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).

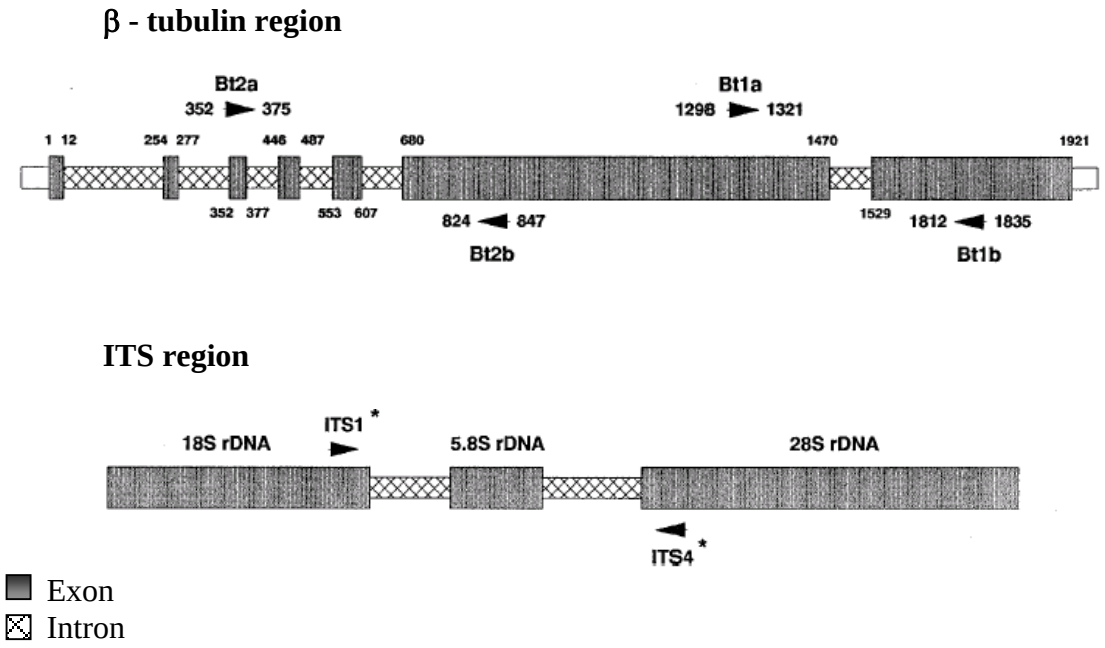


Fig. 2.1 Schematic representation of the ITS and β-tubulin regions as well as the primers used to amplify sections of these regions (Glass *et al*, 1995).

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.

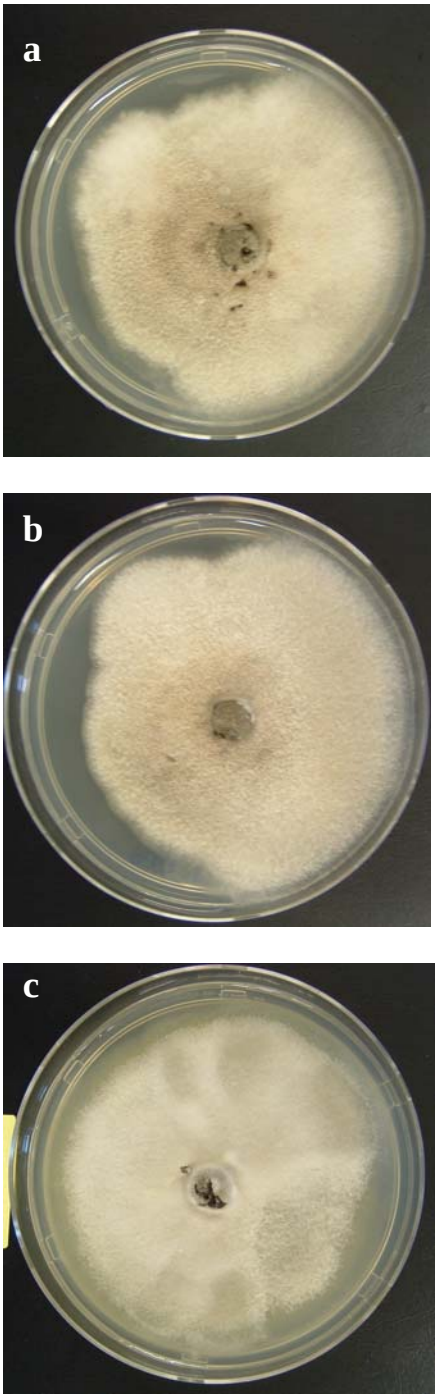


Fig. 2.2 Culture morphology displayed when *Colletotrichum* SHK 2148 (a) and *Colletotrichum lupini* var. *setosum* (b) and *Colletotrichum lupini* var. *lupini* (c) were grown on PDA in total darkness at 20 °C (top view of plates).

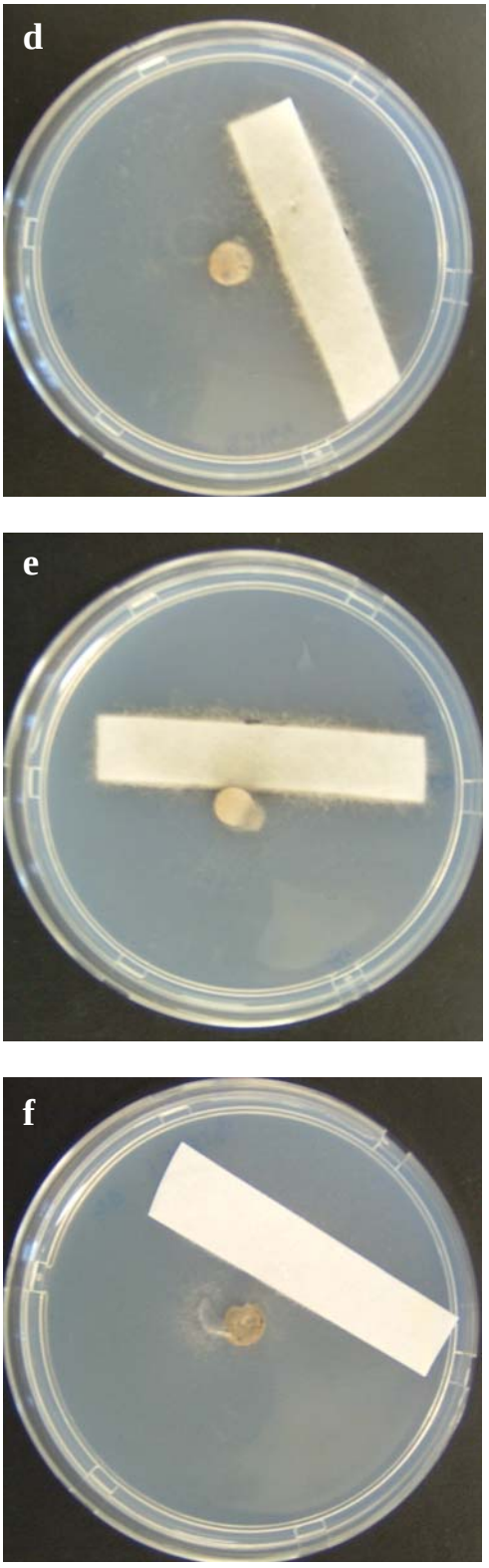


Fig. 2.3 Culture morphology displayed when *Colletotrichum* SHK 2148 (d) and *Colletotrichum lupini* var. *setosum* (e) and *Colletotrichum lupini* var. *lupini* (f) were grown on SNA, with strips of filter paper, in total darkness at 20 °C (top view of plates).

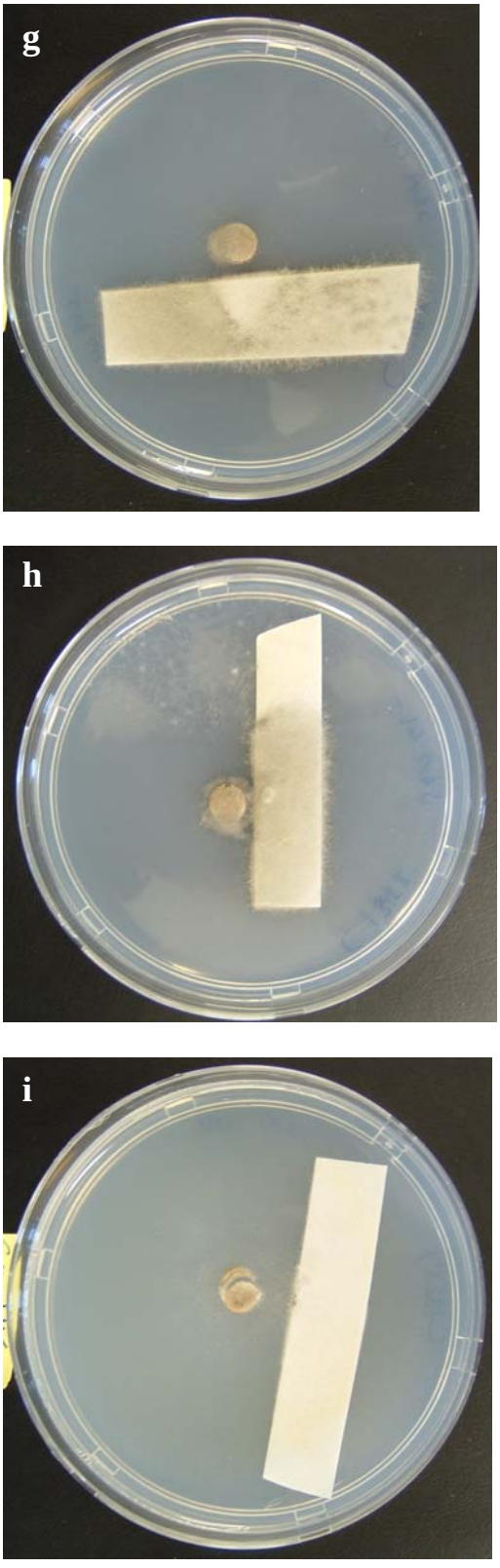
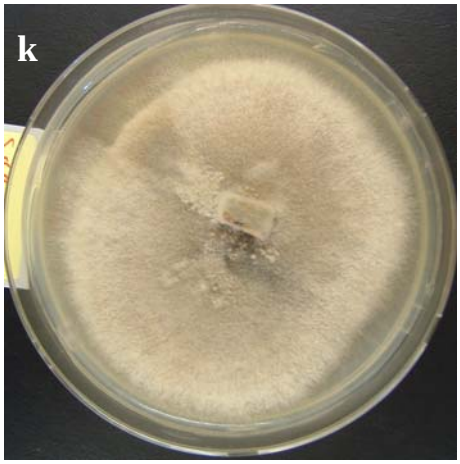
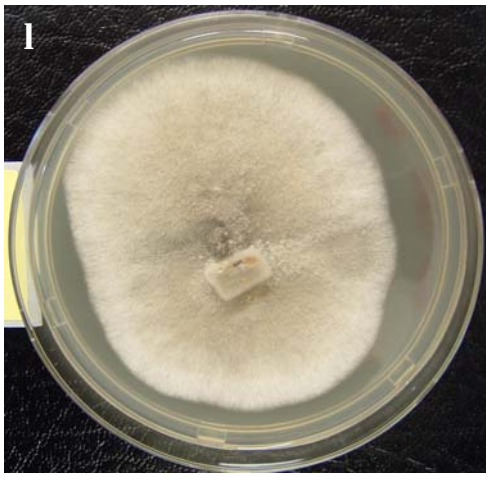


Fig. 2.4 Culture morphology displayed when *Colletotrichum* SHK 2148 (g) and *Colletotrichum lupini* var. *setosum* (h) and *Colletotrichum lupini* var. *lupini* (i) were grown on SNA, with strips of filter paper, under constant light at 20 °C (top view of plates).

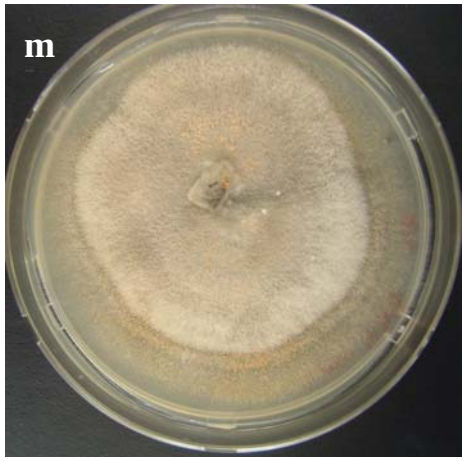
14 Days



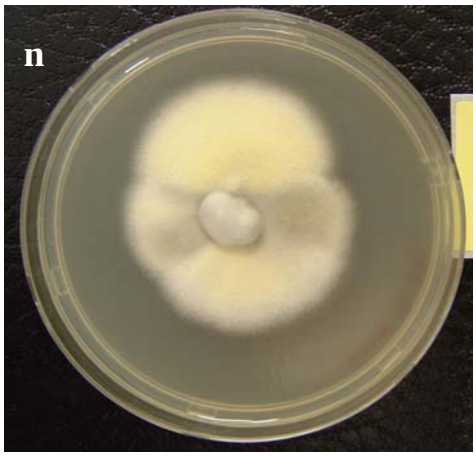
21 Days



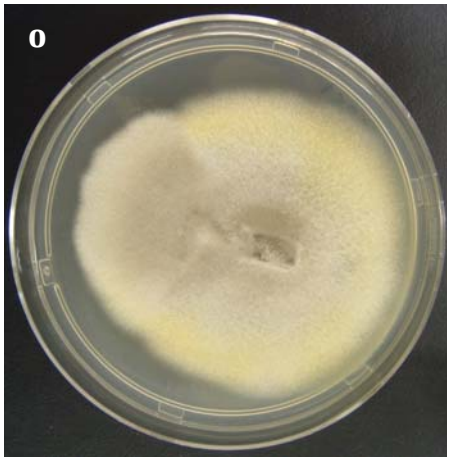
14 Days



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).

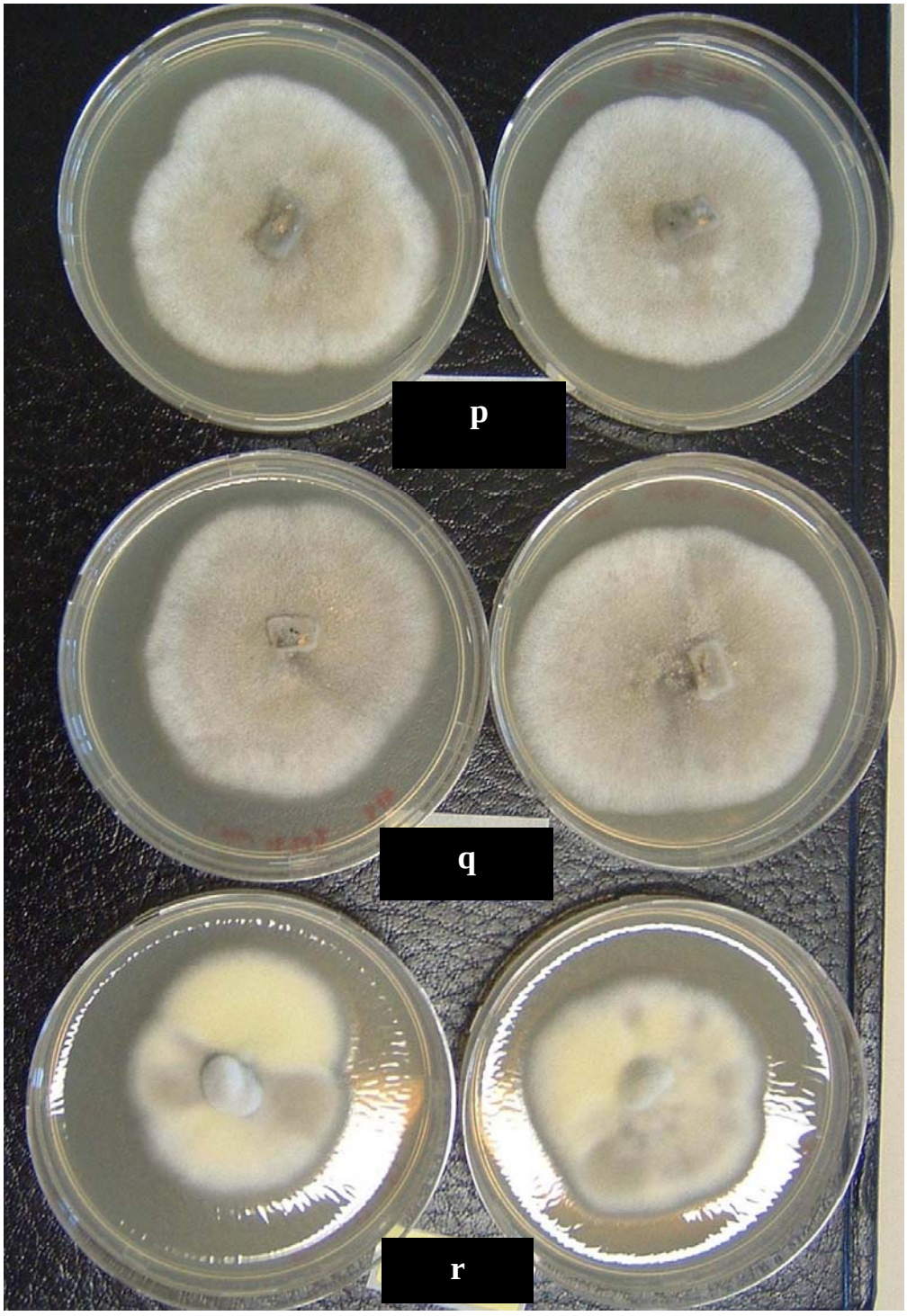


Fig. 2.6 Culture morphology displayed when *Colletotrichum* SHK 2148 (p) and *Colletotrichum lupini* var. *setosum* (q) and *Colletotrichum lupini* var. *lupini* (r) grown on PDA under natural day night rhythms after 14 days (top view of plates).

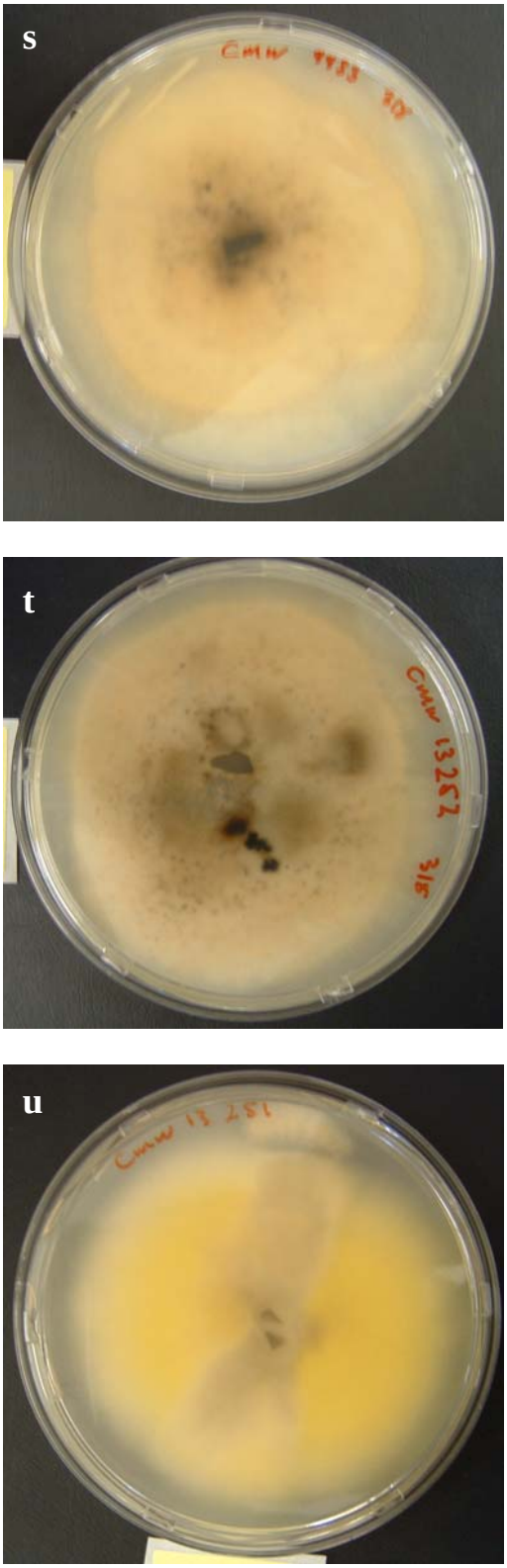
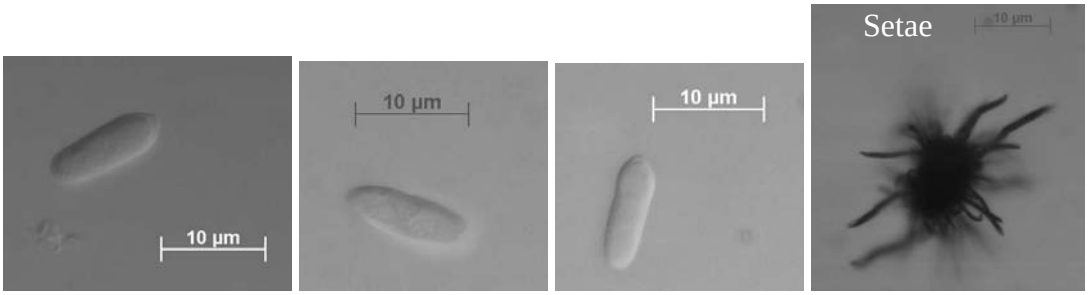


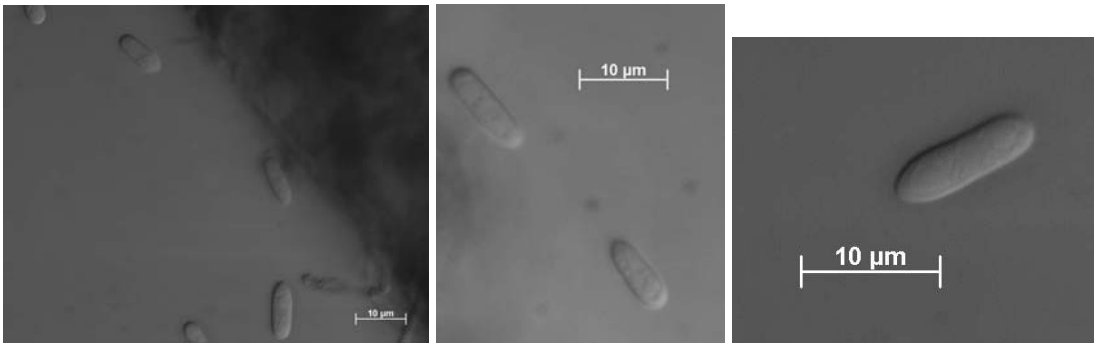
Fig. 2.7 Culture morphology displayed when *Colletotrichum* SHK 2148 (s) and *Colletotrichum lupini* var. *setosum* (t) and *Colletotrichum lupini* var. *lupini* (u) grown on PDA under natural day night rhythms after 21days (bottom view of plates).

3.2.2 Spore characteristics

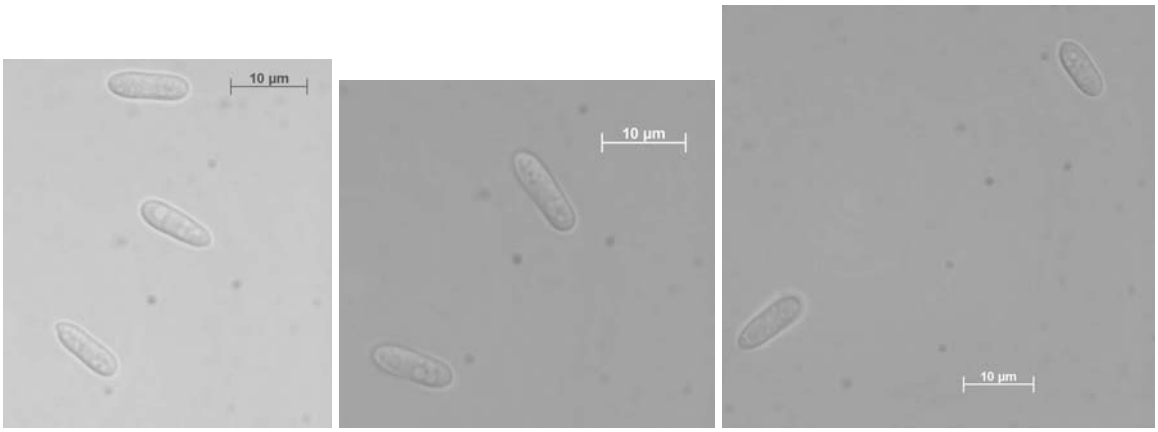
There was no significant difference between the size and shape of the conidia of the different isolates or under different growth conditions. The majority of them displayed the description of Nirenberg *et al* (2002) as being pointed at one end and rounded at the other end (Fig. 2.8, Fig. 2.9 and Fig. 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.



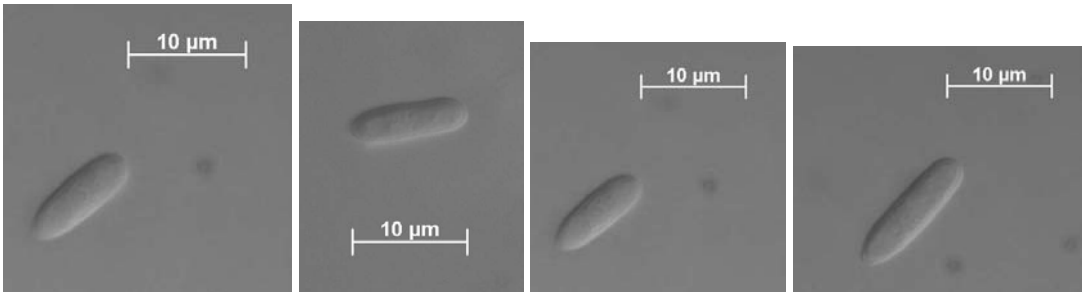
A: PDA, Darkness, 20°C



B: SNA, Darkness, 20°C

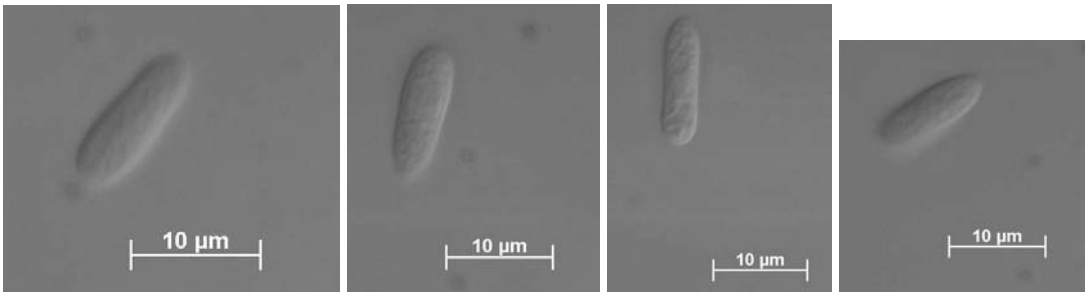


C: SNA, Constant light, 20°C

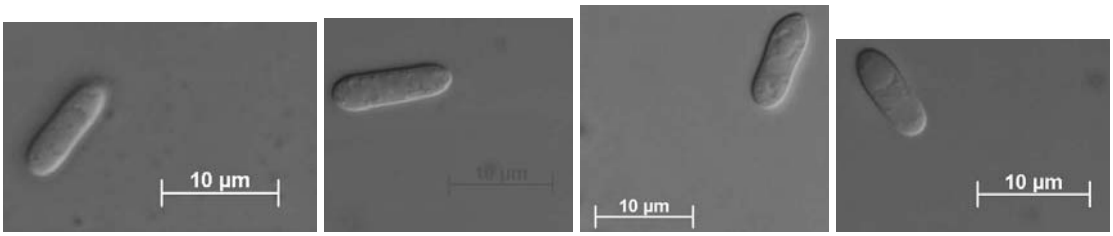


D: PDA, Natural day night rhythm, 20- 22°C

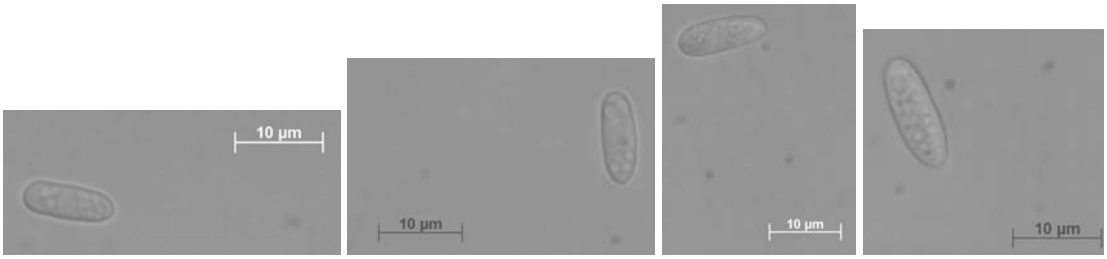
Fig. 2.8 Spores presented for *Colletotrichum* SHK 2148 grown under conditions A, B, C and D.



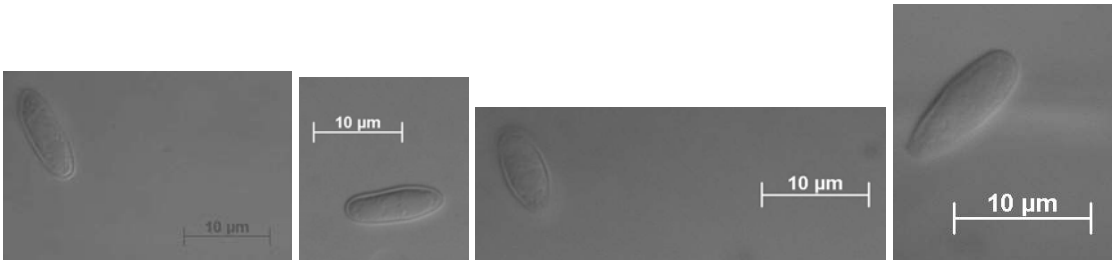
A: PDA, Darkness, 20°C



B: SNA, Darkness, 20°C

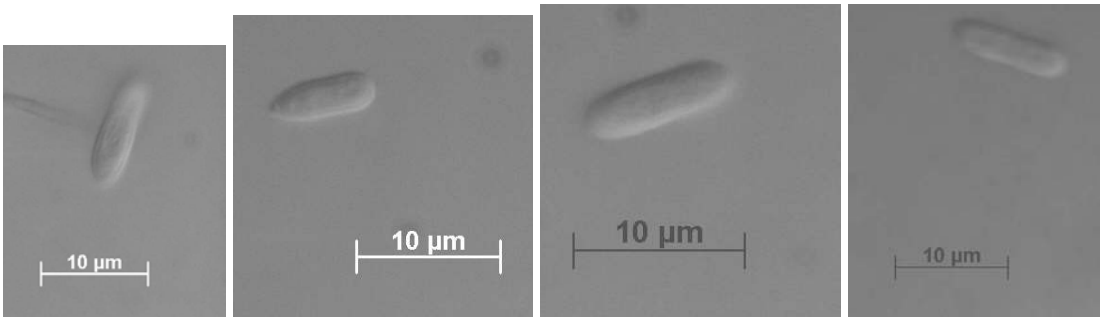


C: SNA, Constant light, 20°C

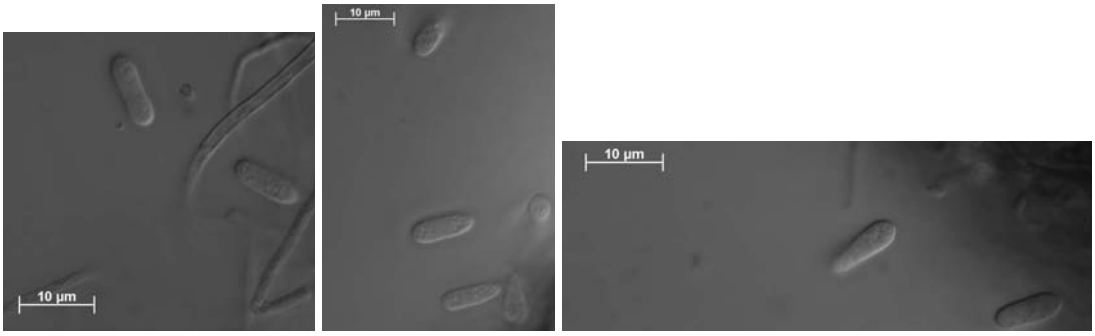


D: PDA, Natural day night rhythm, 20- 22°C

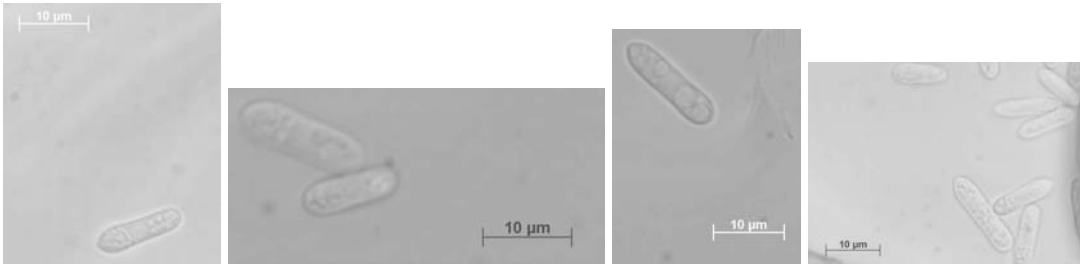
Fig. 2.9 Spores presented for *C. lupini* var. *setosum* grown under conditions A, B, C and D.



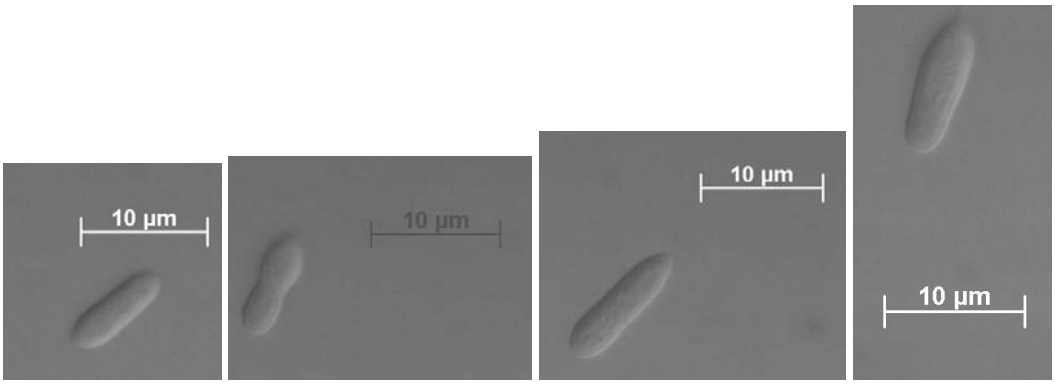
A: PDA, Darkness, 20°C



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).

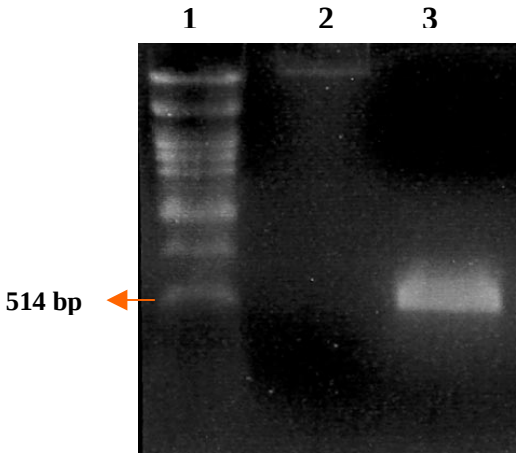


Fig. 2.11 PCR amplification of the ITS regions from *Colletotrichum* SHK 2148
Amplified ITS fragment from the genome of *Colletotrichum* SHK 2148 (lane 3), electrophoresed on a 1%TAE agarose gel together with 20ng λ DNA (lane 2) and aλ *PstI* generated molecular marker (lane 1, Appendix A).

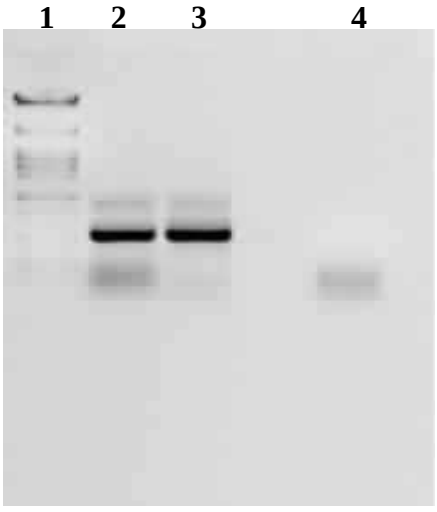


Fig. 2.12 PCR amplification of the ITS regions from isolates *C. lupini* var. *lupini* and *C. lupini* var. *setosum.*
ITS fragments were amplified from the genomic DNA of isolates *C. lupini* var. *lupini* (lane 2) and *C. lupini* var. *setosum* (3) and electrophoresed with the PCR water control (lane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

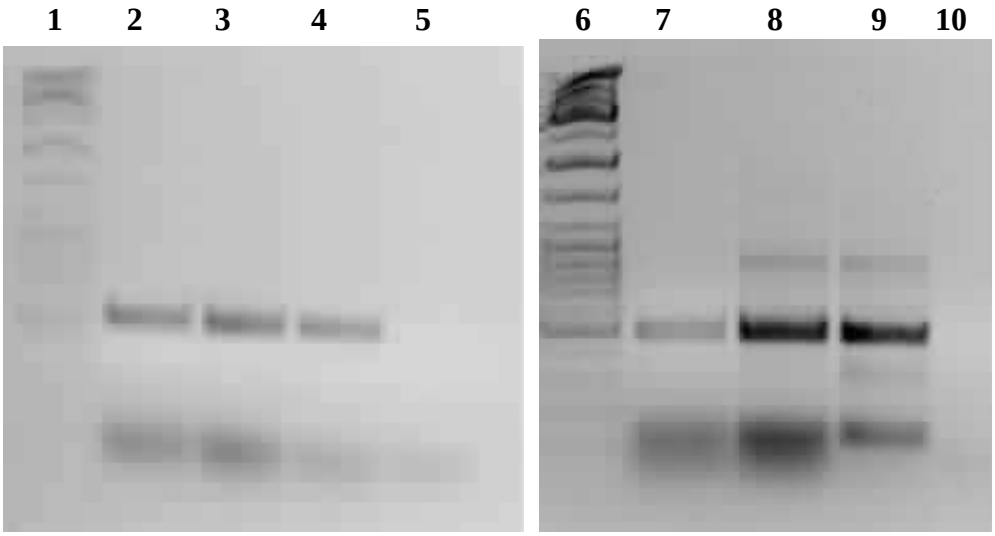


Fig. 2.13 PCR amplification of β-tubulin regions from test isolates *Colletotrichum* SHK 2148, *C. lupini* var. *lupini* and *C. lupini* var. *setosum*.
PCR amplification of β-tubulin region-2 from isolates *Colletotrichum* SHK 2148 (lane2), *C. lupini* var. *setosum* (lane 3) and *C. lupini* var. *lupini* (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.

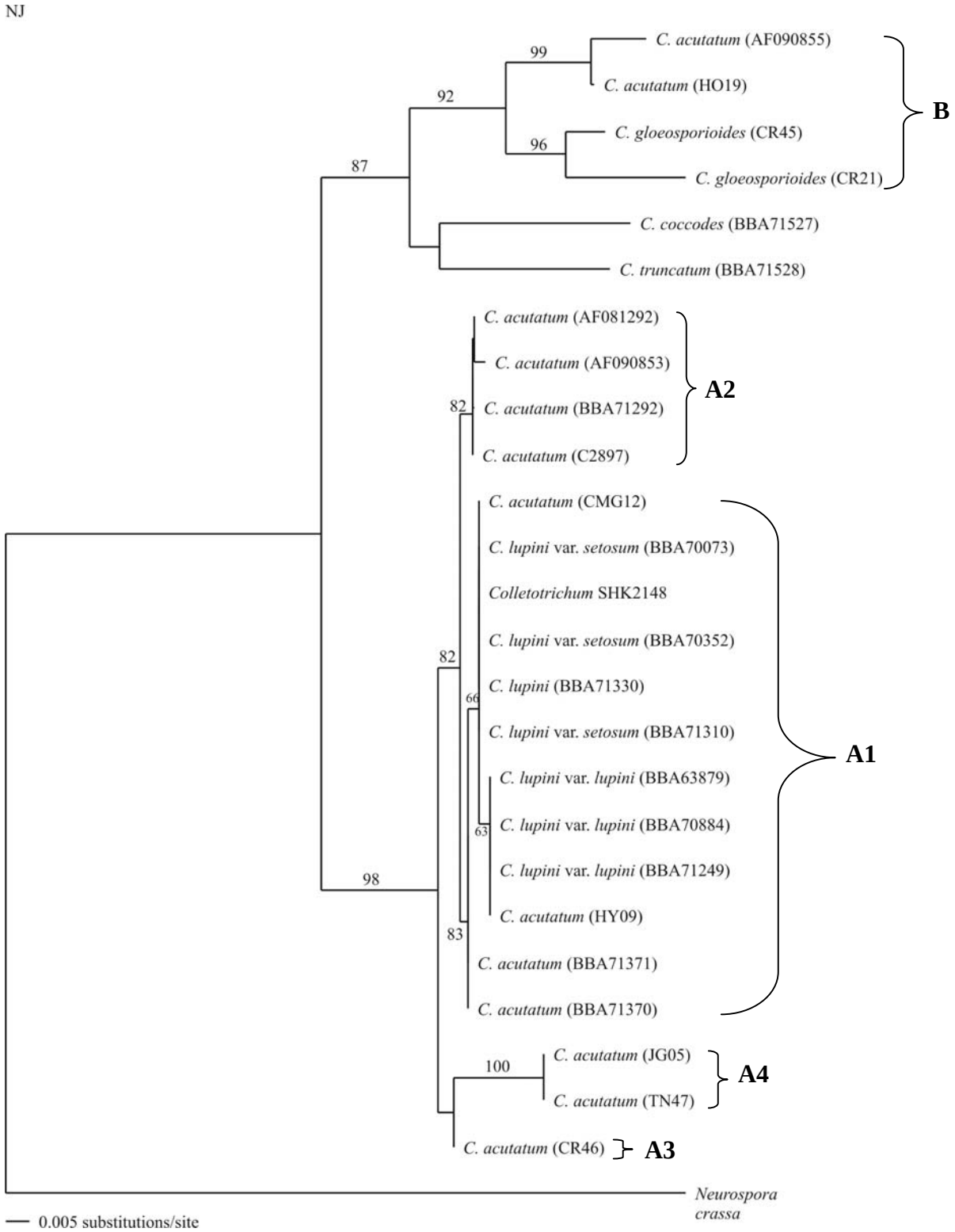


Fig. 2.16 Neighbour joining tree showing the reconstruction of the molecular phylogeny of different *Colletotrichum* isolates based on the ITS1, ITS2 and 5.8S rDNA operon. *Neurospora crassa* was included as the outgroup. (Bootstrap values are indicated on the tree). The grouping of Talhinas *et al* (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]
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                              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]
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                              222222222222222222222222222222222222222222222222222222222333
                              111111122222223333334444455555566666777777788888889999999000
Taxon/Node                    234567902467891235671346701345705689013467801234580135689145
--------------------------------------------------------------------------------------------
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]
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                              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.

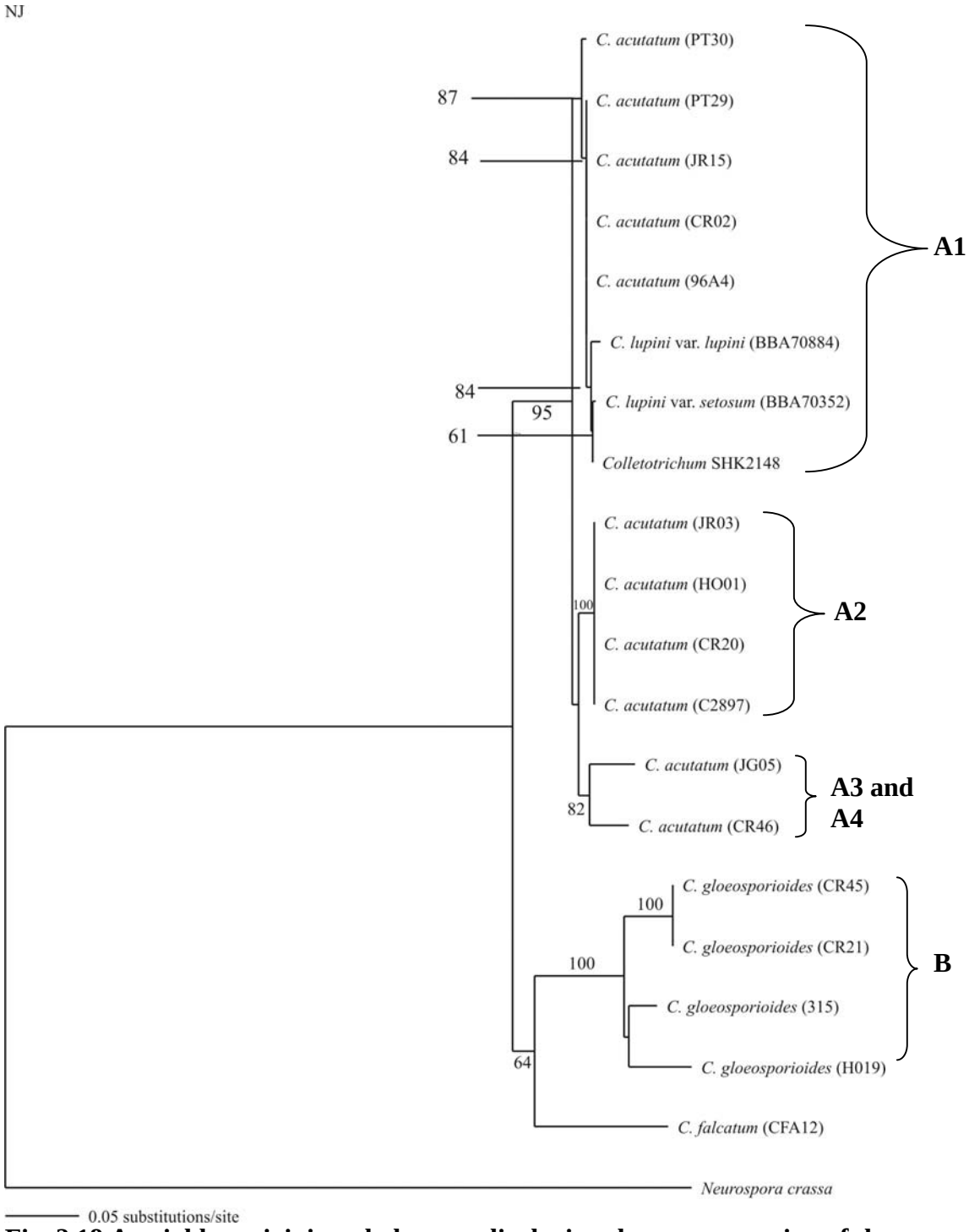

Fig. 2.18 A neighbour joining phylogram displaying the reconstruction of the molecular phylogeny of *Colletotrichum* isolates based on the β-tubulin region using *Neurospora crassa* as outgroup. (Bootstrap values are indicated on the tree). The brackets indicate the groupings from Talhinas *et al* (2002).

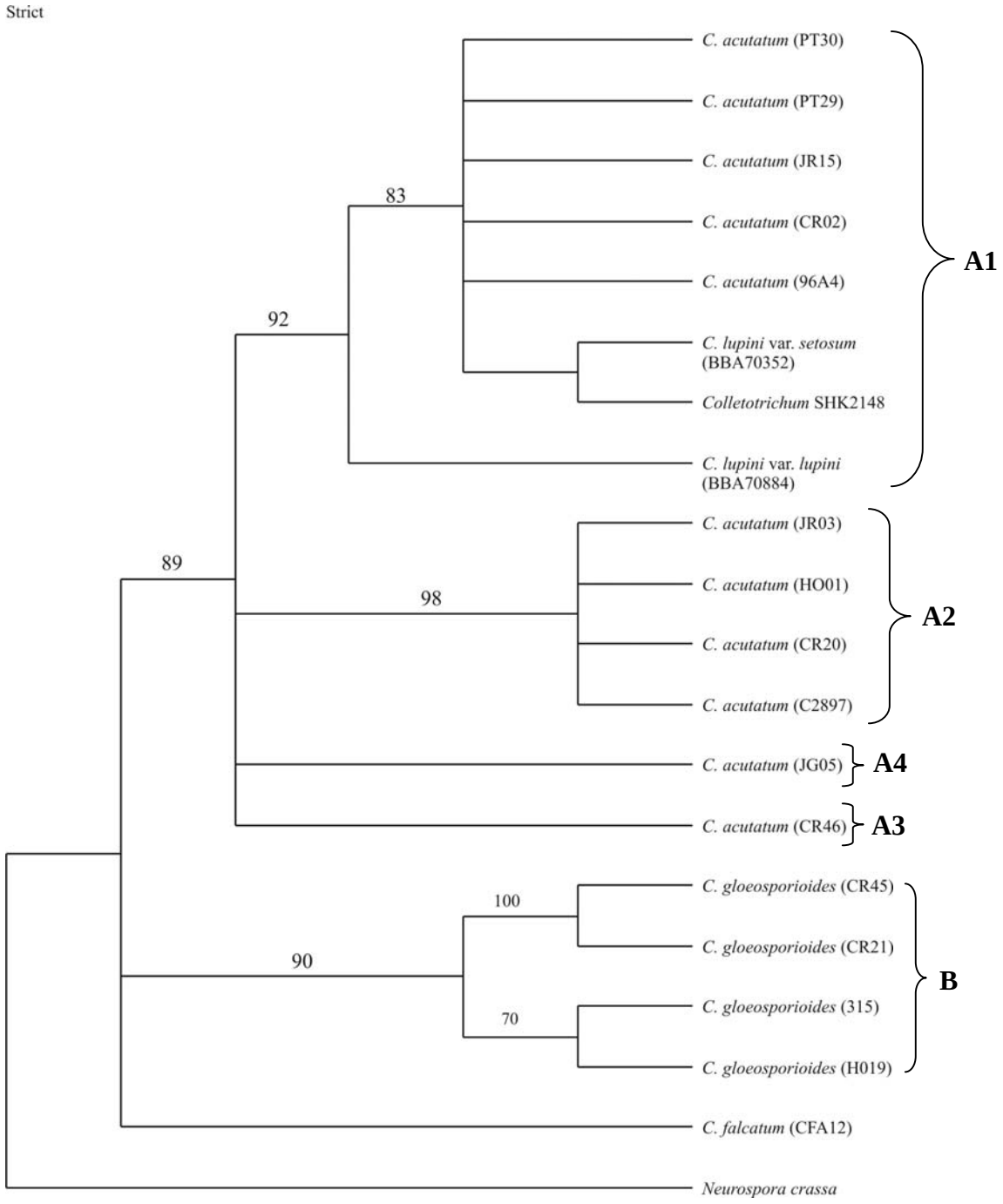

Fig. 2.19 The parsimony strict consensus tree, with bootstrap values, displays the reconstruction of the molecular phylogeny of different *Colletotrichum* isolates, based on the analysis of the β-tubulin region. (Bootsrap values are indicated on the tree).

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.