum(CR02)                ................................   [836]
C. acutatum(96A4)                ................................   [836]
C.lupini var setosum(CW52)       ????????????????????????????????   [836]
C.lupini var lupini(CW51)        ????????????????????????????????   [836]
Colletotrichum SHK2148           ????????????????????????????????   [814]
C. acutatum(JR03)                ................................   [836]
C. acutatum(HO01)                ................................   [836]
C. acutatum(CR20)                ................................   [836]
C. acutatum(C2897)               ................................   [836]
C. acutatum(JG05)                ................................   [834]
C. acutatum(CR46)                ................................   [834]
C. gloeosporioides(CR45)         ................................   [827]
C. gloeosporioides(CR21)         ................................   [827]
C. gloeosporioides(315)          ................................   [826]
C. gloeosporioides (HO19)        ................................   [975]
C. falcatum(CFA12)               .....G..........................   [835]
Neurospora crassa(M13906)        ????????????????????????????????   [800]
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