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.