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.