MOLECULAR AND PATHOLOGICAL DIFFERENTIATION OF *COLLETOTRICHUM TRUNCATUM* FROM SCENTLESS CHAMOMILE AND LEGUME CROPS

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ABSTRACT

The fungus *Colletotrichum truncatum* is a potential biocontrol agent (BA) against the noxious weed scentless chamomile (*Metricaria perforata* Mérat*;* syn.: *Tripleurospermum perforatum* (Mérat) Lainz) in western Canada. This potential BA, however, is taxonomically related to the anthracnose pathogen on lentil, raising questions about crop safety. Ribosomal DNA (rDNA) internal transcribed space (ITS) regions of *C. truncatum* isolates collected from different plant hosts were examined, and compared with additional *Colletotrichum* species. Sequences were amplified with the universal primers its4 and its5, and *C. truncatum* isolates from scentless chamomile and selected legume crops were differentiated consistently. All scentless chamomile isolates fell within a single cluster in phylogenetic trees, regardless of their geographic origins. These isolates were more closely related to lentil isolates of *C. truncatum* than to isolates from the other host species. Soybean isolates, with more falcate and slender conidia and slightly bigger appressoria, were distinguishable from other *C. truncatum* isolates, while the isolates from scentless chamomile, lentil and pea were morphologically more similar. Based on sequence information, strain-specific PCR primers were designed for *C. truncatum* isolates from these hosts and used to amplify specific DNA bands (markers) from isolates of *C. truncatum*. This technique may be used for rapid detection and differentiation of *C. truncatum* from scentless chamomile and designated legume species, as well as for tracking the BA after release. Inoculation trials were conducted using detached leaves and whole plants to determine potential cross infection of these *C. truncatum* isolates. Isolates from scentless chamomile caused disease only on their original host, but not on lentil, pea, soybean or alfalfa. In contrast, lentil isolates caused severe disease on lentil

and pea, light symptoms on alfalfa, but no disease on the other hosts tested. Potential penetration of lentil leaves by scentless chamomile isolates was tested, with 2-23% incidence of the fungus from inoculated detached, senescence leaves but disease symptoms were not observed on either detached leaves or whole plants. Examination of the infection process revealed that scentless chamomile and lentil isolates had a similar pattern of infection and disease development on their respective hosts; infection vesicles were produced 24 h after inoculation, both primary and secondary infection hyphae were present, and the onset of disease symptoms tended to coincide with the development of secondary hyphae. The current study provided molecular and pathological evidence that differentiates the potential BA of scentless chamomile from *C. truncatum* isolates from lentil, pea and soybean.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

AAFC---------Agriculture and Agri-Food Canada

AI----------Active Ingredient

ANOVA------Analysis of Variance

BA----------Biocontrol Agent

CDC----------Crop Development Centre

DGGE---------Denaturing Gradient Gel Electrophoresis

Dpi----------Days Post Inoculation

DS----------Disease Symptom

IGS----------Intergenic Spacer Region

IPM---------- Integrated Pest Management

ITS----------Internal Transcribed Spacer

IV----------Infection Vesicle

LI----------Latent Infection

NJ---------- Neighbor-Joining

PAUP*---------Phylogenetic Analysis Using Parsimony

PCR----------Polymerase Chain Reaction

PH----------Primary Hyphae

RAPD----------Random Amplified Polymorphic DNA

RFLP----------Restriction Fragment Length Polymorphism

SH----------Secondary Hyphae

I. INTRODUCTION

Scentless chamomile (*Metricaria perforata* Mérat*;* syn.: *Tripleurospermum perforatum* (Mérat) Lainz), also known as mayweed, is a noxious weed in western Canada (Kessler 1989). This weed is difficult to control with conventional strategies due to herbicide tolerance and limited tillage options. Most post-emergent herbicides are ineffective at crop tolerant rates (Bowes et al. 1994) and widespread adoption of conservation tillage on the prairies limits the use of tillage in weed control (Blackshaw and Harker 1997). Scentless chamomile has the potential to cause substantial crop yield losses (Douglas et al. 1991). A fungal pathogen has been found as a promising biocontrol agent (BA) against this weed (Peng and Bailey 2002). Based primarily on conidial morphology, this fungus was identified as *Colletotrichum truncatum* (Schwein) Andrus & W.D. Moore. However, fungal species under the same name also causes anthracnose on several important legume crops including lentil, pea and soybean (Hartman et al. 1986**;** Bailey and Jeger 1992; Chongo et al. 2002), and the relatedness of this BA to *C. truncatum* as crop pathogen has not been determined.

The current study was conducted as part of a risk assessment, to compare the BA with *C. truncatum* isolates from crop species for relatedness and crop safety. The objectives were to: 1) determine the relatedness of scentless chamomile isolates of *C. truncatum* to those from crop species (lentil, pea, and soybean) as well as other *Colletotrichum* species using rDNA-ITS sequencing; 2) based on sequence data, develop strain-specific PCR primers for scentless chamomile and crop isolates of *C. truncatum* as

genetic markers for rapid detection and differentiation of specific strains; and 3) assess the safety of BA on important legume crop species based on disease development and infection process.

II. LITERATURE REVIEW

2.1. *Colletotrichum* - an Important Genus of Plant Pathogens

Colletotrichum is a large genus of the class Pyrenomycetes belonging to the Ascomycota family of fungi (Freeman et al. 2000; Curry and Baird 2004), with many species that cause anthracnose on a wide range of crop and ornamental plant species (Bailey and Jeger 1992). At least nine species have been recorded on legume crops worldwide, including *C. capsici, C. coccodes, C. crassipes, C. dematium, C. destructivum, C. gloeosporiodes, C. lindemuthianum, C. trifolli* and *C. truncatum* (Lenne 1992). Legume crops such as bean (*Phaseolus vulgaris* L.), cowpea (*Vigna unguicultata* L. Walp), soybean (*Glycine max* (L) Merr.), peanut (*Arachis hypogaea* L.), lentil (*Lens culinaris* Medik.), and alfalfa (*Medicago sativa* L.) have been reported as hosts of *Colletotrichum* species (Bailey and Jeger 1992). Pathogen-host interactions have been documented for *C. lindemuthianum* on bean (O'Connell et al. 1991), *C. truncatum* on pea (O'Connell et al. 1993), *C. destructivum* on cowpea (Latunde-Dada et al. 1996), and *C. truncatum* on lentil (Anderson 2000; Chongo et al. 2002).

Several diseases caused by *Colletotrichum* pathogens are of economic and social importance. Lentil anthracnose, caused by *C. truncatum*, has been reported in the United States (Ventte et al. 1994), Bulgaria (Kaiser 1998), Brazil and Pakistan (Bellar and Kebabeh 1983; Morrall 1997). In Canada, the disease was first reported in Manitoba (Morrall 1988), and then in Saskatchewan (Morrall and Pederson 1991). In 1999, an epidemic of lentil anthracnose developed under unusually wet and warm weather

conditions, causing severe losses in yield and seed quality (Anderson et al. 1999). Two pathogenic races (Ct0 and Ct1) of the pathogen have been identified, with roughly equal frequency observed in commercial fields (Buchwaldt et al. 2004). Some lentil cultivars are more resistant to race Ct1, but none of the lines tested so far is resistant to Ct0 (Anderson 2003). Some partially resistant cultivars may perform better when combined with fungicide application (Chongo 1998). Recently, a study using a collection of lentil germplasms from Germany and North America found that 16 lines showed resistance to Ct1, but only the wild *Lens ervoides* (Brign.) demonstrated a level of resistance to Ct0 (Tullu et al. 2006).

Cowpea is an important source of protein and other essential nutrients in the diet to many people living in the semi-arid regions of the tropics and subtropics (Singh and Mare 1985). It is subject to attack from seedling to harvest by *C. lindemuthianum* (Onesirosan and Barker 1971; Williams 1975). The disease is a major limiting factor in cowpea production in Nigeria (Emechebe et al. 1985) and yield losses of up to 50% have been reported on susceptible cultivars under wet and humid conditions (Williams 1975; Latunde-dada et al. 1996). Similarly, yam (*Dioscorea* spp.*)* is an important food source for millions of people in the tropical and subtropical areas such as the Caribbean and Nigeria (McDonald et al. 1998; Abang et al. 2003). Anthracnose caused by *C. gloeosporiodes* (Penz.) Sac. has a dramatic impact on yam production, and yield losses of up to 80% have been reported in Nigeria (Amusa et al. 2003). Many diseases on cereal, vegetable and fruit crops are also caused by *Colletotrichum* species.

2.2. Biological Control of Weeds with *Colletotrichum* spp.

Integrated pest management (IPM) calls for multiple pest-control measures to minimize the damage from diseases, insects or weeds (Cook 2000). Use of plant pathogens for weed control is part of many IPM strategies, and products with fungi as the active ingredients (a.i.) are often referred to as mycoherbicides. To date, *Colletotrichum* spp. have been studied most extensively as potential mycoherbicide agents, largely due to their high virulence and host specificity. The first commercial mycoherbicide, LUBAO, use the a.i. of *C. gloeosporioides*, was used to control parasitic dodder (*Cuscuta* sp.) in China (Wang 1990). It was discovered in 1963 and, by the late 1970s, the product had been applied to 670,000 ha of soybean in 10 provinces (Templeton 1992). COLLEGO® is a mycoherbicide with *C. gleosporioides* f. sp. *aeschynomene* as the active ingredient (a.i.) and is used to control northern jointvetch [*Aeschynomene virginica* (L.) B.S.P.] in rice paddies in the southern United States (Templeton et al. 1984). BIOMAL® (a.i. *C. gloeosporioides* f. sp. *malvae*) was developed for control of round-leaved mallow (*Malva pusilla* Sm.) in the prairie provinces of Canada and the northern great plains of the United States (Mortensen 1988). A wettable-powder formulation was registered in Canada in 1992. Used properly, mycoherbicides may provide effective weed control while causing minimal disruption of the environment (Charudattan 2001; Boyetchko et al. 2003).

2.3. Biocontrol of Scentless Chamomile with *Colletotrichum truncatum*

2.3.1. The Weed

Scentless chamomile was introduced to North America from Europe at the beginning of 20th century and has since spread rapidly in Canada. It can now be found in all 10 provinces and the Northwest Territories (Woo et al. 1991). Scentless chamomile is

a very adaptable species on the Canadian prairies; it can be an annual, biennial, or even short-lived perennial that reproduces itself by seeds (McClay and De Clerck-Floate 1999). The heaviest infestations are reported in the black soil zone of Saskatchewan and Alberta (Bowes et al. 1994). At a density of 25 plants per m^2, scentless chamomile caused 55% yield loss in spring wheat (Douglas et al. 1991).

2.3.2. The Biocontrol Agent (BA)

After evaluation of microorganisms from Saskatchewan and Europe, a fungus was found to be moderately effective as a biocontrol agent for scentless chamomile (Peng and Bailey 2002; Peng et al. 2005). Based primarily on conidial morphology and cultural characteristics, it was identified as *Colletotrichum truncatum*. It demonstrated strict host specificity towards scentless chamomile species (including *Matricaria recutita* L. - German chamomile) and caused no disease on field crop species commonly grown in Canada (Peng et al. 2005). Applied at 2×10^7 conidia ml^{-1} and a carrier volume of 200 l ha^{-1}, this fungus reduced the fresh weight of scentless chamomile by approximately 50% compared to untreated controls (Graham et al. 2006). For maximum efficacy, this BA can be tank mixed with several partially effective herbicides for better control of this weed.

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2.4. Infection Characteristics of *Colletotrichum* Pathogens

The infection process of *Colletotrichum* spp. has been studied extensively, with many distinctive characteristics noticed even in early investigations (Walker 1921). Luttrell (1974) later categorized *Colletotrichum* pathogens as intracellular hemibiotrophic, subcuticular intramural, or a combination of both mechanisms depending

on the initial mode of infection. Regardless of the initial strategy, the final phase of all three types of infection is always necrotrophic growth (Bailey and Jeger 1992), leading to the death of plant cells and tissues.

2.4.1. Intracellular Hemibiotrophic Infection

Many *Colletotrichum* pathogens show two distinctive phases during plant infection and colonization, with the early stage being biotrophic and symptomless. This phase is followed by a destructive necrotrophic phase that results in disease symptoms (O'Connell et al. 1985; O'Connell et al. 1993; Latunde-Dada et al. 1996, 1997). In the biotrophic phase, the pathogen colonizes living tissues without killing them until the necrotrophic phase is triggered. This type of infection was described as hemibiotrophic by Luttrell (1974), and O'Connell and Bailey (1991) referred these *Colletotrichum* species to 'intracellular hemibiotrophic' pathogens. This two-phased infection was first described for *C. lindemuthianum* by Leach (1922) and later illustrated in more details by Skipp and Deverall (1972), Mercer et al. (1974), and O'Connell et al. (1985). With the necrotrophic phase, anthracnose symptoms start to appear with the development of intr and inter-cellular infection hyphae throughout host tissues. Eventually, conidiophores rupture through the host cuticle and form acervuli on the tissue surface.

2.4.2. Subcuticular Intramural Infection

Some *Colletotrichum* spp. exhibit subcuticular-intramural infection; host penetration is followed by hyphal growth beneath the cuticle layer and within periclinal walls of epidermal cells. Continued subcuticular growth of fungal hyphae may cause degradation of the host cell wall (Pring et al. 1995). This mode of infection was first

described by Walker (1921) for *C. circinans* on onions, in which the initial phase was also symptomless. However, an extensive network of intramural hyphae formed within days and, as a result, lesions appeared. Although intracellular hemibiotrophic and subcuticular intramural invasion are common during early infection for many *Colletotrichum* spp., the distinction can be less clear with other species that appear to exhibit both strategies. Such examples include infection of several *Stylosanthes, Citrus* or *Hevea* species by *C. gloeosporioides* (Brown 1977; Irwin et al. 1984; Sénéchal et al. 1987; Vinijsanun et al. 1987; Trevorrow et al. 1988; Zakaria 1995).

The majority of *Colletotrichum* species exhibit intracellular colonization, although the duration of this phase may vary. This may be exemplified by the infection process for *C. lindemuthianum* on bean (O'Connell et al. 1985). Following penetration, fungal hyphae grew between plant plasma membranes and cell walls. After colonizing one or more host cells, primary hyphae produced secondary hyphae and resulted in initial tissue necrosis (O'Connell et al. 1985; Bailey and Jeger 1992; Latunde-Dada et al. 1996).

For *C. truncatum*, studies on infection of pea (*Pisum sativum* L.) indicated an intracellular hemibiotrophic strategy (Manandhar et al. 1985; O'Connell et al. 1993). The pathogen showed a brief biotrophic phase that lasted about 24 h, and then changed to the necrotrophic phase with the production of secondary hyphae. In the biotrophic phase, primary hyphae were restricted to the epidermal cells penetrated initially (O'Connell et al. 1993). Based on these studies, O'Connell et al. (1993) suggested that the initial intracellular, biotrophic phase may be important to host specificity of *Colletotrichum* pathogens. Some *Colletotrichum* spp. showed latent infection (Cerkauskas 1988), a

period of quiescence phase prior to disease expression (Fernando et al. 1994). In these cases, there is a substantial delay between host penetration and symptom appearance.

2.5. Advantages of *C. truncatum* as a Biocontrol Agent

Strict host specificity and high virulence are the most important features for a mycoherbicide (Goodwin 2001). The hemibiotrophic pathogen *C. truncatum* has an initial biotrophic phase, which requires a specific metabolic interaction with the host for recognition, followed by a necrotrophic phase that can be highly destructive (Manandhar et al. 1985; O'Connell et al. 1993; Kolattukudy et al. 1995; Wei et al. 1997). In addition, protocols for mass production of the fungus have been studied using submerged culture fermentation (Jackson et al. 1996; Dokken 2003), providing a ready technology for commercial development.

2.6. Host Range of *C. truncatum*

Most isolates of *C. truncatum* from scentless chamomile were non-pathogenic to crop species grown Canada, while two isolates caused slight disease symptoms on lentil and/or flax (*Linum usitatissimum* L.) under conditions of prolonged leaf wetness (Peng et al. 2005). Different strains of *C. truncatum* have been reported to infect a wide range of plant species belonging to the genera *Cicer, Indigofera, Lathyrus, Lens, Lupinus, Pisum* and *Vicia* (Weidemann et al. 1988) and cause anthracnose on *Medicago* (Graham et al. 1976), *Vigna* (Adebitan et al. 1992), *Phaseolus* (Han and Lee 1995), *Glycine* (Tiffany and Gilman 1954, Hartman et al. 1986), *Vigna mungo* (Kausaul and Sharna 1998) and some dicotyledonous weeds (Hartman et al. 1986; Sinclair 1988). Other strains of *C. truncatum* have been investigated as mycoherbicide candidates on hemp sesbania

[*Sesbania exaltata* (Raf.) Rydb. ex A. W. Hill] (Silman and Nelsen 1993; Boyette et al. 1991), purple loosestrife (*Lythrum salicaria* L.) (Nyvall and Hu 1997) and Florida beggarweed [*Desmodium tortuosum* (S.W.) DC] (Caulder and Stowell 1988).

Many *C. truncatum* pathogens showed latent infection with symptomless host colonization (Fernando et al. 1994) until a change occurs in the physiological state (often senescence) of the host tissue (Cerkauskas and Sinclare 1980; Hartman et al. 1986). This makes pathogenicity assessment a challenge. Strict host specificity is required for the scentless chamomile BA because, in the region of intended application, fungal pathogen also called *C. truncatum* causes anthracnose on lentil (Morrall and Pedersen 1991) and pea (Anderson et al. 2000). Currently anthracnose has become widespread in lentil-growing areas of the prairie region (Chongo et al. 2002; Anderson 2003). In other areas, this fungal species causes anthracnose on soybean (Manandhar et al. 1985; Roy 1982). Due to the importance of several legume crops on the Canadian prairies, any non-host specific *C. truncatum* isolate will likely be rejected as a mycoherbicide candidate (Mortensen and Makowski 1994). It is also essential that scentless chamomile isolates are tested on multiple cultivars of lentil due to the variation of variety resistance (Buchwaldt et al. 2004). Additionally, cross inoculation with *C. truncatum* isolates from several crop hosts, together with an examination of the infection process, should provide direct evidence of the pathogenicity spectrum, and important insights into potential variation of *C. truncatum* isolates with respect to their host range.

Although most isolates from scentless chamomile are reported to be non-pathogenic to crop species, the fact that two isolates caused minor infection on lentil is of concern due to perceived risks to a region with the world's largest lentil production for

export markets. Besides causing disease on plants, some *Colletotrichum* spp. can be mammalian pathogens; *C. coccodes*, *C. crassipes*, *C. dematium*, C. *gloeosporioides, C. graminicola* and *C. acutatum* have been reported to cause infection in humans or animals (De Hoog et al. 2000; Fernandez et al. 2002; Cano et al. 2004). As a result, a clear differentiation of BA from others strains or species is an essential component of a risk assessment.

2.7. Taxonomy of *C. truncatum*

It is widely recognized that conventional identification/classification methods based on conidial morphology and colony characteristics are often unable to differentiate closely related *Colletotrichum* species. For example, it is almost impossible, on the basis of fungal morphology, to separate scentless chamomile isolates from several other *C. truncatum* pathogens or even different *Colletotrichum* species in the capsici group, including *C. capsici* (Syd.) Butl. & Bisby, *C. circinans* (Berk.) Vogl., and *C. lini* (Westerd.) Tochinai (R.A. Samsom, Centraalbureau Voor Schimmelcultures, the Netherlands and P.F. Cannon, CABI Bioscience, England, personal communications).

2.7.1. Classical Methods

The taxonomy of *Colletotrichum* spp. has traditionally been based on morphological characteristics such as conidial shape and size, the shape of appressoria, and presence or absence of setae. The species *C. truncatum* was first described in 1935 as a forma specialis within *C. dematium,* but was later taken out based on conidial size and host range variations (reviewed by Sutton 1992; Bailey et al. 1996). However, conidial morphology can sometimes be affected by environmental and nutritional

conditions in culture (Russo et al. 1980; Memmott et al. 2002), making definitive identification a challenge. Initially, there were a total of 900 *Colletotrichum* species which were reduced later to 39 (Sutton 1992). Conidia of *C. truncatum* are described as hyaline and one celled, with slightly falcate shape and a size of 17.0-31.5 x 3.0-4.5µm (length x width). Dark brown to black setae are generally produced in abundance. Irregular-shaped microsclerotia are submerged and may be confluent (Tiffany and Gilman 1954; Sutton 1992). The teleomorph of *Colletotrichum truncatum* has recently been reported belonging to *Glomerella* sp., based on a study of the pathogen causing lentil anthracnose (Armstrong-Cho and Banniza 2006).

2.7.2. Molecular Methods

Due to the lack of morphological variability among *Colletotrichum* species, additional criteria are needed that differentiate the BA from crop pathogens more reliably. In fungal genomes, ribosomal DNA (rDNA) genes include the 18S, 5.8S and 28S segments that code for ribosomal RNAs (rRNA). These are highly conserved genes that are separated by two less conserved regions, the internal transcribed spacers 1 and 2 (ITS1 and ITS2) (Figure 2.1, Muirl and Schlotterer 1999). Although not translated into proteins, these ITS regions have a critical role in the development of functional rRNA (Zimmerman and Dahlberg 1996). ITS sequences generally vary among different species, and are used widely as informative regions for PCR assays (White 1990; Kendall et al. 2005).

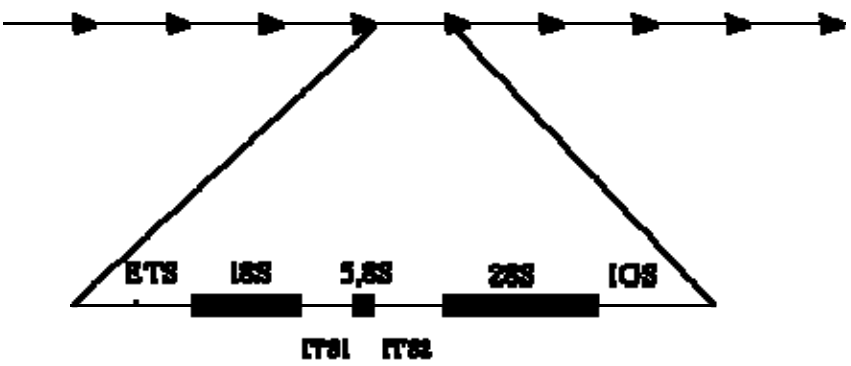

Figure 2.1. Illustration of ribosomal DNA (rDNA) and ITS locations on the chromosome. Single repeat units (arrows) are tandemly organised, consisting of the rRNA genes 18S, 5.8S and 28S. Spacers separate these genes, namely the external transcribed spacer (ETS), the internal transcribed spacers (ITS 1 and ITS 2) and the intergenic spacer (IGS).

These ITS regions have several advantages for sequencing and phylogenetic analysis of fungal species (Frothingham and Wilson 1993; Roth et al. 1998). First, the rate of change is appropriate for studies at the species and genus levels. Second, the alignment of the sequences is relatively simple and results can be interpreted phylogenetically. These regions are large enough to provide potential characteristics for phylogenetic reconstruction. Third, because these ITSs are flanked by regions that are highly conserved within genera and species, PCR amplification and sequencing are much easier than other parts of genomic DNA (White et al. 1990). ITS sequences have been used to study phylogenetics and systematics of *Colletotrichum* spp. (Sreenivasaprasad et al. 1996), assist with detection and diagnosis of clinical *Colletotrichum* specimens (Cano et al. 2004), investigate host-pathogen interactions (Casimiro et al. 2004), and conduct molecular typing (Iwen 2002) or develop markers for tracking purposes (Dauch et al. 2003). Other molecular techniques, including nuclear DNA polymorphism, mitochondrial DNA (mtDNA) analysis, random amplified polymorphic DNA (RAPD),

sequence analyses of gene introns and intron restriction fragment length polymorphism (RFLP) and rDNA analysis and sequencing, have also been used to differentiate *Colletotrichum* spp. (Sreenivasaprasad et al. 1996; Johnston 1997; Prusky 2000; Nirenberg et al. 2002; Guerber et al. 2003; Martinez-Culebras 2003). Some studies have shown that ITS1 is more divergent in *Colletotrichum* spp. than ITS2 (Screenivasaprasad et al. 1996; Cano et al. 2004). A number of *C. truncatum* isolates from lentil in Canada were studied for genetic similarity to Australian isolates of *C. truncatum* from hosts other than lentil (Ford et al. 2004), and found to be different based on RAPD markers and 18-25S rDNA sequences. The 18S rDNA gene analysis and denaturing gradient gel electrophoresis (DGGE) methods have also been used to delineate morphologically similar *Colletotrichum* species (Green et al. 2004). These results indicated that analysis of the ITS region was more informative than the 18S rDNA, providing an ideal tool for differentiation of closely related species.

In summary, identification and classification of *Colletotrichum* strains are frequently a challenge due to subtle differences in fungal morphology, but safe use of a *Colletotrichum* BA requires clear discrimination of the agent from closely related crop pathogens. Fungal genetic information, especially the ITS1 and ITS2 sequences, are being used increasingly to separate fungal strains at sub-species levels, which would otherwise be impossible to differentiate based on morphological variations. Molecular markers may also be used to track BA after release into the environment.

III. MOLECULAR AND MORPHOLOGICAL DIFFERENTIATION OF *COLLETOTRICHUM TRUNCATUM*

3.1. Introduction

Several *C. truncatum* isolates were considered promising for biocontrol of scentless chamomile due to moderate weed-control efficacy and high host specificity (Peng et al. 2005). In the same study, however, two isolates also caused slight infection on lentil and/or flax, raising a question on crop safety. This fungus is currently being studied for potential uses as a biocontrol agent (BA) in western Canada. The species *C. truncatum* is also an important pathogen on lentil in Manitoba and Saskatchewan (Morral 1988; 1991; Chongo and Gossen 2002), causing serious losses when disease epidemics occur (Anderson et al. 1999). This pathogen has also been reported to cause anthracnose diseases on several legume crops worldwide, including lentil, pea and soybean (Kaiser et al. 1998). Morphological and pathological characteristics often are insufficient for discrimination of different *C. truncatum* strains, and maybe unreliable for separating *C. truncatum* from closely related species such as *C. capsici*, *C. linicola*, and *C. circinans* (Dr. R.A. Samson, Centraalbureau Voor Schimmelcultures, Institute of the Royal Academy of Arts and Sciences, the Netherlands, personal communications). More definitive criteria are required to differentiate scentless chamomile isolates of *C. truncatum* from crop pathogens.

Classification of *Colletotrichum* species is based primarily on conidial and appressorial size and shape, and sometimes on pathogenic and biochemical traits.

Molecular techniques such as PCR amplification and sequence of ITS regions have been explored for differentiation of closed related fungal species and applications to characterizing different *Colletotrichum* species or *C. truncatum* strains were generally satisfactory (Bailey et al. 1996; Sreenivasaprasad et al. 1996; Ford et al. 2004).

The objectives of this study were to: 1) compare rDNA-ITS sequences for isolates of *C. truncatum* from scentless chamomile and pulse crops grown in western Canada; 2) determine phylogenetic relationship of these *C. truncatum* isolates to selected *Colletotrichum* spp.; and 3) examine morphological traits of *C. truncatum* isolates from different hosts to aid in strain discrimination at the sub-species level.

3.2. Materials and Methods

3.2.1. Isolates of *Colletotrichum* spp.

About 70 isolates of *Colletotrichum* spp. were selected from a wide range of hosts and grown in pure culture. Of these, 33 were *C. truncatum* isolates from scentless chamomile, lentil, pea, or soybean. The origin of these isolates is provided in Table 3.1. Unless otherwise noted, all isolates were collected from the Canadian prairies. Most isolates were from the Weed Biocontrol Cultural Collection of Agriculture and Agri-Food Canada (AAFC). Dr. Bruce Gossen (AAFC) provided most of the *C. truncatum* isolates from lentil, pea and soybean. The lentil Ct1 and Ct0 races of *C. truncatum* were provided by Dr. Sabine Banniza, Crop Development Centre, University of Saskatchewan. Additional isolates of *Colletotrichum* spp. were obtained from the Fungal Biodiversity Centre at the Centraalbureau Voor Schimmelcultures, the Netherlands. Scentless chamomile isolates were collected from Saskatchewan and Germany, and were treated as

two different populations. Each isolate was cultured in potato dextrose broth (PDB) at 20ºC for 3 d and the mycelial mass was harvested for DNA extraction.

3.2.2. Fungal DNA Extraction

Total genomic DNA was isolated and purified using the methods described by Talbot (2001). Mycelia were collected from liquid culture with filter paper and ground in liquid nitrogen. An aliquot of 400 µl extraction buffer was added to a 1.5 ml Eppendorf tube containing 50 mg of mycelium powder. The mix was vortexed, added with 400 µl of chloroform: isoamylethanol (24:1), then heated at 65ºC for 5 min, vortexed and centrifuged at 14000 rpm for 5 min. The supernatant was transferred to a new 1.5 ml Eppendrof tube, and added with 400 µl of iso-propanol was added to precipitate DNA at room temperature (23ºC). DNA was further centrifuged and washed with 70% ethanol, air-dried and the pellet was resuspanded in 20 µl of sterilized distilled water. These DNA suspensions were diluted to a final concentration of 10 ng /µl, and 2 µl of the DNA suspension from each isolate was used for PCR amplification of the ITS-rDNA region.

3.2.3. Conidial Production and Morphology

Isolates were grown on V8-juice agar medium at 20ºC for 10 d. Sporulating cultures were flooded with sterilized water amended with 0.1% Tween® 80 (Sigma Co., Oakville, ON, Canada) and scraped with a bent glass rod to dislodge spores. Conidial suspensions were filtered through two layers of cheesecloth and diluted to $1x10^5$/ml. Conidia were stained with tryphan blue (0.1%) and observed under light and confocal microscope for conidial morphology and spore size. Appressoria produced on host leaves were also stained with tryphan blue. Stained samples were examined initially

under a light microscope (Lertz Diaplan, Germany). A confocal laser-scanning microscope (Zeiss LSM 510 META, Jena, Germany) was used and images were produced under the following conditions: excitation wavelength was 543 nm. 25x water immersed objective; and the emission was collected from 603 nm to 625 nm by META channel. The pinhole size was 145 micrometer. Different emission images were sometimes combined for optimal visual effects. Spore and appressorium size was measured with a micrometer for length and width, and the mean size was calculated for each isolate based on 100 spores and appressoria. Treatment replicates for each isolate were first subjected to analysis of variance, followed by mean comparisons by the least-significant-difference test. Significance of differences in sample means for isolates was determined by one-way ANOVA analysis.

3.2.4. DNA Amplification and ITS Sequencing

The two ITS regions plus the 5.8 rDNA of *Colletotrichum* spp. were amplified using the universal primers ITS4 and ITS5 (Table 3.2). PCR procedures for taxon-specific amplification were performed in a total volume of 25 µl, containing 10 mM Tris-HCL, 50 mM KCL, 3.5 mM $MgCL_2$, 80 mM (each) of dATP, dCTP, dGTP, dTTP, 0.1 µl (1µM) each of the primers, 0.1 µl Taq (Invitrogen, 5U/µl) polymerase, and 0.1µl Pfu (Stratagene, 2.5 U/µl), 2 µl genomic DNA. The reaction mixtures were incubated in an Eppendorf Thermocycler (Eppendorf Co., NY, USA), DNA was denatured at 94˚C for 3 min, followed with 35 cycles consisting of 30 s at 94˚C, 20 s at 58˚C, and 1 min at 72˚C. PCR products were separated on 1% agarose gels with 0.5X TAE buffer. Gels were stained with ethidium bromide and DNA bands visualized with UV light. DNA bands were estimated with the aid of a standard DNA ladder (QIAGEN Inc. Mississauga, ON,

Canada). Purification of the amplicons from the gel was performed with QIAGEN purification kits according to supplier's protocols and processed samples were stored at –20ºC. DNA sequencing was performed on contract at the Plant Biotechnology Institute, National Research Council of Canada in Saskatoon.

3.2.5. Phylogenetic Tree Generation

Sequences of the 5.8 rDNA-ITS fragment were aligned using the multiple-sequence alignment program CLUSTAL X, prior to being imported into PAUP* 4.0 (Phylogenetic Analysis Using Parsimony) (Swofford 2002), which used parsimony and neighbor-joining (NJ) methods to generate trees for similarity analysis. Parsimony trees were generated by heuristic search with the bootstrap test (1000 pseudoreplicates). All characters had equal weight and gaps were treated as "missing" values. Bootstrap 50% majority-rule consensus was used to separate isolates. Initially, sequences for the whole 5.8 rDNA-ITS segment were used for a parsimony tree. For the analysis of individual ITS regions, sequences ranging from 50 to 200 base pairs (bp) and 400 to 550 bp were chosen to generate parsimony ITS1 and ITS2 trees, respectively. A NJ tree was built using the ITS1 sequence information only for a comparison with the parsimony method.

Table 3.1. *Colletotrichum* spp. selected for ribosomal DNA sequencing.

Fungal isolates	Species [a]	Host	Origin	Seq. No.
91-166	*C. dematium*	*Chenopodium album* L.	Canada	C5
86-20A	*C. dematium*	*Rumex* sp.	Canada	C16
95-43B	*C. dematium*	*Taraxacum officinale* Weber	Canada	C33

CBS506-97	*C. capsici*	*Vigna unguiculata* L.	Brazil	D3
94SD	*C. trifolii*	*Medicago sativa* L.	Canada	D16
2-6-33	*C. circinans*	*Allium vineale* L.	U.S.A.	E7
CBS172.51	*C. lini*	*Linum ustatissimum* L.	Netherlands	E9.F19
Aust-3	*C. orbiculare*	*Xanthium strumarium* L.	Canada	C29
5-6-33	*C. orbiculare*	*Lagenaria* L.	U.S.A.	E6
4-1-38	*C. orbiculare*	*Cucumis sativus* L.	Canada	F16
CBS142-79	*C. truncatum*	*Stylosanthes hamata* L.	Australia	F17
93-121-A1	*C. destructivum*	*Convolvulus arvensis* L.	Canada	C25.F9
97-17-C2	*C. destructivum*	*Trifolium pratense* L.	Canada	C17
CBS509-97	*C. acutatum*	*Lupinus albus* L.	France	F18
94-392	*C. caudatum*	*Setaria viridis* (L.) Beauv.	Canada	F7.D4
CBS-330-75	*C. acutatum*	*Cothea Arabica* L.	Netherlands	C36
94-273A1	*Colletotrichum* sp.	*Cirsium arvense* (L.) Scop.	Canada	C31
98-13-A	*Colletotrichum* sp.	*C. arvense*	Canada	C34
00248A1	*C. truncatum*	*Metricaria perforate* Mérat	Canada	C21
01-G1A1-1	*C. truncatum*	*M. perforate*	Germany	C26
01-G3A1-3	*C. truncatum*	*M. perforata*	Germany	C27
0214B1	*C. truncatum*	*M. perforata*	Canada	E5
01-052A1	*C. truncatum*	*M. perforata*	Canada	E4
00193C	*C. truncatum*	*M. perforata*	Canada	E2
003B1	*C. truncatum*	*M. perforata*	Canada	C3
94-97C	*C. truncatum*	*M. perforata*	Canada	C7
21-H-1	*C. truncatum*	*M. perforata*	Canada	C8
01-G3A1-4	*C. truncatum*	*M. perforata*	Germany	C28
026B2	*C. truncatum*	*M. perforata*	Canada	F8
90-50	*C .coccodes*	*Amaranthus* sp.	Canada	C19
184034	*C. coccodes*	*Abutilon theophrasti* Medik	Canada	E12
95-394A2	*C. graminicola*	*Aechinochloa crusgalli* (L.) Beauv.	Canada	D18
89-23A	*C. gloeosporioides*	*Lavatera* sp.	Canada	F15.D20
84-15	*C. gloeosporioides*	*Malva pusilla* Sm.	Canada	D7
Cga060	*C. gloeosporioides*	*Aeschynomene virginica* (L.) B.S.P.	Canada	C1
Jun-9903	*C. truncatum* Ct0	*Lens. culinaris* Medik	Canada	D14
May9904	*C. truncatum*	*L. culinaris*	Canada	C11
May9901	*C. truncatum*	*L. culinaris*	Canada	F13
Jun9902	*C. truncatum* Ct1	*L. culinaris*	Canada	C2
May9930	*C. truncatum*	*L. culinaris*	Canada	C6
Ct8-0202	*C. truncatum*	*L. culinaris*	Canada	D21
Apr-9906	*C. truncatum* Ct0	*L. culinaris*	Canada	D19
Ct095A8	*C. truncatum*	*L. culinaris*	Canada	D12
Ct1-0202	*C. truncatum*	*L. culinaris*	Canada	E8
(May9903)	*C. truncatum*	*L. culinaris*	Canada	F12
apr0102	*C. truncatum*	*L. culinaris*	Canada	C4
May-9914	*C. truncatum* Ct0	*L. culinaris*	Canada	F14
May-9934	*C. truncatum*	*L. culinaris*	Canada	C12
Ct21-0202	*C. truncatum*	*L. culinaris*	Canada	C18
Ct14-0202	*C. truncatum*	*L. culinaris*	Canada	E11

Ct11-0202	*C. truncatum*	*L. culinaris*	Canada	C30
Ct12-0202	*C. truncatum*	*L. culinaris*	Canada	C38
May-9938	*C. truncatum*	*L. culinaris*	Canada	C13
2-5-7	*C. truncatum*	*Glycine max* (L.) Merr.	U.S.A.	D22
Jul0428	*Colletotrichum* sp.	*G. max*	Canada	F6
Jul0429	*Colletotrichum* sp.	*G. max*	Canada	F3
Jul0430	*Colletotrichum* sp.	*G. max*	Canada	F5
Jul0431	*Colletotrichum* sp.	*G. max*	Canada	F2
Jul0433	*Colletotrichum* sp.	*G. max*	Canada	F1
Jul0436	*Colletotrichum* sp.	*G. max*	Canada	F4
Jul0415	*Colletotrichum* sp.	*Pisum sativum* L.	Canada	D13
Jul0402	*Colletotrichum* sp.	*P. sativum*	Canada	F11
Jul0403	*Colletotrichum* sp.	*P. sativum*	Canada	C24
Jul0414	*Colletotrichum* sp.	*P. sativum*	Canada	D1
Jul0413	*Colletotrichum* sp.	*P. sativum*	Canada	D6
94-139-A1	*Colletotrichum* sp.	*Stellaria media* (L.) Vill.	Canada	C23
DQ195708 [b]	*C. graminicola*	unknown	Gene bank	G01
AY539806 [b]	*C. gloeosporiodes*	*Crupina vulgaris* Cass.	Gene bank	G02
AF451902.1 [b]	*C. truncatum*	*L. culinaris*	Gene bank	G03
AB233340 [b]	*C. coccodes*	*P. sativum*	Gene bank	G04
Aj301985 [b]	*C. truncatum*	*G. max*	Gene bank	G05
AJ301976 [b]	*C. truncatum*	*Lupinus* sp.	Gene bank	G06
DQ195718 [b]	*C. circinans*	unknown	Gene bank	G07
DQ195689 [b]	*C. capsici*	unknown	Gene bank	G08
DQ195690 [b]	*C. caudatum*	unknown	Gene bank	G09

[a] Classification based on morphology only. [b] Sequences available at the Gene bank identified by these accession numbers.

Table 3.2. Primers used for sequencing ITS-rDNA regions of *Colletotrichum* spp.[a]

Primers	Oligos
ITS4 (R)	TCC TCC GCT TAT TGA TAT GC
ITS5 (F)	TGG AAG TAA AAG TCG TAA CAA GG

[a] From White et al. 1990.

3.3. Results

3.3.1. ITS Sequencing

PCR amplicons from a total of 70 *Colletotrichum* isolates were sequenced, and the size of ITS-rDNA fragments ranged from 471 to 666 bp. An example of PCR amplification of scentless chamomile isolates is presented in Figure 3.1.

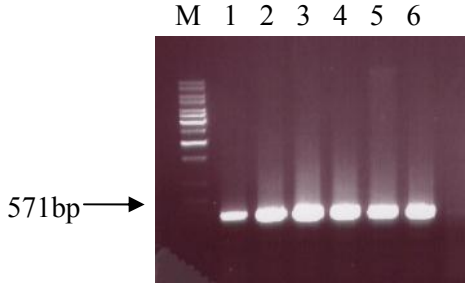

Figure 3.1. PCR amplicon of ITS regions using ITS4/ITS5 primers. Lane 1-6: isolates of *C. truncatum* from scentless chamomile; Lane M: a DNA ladder.

Based on nucleotide homology in whole 5.8S rDNA-ITS segments, isolates from different hosts were separated into four groups (Figure 3.2) with high similarity within each group of scentless chamomile, lentil, and soybean. Isolates from pea showed slightly greater divergence (4%) and were further divided into two subclusters within which the nucleotide similarity was 98% and 99%, respectively. Within one of the subclusters, two pea isolates (C24, F11) were 97% and 99% similar to a *C. circinans* isolate from wild garlic (E7), while isolates in the other subcluster were highly similar to a *C. coccodes* isolate (G04). Among host groups, scentless chamomile isolates were 96% similar to lentil isolates, 95% to soybean isolates, 94% identical to the *C. circinans*-like pea isolates, and 95% to *C. coccodes*-like pea isolates. Sequences of lentil and pea isolates were also 95% similar, whereas lentil and soybean isolates were 96% similar.

One scentless chamomile isolate (C21) showed a slight variation from others (8% divergence), but was still closest to the cluster of scentless chamomile isolates (Figure 3.2). Two *C. truncatum* isolates, one from soybean (D22) and the other from Caribbean stylo (F17), exhibited >10% divergence when compared to the *C. truncatum* isolates from scentless chamomile, lentil, pea or soybean (Figure 3.2). The isolate D22 was most closely related to *C. capsici*, and F17 was identical to *C. acutatum* with >99 % similarity in both cases. Other *Colletotrichum* spp. were more clearly separated from *C. truncatum* isolates, with 3% to 10% divergence.

When the two ITS regions were examined separately, the size of ITS1 fragments ranged from 97 to 130 bp, and that of ITS2 from 120 to 150 bp. There was a slightly greater degree of nucleotide variability in the ITS1 region than in ITS2, resulting in different placement of several isolates (Figures 3.3, 3.4, 3.5). The phylogenetic tree derived from ITS1 data was more similar to that based on sequences of whole rDNA-ITS fragments. In contrast, sequences of the ITS2 failed to differentiate two types of pea isolates (97% homology), and placed a scentless chamomile isolate of *C. truncatum* (C21*)* in the cluster of mixed species (Figure 3.5). Based on ITS1 sequences, phylogenetic trees generated with both parsimony and NJ methods showed a similar pattern for isolate separation (Figures 3.3; 3.4).

The size of rDNA-ITS segments sequenced with the primer ITS4 and ITS5 ranged from 471 bp to 666 bp. All of the isolates from scentless chamomile showed the same DNA sequence regardless of their origin (from Canada or Germany), with a total of 571 bp of rDNA-ITS amplified except the isolate C21 (572 bp). Lentil isolates, with 666 bp

amplified, also showed the same sequence consistently. Complete sequence region data for all tested *Colletotrichum* species is provided in Appendix A.

3.3.2. Phylogenetic Analysis

Based on the rDNA-ITS sequence, the phylogenetic tree derived from parsimony analysis consisted of two major branches that separated the isolates D3, D22, and G08 from the rest with 100% bootstrap support (Figure 3.2). Several sub- or sub-sub-branches under one of the major branches further divided the remaining isolates into lentil, scentless chamomile, soybean, pea and other distinctive clusters with 82% to 100% bootstrap support. C16 and F17, previously identified as *C. dematium* and *C. truncatum*, were grouped with two isolates of *C. acutatum* with 100% bootstrap support. One isolate of *C. gloeosporioides* was placed with three isolates of *C. orbiculare* (89% similarity) with 82% bootstrap support.

On the basis of bootstrap values and paired comparisons of nucleotide variability with selected species/isolates (Table 3.4), scentless chamomile isolates were separated from other *C. truncatum* strains or *Colletotrichum* spp. with 3 to 13% divergence and 99% bootstrap support (Table 3.4, Figure 3.6). Two lentil isolates (C30, G03) were identical with 100% similarity and were separated from others with 97% bootstrap support (Figure 3.6). Two isolates of *C. truncatum* (G05, G06) from soybean and lupine were 99% similar to another soybean isolate F1 (Table 3.4), and together they formed a distinct group with 100% bootstrap support (Figure 3.6). Two groups of pea isolates were separated from each other and from others with 92% and 100% bootstraps (Figure 3.6). These pea isolates showed 96% to 99% similarity to *C. coccodes* and *C. circinans*, respectively (Table 3.4). Lentil and scentless chamomile isolates are most closely related

among different *C. truncatum* isolates tested (Figure 3.6). Although the scentless chamomile isolate C21 was 13% different from the scentless chamomile isolate C3 (Table 3.4), it was still most similar to the C3.

3.3.3. Conidial Morphology

Conidia of *C. truncatum* isolates from scentless chamomile and lentil were slightly falcate, but spores of the scentless chamomile isolates were slightly more truncated (Figure 3.7). Conidia of the soybean isolates were most typically falcate, whereas those of the alfalfa isolates were more ovoid (Figure 3.7). Isolates of *C. lini* and *Colletotrichum* spp. from pea produced conidia with a similar shape to that of scentless chamomile and lentil isolates (data not shown). Appressoria of various *Colletotrichum* spp. were generally globose (Figure 3.8) with only slight differences in size.

Conidia of the soybean isolates were approximate 50% longer but 20% narrower than those of other isolates examined (Table 3.3), and therefore were distinguishable from others. The soybean isolate produced appressoria, which were about 40% bigger than those of other isolates. The size of conidia and appressoria were similar among other isolates (Table 3.3).

Table 3.3. Average size of conidia and appressoria for selected *Colletotrichum* isolates [a]

Isolate (Species)	Host	Size of conidia (μm) [b]	Size of appressoria (μm) [b]
94SD (*C. trifolii*)	Alfalfa	17.5 ± 2.3 x 4.9 ± 0.4	6.2 ± 0.5 x 5.4 ± 0.4
00248A1(*C. truncatum*)	Chamomile	17.4 ± 3.6 x 4.6 ± 0.5	6.0 ± 1.3 x 5.1 ± 0.5
Ct110202 (*C. truncatum*)	Lentil	17.3 ± 1.8 x 4.6 ± 0.5	6.3 ± 1.0 x 5.4 ± 0.6
Jul0414 (*Colletotrichum* sp)	Pea	16.1 ± 0.2 x 4.5 ± 0.5	5.8 ± 1.6 x 4.6 ± 1.2
Jul0435 (*Colletotrichum* sp)	Soybean	26.4 ± 2.1 x 3.8 ± 1.0	10.1 ± 0.1 x 5.0 ± 0.2

[a] Based on 100 single spores or appressoria of each isolate (mean ± standard deviation). No significant differences were observed in the size of conidia and appressoria between scentless chamomile, lentil, pea and alfalfa isolates, whereas conidia of the soybean isolate were substantially longer but narrower, and appressoria were larger ($P > 0.05$).
[b] Length and width of conidia, or maximum and minimum diameters of appressoria.

Table 3.4. Similarity matrix of rDNA-ITS sequence for representative isolates of *Colletotrichum* spp. [a]

	F18	*D3*	*G08*	*G09*	*E7*	*G07*	*G04*	*G02*	*G01*	*E9*	*E6*	*D1*	*F11*	*C3*	*C21*	*C30*	*D22*	*F1*	*F17*	*G03*	*G05*	*G06*
F18 *C. acutatum*	100																					
D3 *C. capsici*	88	100																				
G08 *C. capsici*	89	99	100																			
G09 *C. caudatum*	93	92	92	100																		
E7 *C. circinans*	93	93	93	96	100																	
G07 *C. circinans*	93	93	88	89	99	100																
G04 *C. coccodes*	92	91	92	95	96	96	100															
G02 *C. gloeospo*.	92	91	91	90	96	96	93	100														
G01 *C. graminicola*	92	92	93	96	96	95	94	95	100													
E9 *C. lini*	91	91	91	96	96	96	93	99	93	100												
E6 *C. orbiculare*	88	87	88	79	89	88	88	89	81	89	100											
D1 *Colletotrichum* sp.	93	92	92	95	97	96	99	96	94	96	89	100										
F11 *Colletotrichum* sp.	93	93	93	96	99	99	96	96	96	96	89	97	100									
C3 *C. truncatum*	92	90	91	95	94	94	91	97	94	97	88	94	94	100								
C21 *C. truncatum*	82	81	81	84	84	84	82	86	84	86	80	85	84	87	100							
C30 *C. truncatum*	93	91	91	96	96	96	94	98	95	98	90	96	96	96	86	100						
D22 *C. truncatum*	89	99	99	92	93	93	91	92	92	92	87	93	93	91	81	92	100					
F1 *C. truncatum*	93	92	91	93	96	96	95	96	93	96	88	96	96	94	84	96	93	100				
F17 *C. truncatum*	96	85	86	90	90	90	89	90	90	89	86	91	90	90	80	90	86	90	100			
G03 *C. truncatum*	93	91	91	96	96	96	96	96	95	98	90	96	96	96	85	100	92	96	90	100		
G05 *C. truncatum*	92	92	91	93	95	96	96	96	90	96	87	96	95	94	85	96	92	99	90	96	100	
G06 *C. truncatum*	92	92	91	93	95	96	96	95	93	95	83	96	95	93	85	95	92	99	90	95	100	100

a. Based on ITS-rDNA sequence with multiple alignment including gaps.

3.4. Discussion

In this study, sequences of the 5.8S rDNA-ITS fragments were used to differentiate among several strains of *Colletotrichum truncatum,* as well as closely related species that could not be separated based on morphological characteristics alone. In most cases, there were no clear differences in conidial or appressorial size and shape among these strains or species. Also, it was not possible to separate scentless chamomile and lentil isolates of *C. truncatum* based on spore shape and dimension. In contrast, there was sufficient intraspecific divergence in ITS nucleotides between these two groups to allow their separation with confidence. Similar techniques have been used previously to evaluate systematics of *Colletotrichum* spp. (Bailey et al. 1996; Sherriff et al. 1994; Sreenivasaprasad et al. 1996), relatedness of anthracnose isolates causing diseases on crops (Johnston and Jones 1997; Sreenivasaprasad et al. 1992), and *C. truncatum* isolates from different hosts (Ford et al. 2004). The separation of various isolates of *C. truncatum* based on the ITS sequence suppports a previous report that *C. truncatum* isolates from different hosts can be discriminated using molecular techniques (Ford et al. 2004). Genetic evidence from the current study showed that despite a high degree of similarity, there were identifiable divergences among the *C. truncatum* isolates originating from scentless chamomile, lentil and soybean that separate them effectively. Although these differences may not necessarily influence the function of the isolates or strains, especially parasitism and pathogenicity on plants, they did help discriminate scentless chamomile isolates more definitively from others as opposed to using morphological traits. As a molecular marker, this differentiation is also highly desirable for testing *C. truncatum* strains that infect lentil. Coupled with data on host range reported earlier (Peng et al. 2005), this information should reduce the concern about

crop safety for using BA strains of *C. truncatum* against scentless chamomile in western Canada.

Previous studies on the phylogeny and systematics of *Colletotrichum* spp. indicate that separate examinations of ITS1 and ITS2 regions yield more information than the whole segement of 5.8 rDNA-ITS (Cano et al. 2004; Sherriff et al. 1994; Sreenivasaprasad et al. 1996). In the current study, phylogenetic grouping based on ITS1 data was similar to that derived from sequencing the whole 5.8 rDNA-ITS region, while ITS2 data generated a tree with slight variations from those based on 5.8 rDNA-ITS or ITS1 sequences. In general, less variability in nucleotides was obserbed in ITS2, and this was largely responsible for failing to separate putative isolates of *C. circinans* and *C. coccodes* from pea (> 97% homology). These two species could be discriminated clearly based on ITS1 sequence data.

Varying sequence characteristics were observed for *C. truncatum* isolates from different hosts. All lentil isolates, including both Ct0 and Ct1 races that exhibited different levels of virulence on lentil cultivars (Buchwaldt et al. 2004), were very similar in both ITS1 and ITS2 regions, and are indistinguishable in ITS sequences. No substantial variation was observed between scentless chamomile isolates from western Canada and Europe, implying that the two populations likely shared a common ancestor and the fungus might have come with the plant or seeds to North America when the weed was introduced from Europe 100 years ago (Douglas et al. 1991). One isolate (C21) from Canada was sufficiently divergent so it was differentiated from the rest of the scentless chamomile isolate group. This indicates potential diversity within the fungal population. All soybean isolates tested in the study showed similar sequences to those of Genbank

soybean (G05) and lupine (G06) isolates of *C. truncatum*. Although two of the soybean isolates had previously been designated as *C. destructivum* (C17) and *C. dematium* (C33), their ITS sequences were identical to others in this group. Classification of *C. truncatum* has evolved over the years. This species was initially described as a forma specialis in *C. dematium* (Sutton 1992), but morphological plasticity and overlap of phenotype make accurate identification of these closely related species very difficult (Screenivasaprasad et al. 1996). Based on the ITS information, it is believed that all the soybean isolates tested in the current study are similar and should be designated as *C. truncatum.*

Isolates of *C. gloeosporioides* displayed a high degree of variability, which divided them into three separated clusters. Similar variation was also noted for this species in other studies using isolates from a range of hosts (Sreenivasaprasad et al. 1996; Johnston and Jones 1997). Based on ITS1 sequencing of 26 *C. gloeosporioides* isolates, Sreenivasaprasad et al. (1996) found that the divergence ranged from 0 to 3.6%. Sherriff et al. (1994) observed even greater genetic variation within this species and suggested that *C. gloeosporioides* isolates used in various studies represent more than one species. The two putative *C. gloeosporioides* isolates tested in the current study might have been misidentified previously; one of them was closely associated with the group of *C. obiculare* and the other was simiar to the Genbank isolate of *C. caudatum*.

Also based on ITS1 sequences, isolates from pea can be separated into two subgroups; one has almost identical sequences to a *C. circinans* isolate obtained from a crow garlic plant (*Allium vineale* L.) and the other is similar to a Genbank isolate of *C. coccodes*. There has been no report in literature linking these two species to anthracnose on pea. In an earlier study, Anderson et al. (2000) found that lentil *C. truncatum* isolates

were able to infect pea, although the virulence was lower than that observed on lentil. Caution is required when interpreting the results due to a limited number of pea isolates included in the study. A couple of possibilities exist: 1) *C. circinans*, *C. coccodes*, and *C. truncatum* are all capable of infecting pea, and 2) previous designation of *C. circinans* and *C. coccodes* may be incorrect because the three species are morphologically similar. Further information is needed to clarify these issues, but evidence from a later study (Chapter V) does indicate that *C. truncatum* can be highly virulent on pea.

Phylogenetic trees placed the isolates of *C. destructivum* and *C. lini* together with several other species, including *C. coccodes*, *C. gloeosporioides*, and *C. trifolii.* This cluster encompassed a group of *Colletotrichum* species, whose ITS sequences showed reasonably high similarity. A possible explanation may be that some of the isolates might have been misidentified previously. The isolates of *C. destructivum* and *C. lini*, with similar morphological and sequence characteristics, may belong to the same species, as suggested by Shen et al. (2001).

This study was not intended to develop comprehensive molecular systematics for of the genus *Colletotrichum*, but rather to focus on *C. truncatum* and related species to verify a hypothesis derived from an earlier study that *C. truncatum* isolates from scentless chamomile were different from those that cause anthracnose diseases on lentil, pea, or soybean (Peng et al. 2005). It was shown that rDNA-ITS sequences are valuable for differentiating among *C. truncatum* strains from scentless chamomile, lentil, soybean and other host species. This information will be useful, when combined with host-specificity data, to demonstrate crop safety of the BA on major field crops in western Canada.

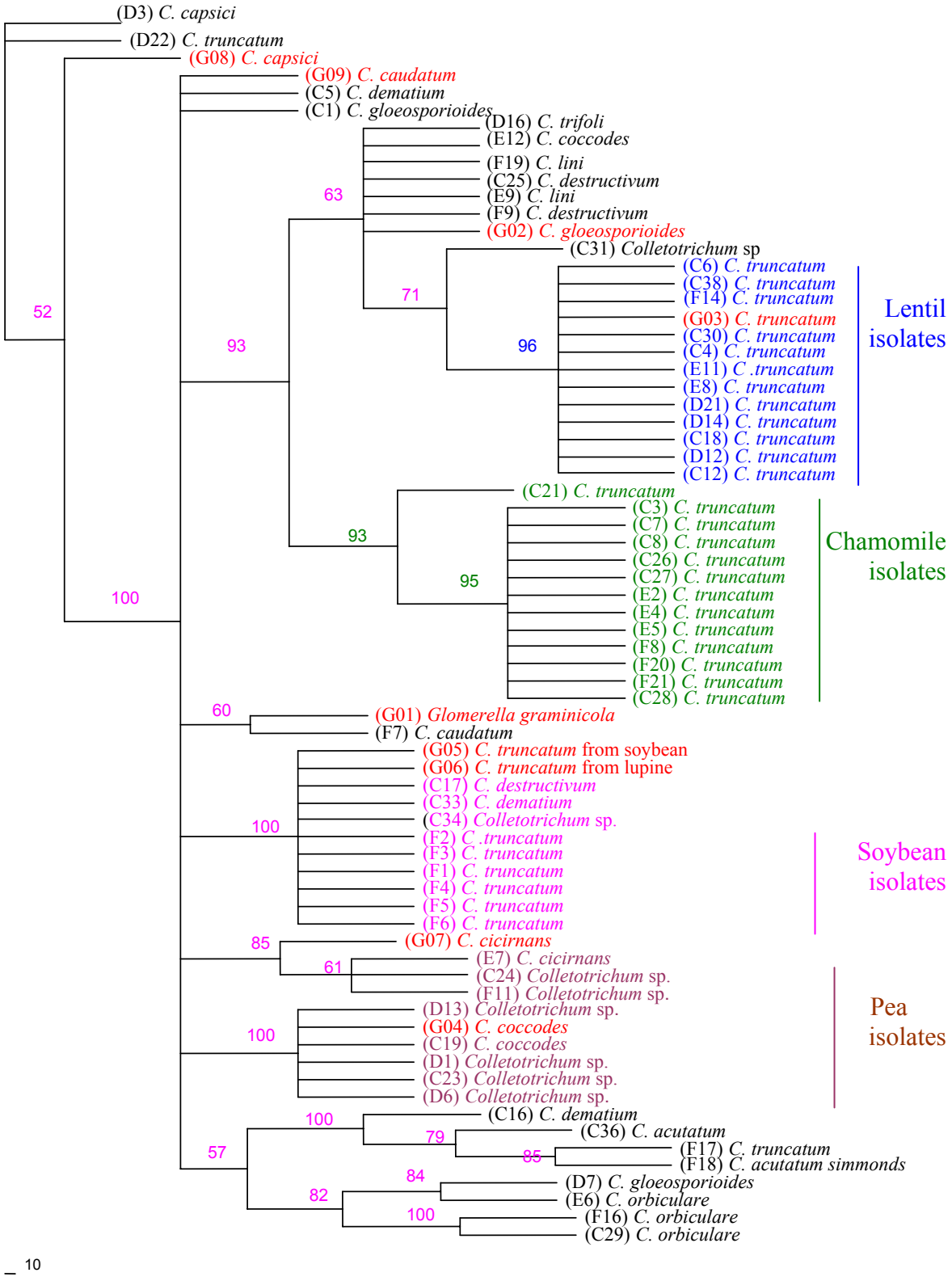

Figure 3.2. Parsimony phylogram of ITS sequences with bootstrap generated with PAUP* Numbers in brackets are isolate I.D. numbers. Bootstrap values >50% are presented on the top of branches. Red colour isolates are from Genbank.

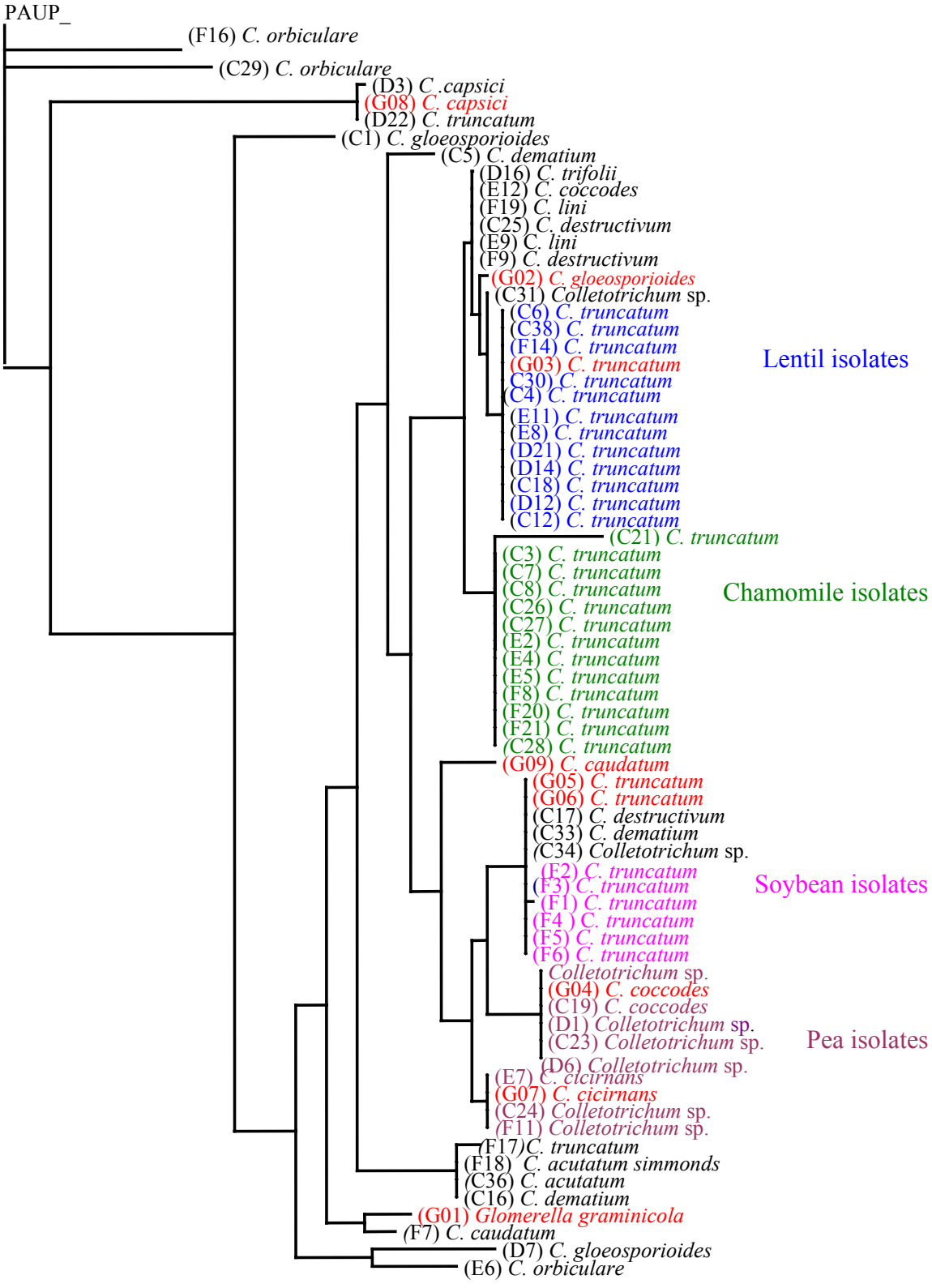

Figure 3.3. Neighbor-joining tree generated with ITS1 sequences. Numbers showed in brackets are isolate I.D.numbers (corresponding to isolate numbers in Table 3.1). Red colour isolates are from GenBank.

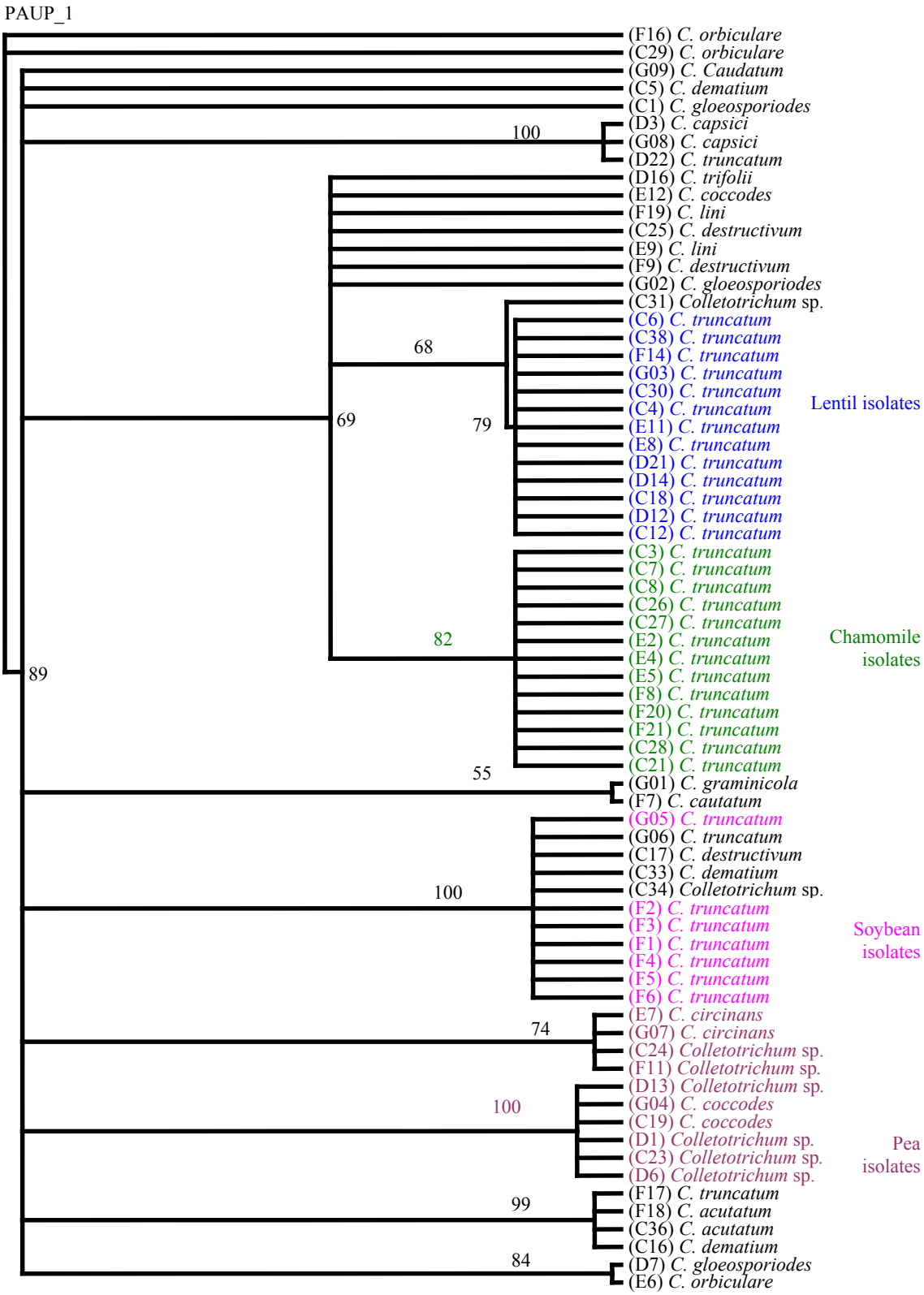

Figure 3.4. Parsimony phylogram tree of ITS1 sequences generated with PAUP* Numbers in brackets are isolate I.D.numbers. Bootstrap values > 50% are presented on the top of branches.

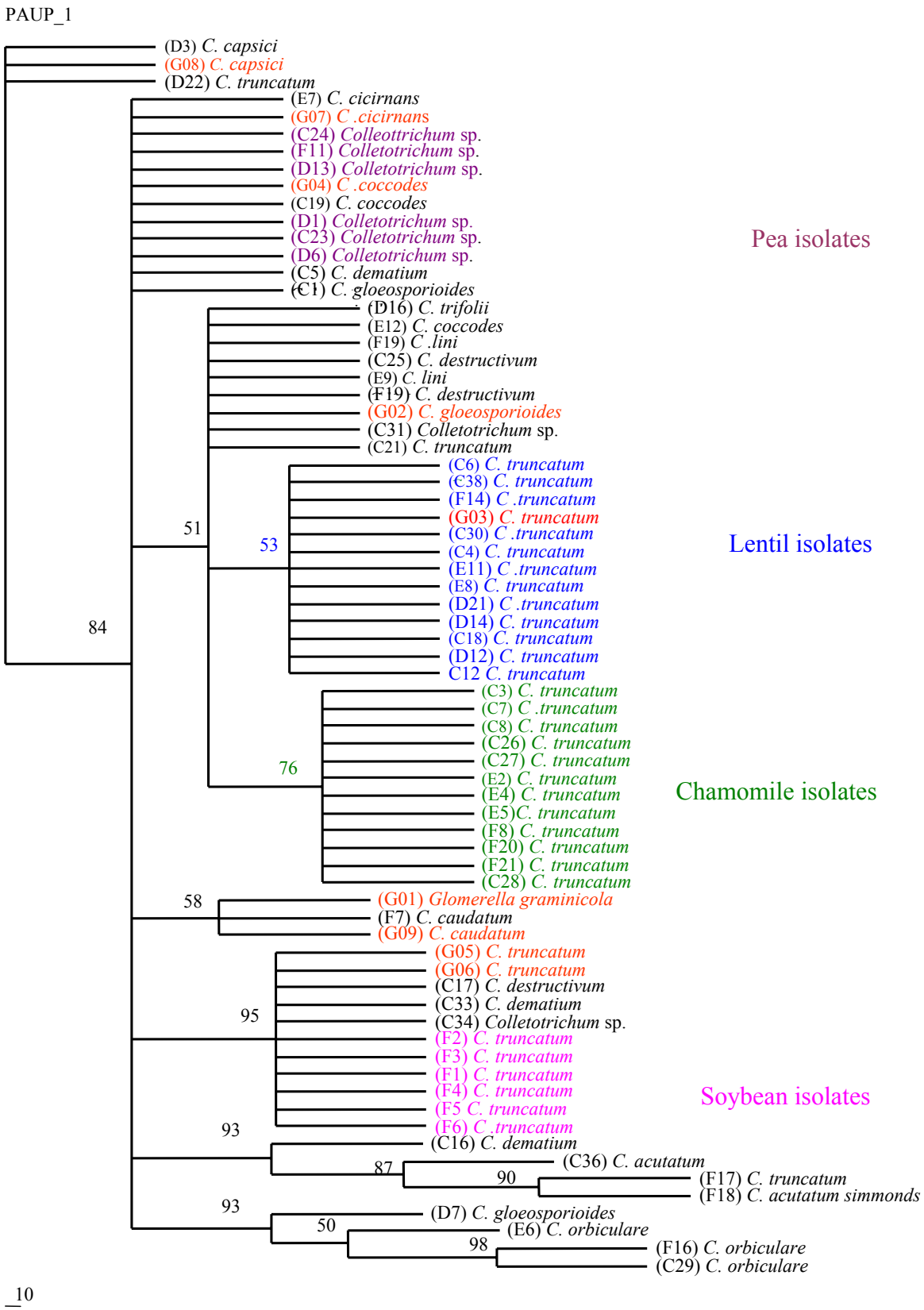

Figure 3.5. Parsimony phylogram tree based on ITS2 sequence data. The numbers in brackets are isolate I.D. numbers. Bootstrap values > 50% are presented on the top of branches. Red colour isolates are from GenBank.

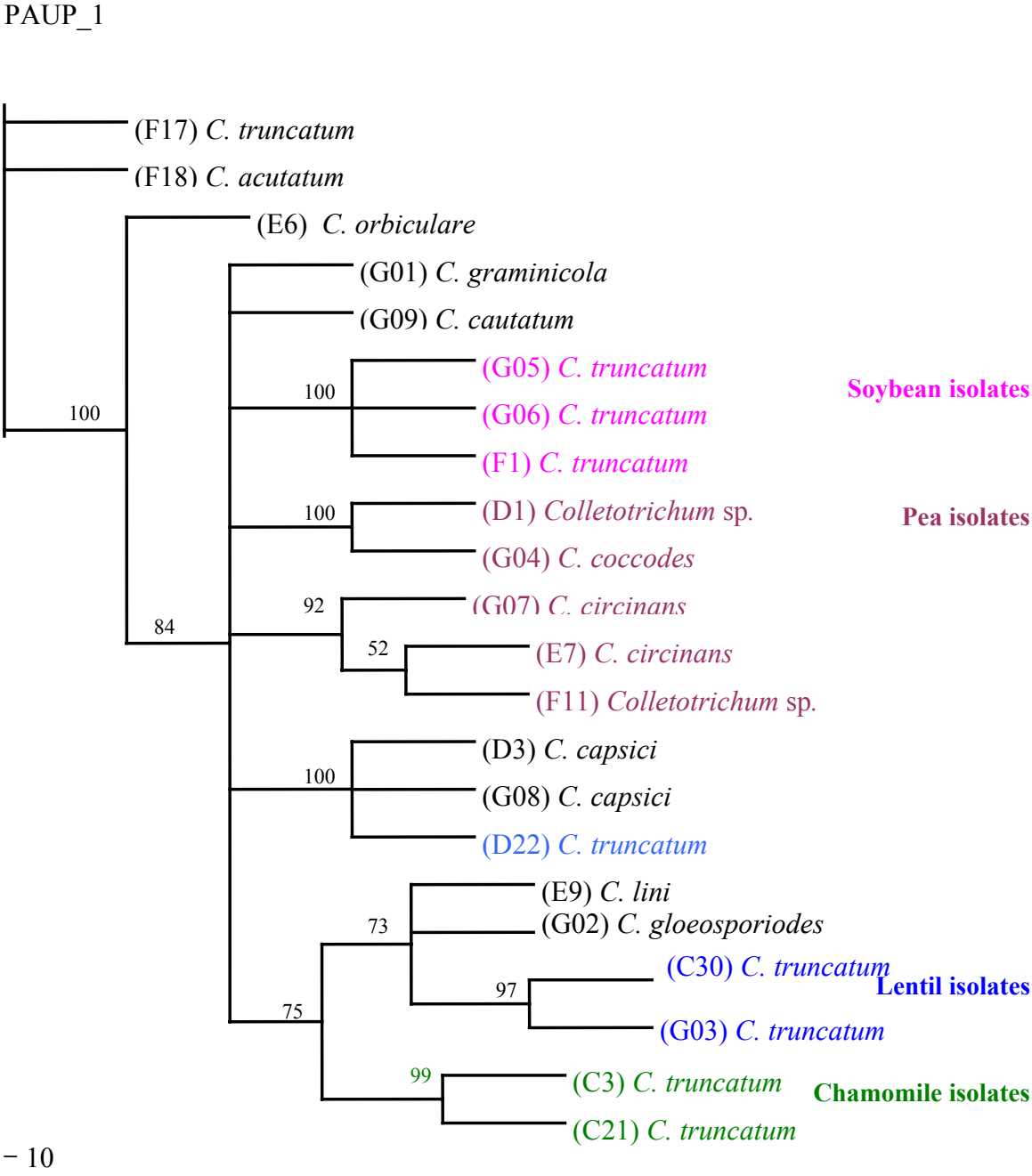

Figure 3.6. Parsimony phylogram tree based on whole 5.8s rDNA-ITS sequence of selected isolates of different *Colletotrichum* spp. included in Table 3.5. Bootstrap values >50% are presented on the top of branches.

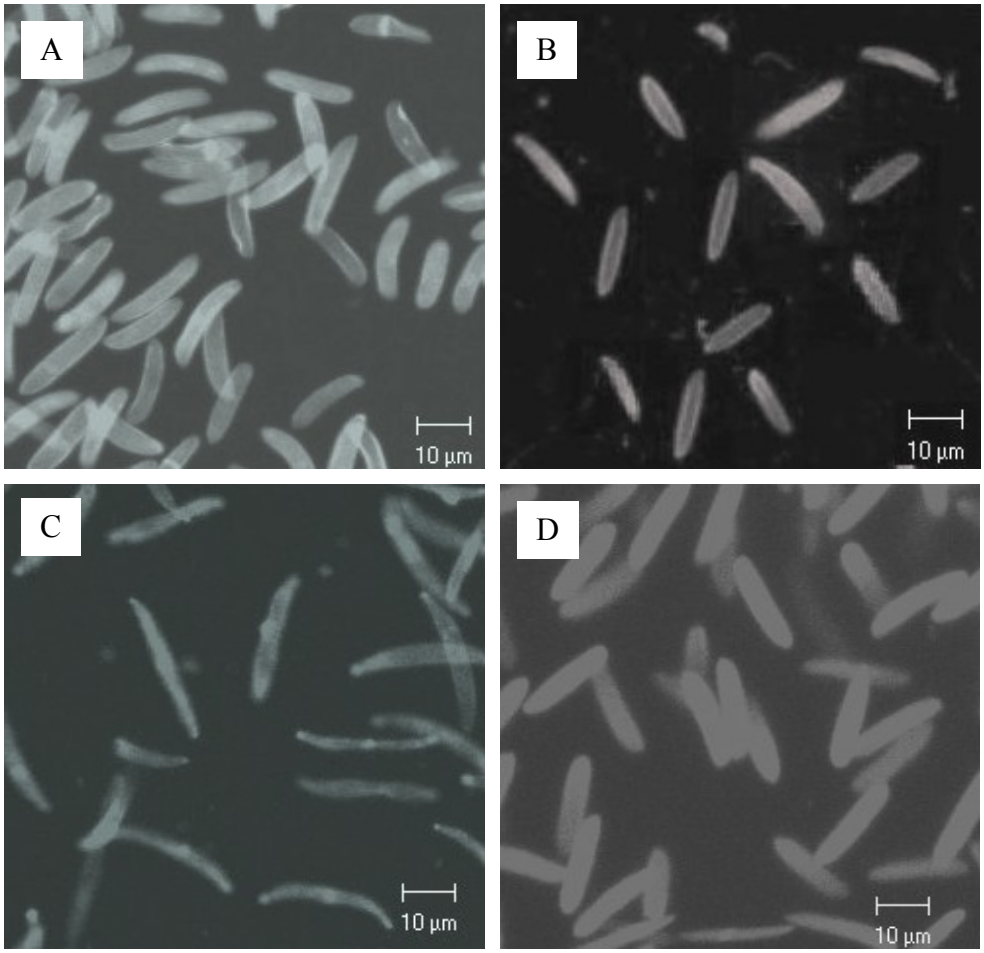

Figure 3.7. Conidia of *Colletotrichum* spp. from: scentless chamomile (A), lentil (B), soybean (C) and alfalfa (D) under a confocal laser-scanning microscope with images in a gray scale. These images showed conidial shape and size of different isolates on their host plants.

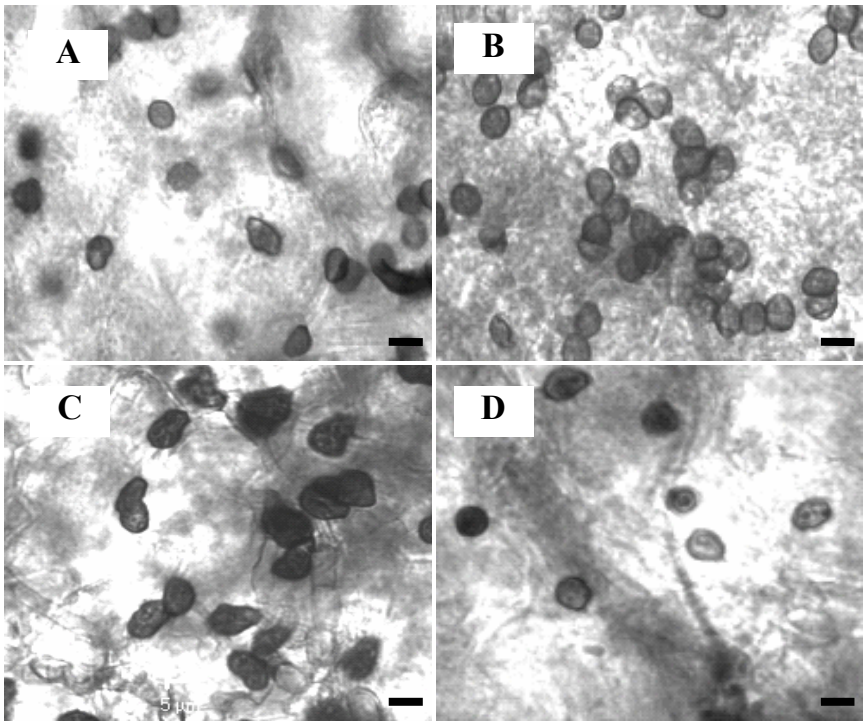

Figure 3.8. Appressoria of *Colletotrichum* spp from: scentless chamomile (A), lentil (B), soybean (C), and pea (D) under a confocal laser-scanning microscope with images in a gray scale. Bars = 5μm.

IV. DEVELOPEMNT OF STRAIN-SPECIFIC PCR MARKERS FOR *COLLETOTRICHUM TRUNCATUM*

4.1. Introduction

Colletotrichum truncatum causes anthracnose diseases on several important legume crops worldwide, while some host-specific strains of *C. truncatum* have been studied for weed biocontrol (Jackson et al. 1990; Boyette 1991). In western Canada, a strain of *C. truncatum* has been under development as a potential biocontrol agent (BA) against scentless chamomile (Peng et al. 2005; Graham at al. 2006). Despite variations in host specificity, isolates of *C. truncatum* from different hosts are often impossible to distinguish based on morphological characters. Molecular methods based on the amplification of DNA sequences using polymerase chain reaction (PCR) have been employed to differentiate strains at a subspecific level (Moriwaki et al. 2002; Cano et al. 2004; Ford et al. 2004), and some of these techniques have previously been used to detect and monitor soil fungi in natural environments (Bridge and Spooner 2001). Compared to other diagnostic methods, PCR-based techniques have the advantages of being simple, sensitive, rapid and specific (Casimiro et al. 2004; Cano et al. 2004; Ford et al. 2004). PCR diagnostic assays can be developed by exploiting DNA-sequence polymorphisms of fungi within internal transcribed spacers (ITS), unique sequences of mitochondrial DNA, cloned restriction fragments of genomic DNA, and sequenced fragments derived from randomly amplified polymorphic DNA (RAPD) markers or universally primed markers (Bulat et al. 1995).

With *C. truncatum* being developed as a BA for scentless chamomile, questions regarding environmental impact of the potential bioherbicide need to be addressed. As part of the risk assessment process, movement and persistence of BA should be determined under various environmental conditions (DeJong and Zadoks 1990; Cook et al. 1996). A rapid and reliable protocol that differentiates target organisms from others will greatly facilitate tracking of the BA. Molecular markers have been previously used for monitoring fungal BA. For instance, Zhou et al. (2004) used both microbiological and molecular techniques to investigate colonization of target and non-target plants by the bioherbicide agent *Phoma macrostoma* Montagne and found that specific PCR markers detected the BA at extremely low doses. Furthermore, with this marker technology, they were able to demonstrate that applied fungal inoculum did not persist in soils at detectable levels beyond 4 months post application on the Canadian prairies. Molecular markers were also used to assess the impact of releasing selected isolates of the bioherbicide agent *Chondrostereum purpureum* (Pers.: Fr. Pouzar) on natural populations of the pathogen in Canadian forest (Hintz et al. 2001). These markers assisted the analysis of gene flow between fungal populations as part of the risk assessment during the development of this BA for management of hardwood species in reforestation sites and utility right-of-way in Canada.

Based on the ITS sequence analysis reported in the previous chapter, the objectives in this study were: to 1) design strain-specific primers for PCR amplification of *Colletotrichum truncatum* DNA; 2) develop PCR markers to differentiate *C. truncatum* strains from scentless chamomile and those from selected field crops; and 3) verify the feasibility and effectiveness of trial protocols for tracking and diagnostic purposes.

4. 2. Materials and Methods

4.2.1. Genomic DNA Extraction from Fungal Cultures

A total of 37 *Colletotrichum* isolates were tested in this study, including 10 isolates of *C. truncatum* from lentil, 10 isolates from scentless chamomile, and 6 isolates from soybean, 6 isolates from pea; and 5 isolates of other *Colletotrichum* species for additional comparisons (Table 4.1). To prepare DNA, isolates were grown in pure culture on potato dextrose agar (PDA) in Petri dishes at 28ºC in the dark for 1 week, then transferred to PDB broth to grow 24h at room temperature. Genomic DNA was extracted from mycelia using the method described by Talbot et al. (2001). Briefly, mycelia were collected from 24 h liquid culture with PDB broth and ground to powder in liquid nitrogen. 400 µl of extraction buffer was added to the mycelium powder, followed by vortex after adding 400 µl of chloroform: isoamylethanol (24:1). The mixture was incubated at 65ºC for 5 min., then it was vortexed and centrifuged at 14,000 rpm for 5 min., and 400 µl of iso-propanol was added to the supernatant to precipitate DNA by centrifugation at 14,000 rpm for 10 min. DNA pellets were washed with 70% ethanol. The pellets were resuspended in 20 µl of sterile distilled water.

For detecting the pathogen in inoculated plants, DNA was extracted from plant leaves 1 week after inoculation. Genomic DNA from both plant and fungal tissues was extracted as described above.

4.2.2. Inoculum Preparation

Conidia were produced by growing *C. truncatum* isolates on V8-juice agar medium in Petri dishes for 1 week without light. Sporulating cultures were flooded with

distilled water amended with 0.1% Tween® 80, and conidia were dislodged by scraping the cultures with a bent glass rod. Conidial suspensions were adjusted to $1x10^5$ spores / ml using a hemacytometer prior to inoculation.

4.2.3. Plant Production and Inoculation

Seeds of scentless chamomile, lentil, pea and soybean were planted in a soil-less growth medium consisting of peat moss/perlite/vermiculite (2/1/1) (Premier Horticulture, Montréal, QC) in 8-cm pots, and kept in a greenhouse (20 ± 3ºC with 14-h photoperiod at 280-360 $\mu E\ m^{-1}s^{-1}$) for about 2 weeks. After emergence, each pot was thinned to one plant. Soybean plants were inoculated at the 2-leaf stage, whereas other species were inoculated at the 4 to 6 leaf stage. Conidial suspensions were applied to plants using an air-brush sprayer (Paasche Airbrush Ltd., Chicago, IL, USA) at 275 kPa constant air pressure to run off. Plants sprayed with water were used as controls. Inoculated plants were kept in a dew chamber (Percival Scientific Inc., Boone, IA, USA) at 20ºC and darkness for 24 h, and then moved back to the greenhouse. The experimental design was a completely randomized design with four replicates per treatment.

To reisolate the causal agent, diseased tissues were surface sterilized in 70% ethanol for 3 s, 0.6% NaOCl for 60 s, and rinsed in sterile water three times prior to being placed on PDA Petri dishes containing 100 ppm of streptomycin. Resulting cultures were transferred to PDA dishes and grown under the same conditions as described above for mycelial production and DNA extraction.

4.2.4. PCR Amplification

Strain-specific primers were designed based on ITS sequences information.

Table 4.1. *Colletotrichum* spp. isolates used for test of specific primers

isolates	Species	Host	Origin
003B1	*C. truncatum*	*Metricaria perforate*	Canada
00248A1	*C. truncatum*	*M. perforate*	Canada
00193C	*C. truncatum*	*M. perforata*	Canada
01-076A2	*C. truncatum*	*M. perforata*	Canada
0214B1	*C. truncatum*	*M. perforata*	Canada
01052A1	*C. truncatum*	*M. perforata*	Canada
01-G1A1-1	*C. truncatum*	*M. perforata*	Germany
01-G3A1-4	*C. truncatum*	*M. perforata*	Germany
026B1	*C. truncatum*	*M. perforata*	Canada
21-H-1	*C. truncatum*	*M. perforata*	Canada
Ct1-0202	*C. truncatum*	*Lens culinaris*	Canada
Ct11-0202	*C. truncatum*	*L. culinaris*	Canada
Ct12-0202	*C. truncatum*	*L. culinaris*	Canada
Ct14-0202	*C. truncatum*	*L. culinaris*	Canada
May9930	*C. truncatum*	*L. culinaris*	Canada
May9934	*C. truncatum*	*L. culinaris*	Canada
May9991	*C. truncatum*	*L. culinaris*	Canada
Apr0102	*C. truncatum*	*L. culinaris*	Canada
Apr9906	*C. truncatum*	*L. culinaris*	Canada
Jun9903	*C. truncatum*	*L. culinaris*	Canada
Jul0439	*C. truncatum*	*Glycine max*	Canada
Jul0436	*C. truncatum*	*G. max*	Canada
Jul0433	*C. truncatum*	*G. max*	Canada
Jul0428	*C. truncatum*	*G. max*	Canada
Jul0431	*C. truncatum*	*G. max*	Canada
Jul0438	*C. truncatum*	*G. max*	Canada
Jul0403	*C. coccodes*	*Pisum sativum*	Canada
Jul0414	*C. circinans*	*P. sativum*	Canada
Jul0413	*C. circinans*	*P. sativum*	Canada
Jul0412	*C. circinans*	*P. sativum*	Canada
Jul0402	*C. coccodes*	*P. sativum*	Canada
Jul0416	*C. circinans*	*P. sativum*	Canada
Cga060	C. *gloeosporioides*	*Aeschynomene virginica*	USA
CBS172.51	*C. lini*	*Linum ustatissimum*	Netherlands
CBS509-97	*C. acutatum*	*Lupinus albus*	France
5-6-33	*C. orbiculare*	*Lagenaria* sp	USA
2-6-33	*C. circinans*	*Allium vineale*	USA

The primers ChF/ChR (Table 4.2) were used to detect scentless chamomile isolates of *C. truncatum* under the following conditions: PCR amplification reactions were performed in the total volume of 25 µl mixture containing 2.5 µl of buffer (10 mM Tris-HCL at pH 8.8, 50 mM KCL, 3.5 mM $MgCL_2$), 0.5 µl of 10 mM dNTP (dATP, dCTP, dGTP, dTTP), 1 µl of 10 mM primers, 0.25 µl Taq polymerase (Invitrogen Co., California, USA), 0.1 µl Pfu (Invitrogen Co., USA) and 1.0 µl of DNA template (10 ng/µl). DNA amplification was conducted in an Eppendorf Thermocycler, the cycle parameters were an initial denaturation at 94ºC for 3 min, followed by 30 cycles of 94˚C for 30 s, 56˚C - 65˚C (depend on primers) for 30 s, and 72˚C for 40 s, and a final extention for 1 min at 72ºC. PCR amplification from diseased plant tissue also used the same conditions as described above.

PCR products were separated on 1% agarose gel running in 0.5X TBE buffer. Sequences of all the primers were shown in Table 4.2.

Table 4.2. Primers designed for detecting *Colletotrichum truncatum* isolates from different hosts

Primer	Primer sequence	Origin of host
ChF	5’ –GCAGGAGGACAACCCCCCC-3’	Scentless chamomile
ChR	5’-TCCGAGGTCAACCTGGT-3’	Scentless chamomile
LenF	5’-GCAGGAGGACGACCCCCT-3’	Lentil
LenR	5’-GATCCGAGGTCAACCT-3’	Lentil
SoyF	5’-CGGGCAGAGGTTCCCTC-3’	Soybean
SoyR	5’-ATCCGAGGTCAACCTTA-3’	Soybean
PeaF1	5’-GGCAGGGGGTGCCGCCT-3’	Pea
PeaR1	5’-GATCCGAGGTCAACCTTT-3’	Pea
PeaF2	5’-CGGGCAGGGGGTCCCCTC-3’	Pea
PeaR2	5’-ATCCGAGGTCAACCATTAGA-3’	Pea

4.3. Results

4.3.1. Detection of *C. truncatum* from Scentless Chamomile

Genomic DNA of *C. truncatum* isolates from scentless chamomile was used as a template for PCR amplification. The primers ChF/ChR amplified a single band from all DNA samples of scentless chamomile isolates (Figure 4.1). The lowest detectable amount of DNA was 5 ng (lane 5 and 6), but bands from samples with more than 50 ng DNA were substantially more visible for detection.

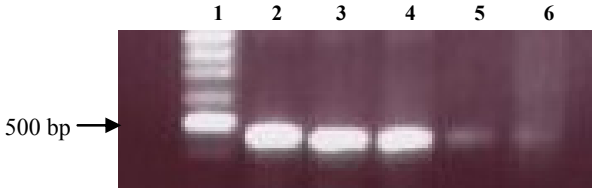

Figure 4.1. PCR amplification of genomic DNA of a *Colletotrichum truncatum* (003B1) isolate from scentless chamomile with primers ChF/ChR. Lane 1: DNA ladder; Lanes 2 - 6: genomic DNA at 750, 500, 250, 50 and 5 ng, respectively.

PCR amplification of DNA from infected scentless chamomile tissues with the primers ChF/ChR generated a single 421 bp amplicon (Figure 4.2), similar to that amplified from pure cultures of *C. truncatum*. These primers did not amplify any of the *C. truncatum* isolates from lentil, soybean or *Colletotrichum* spp. from pea.

Figure 4.2. PCR amplification with the ChF/ChR primers for detection of *Colletotrichum truncatum* from diseased scentless chamomile plants. Lane 1: DNA ladder; Lanes 2 and

3: DNA extracted from pure culture of lentil isolates Ct11-0202 and Ct12-0202; Lanes 4 and 5: DNA extracted from pure culture of soybean isolates Jul0431 and Jul0436; Lane 6: DNA extracted from a pure culture of scentless chamomile isolate 01-G3A1-4; Lane 7: DNA extracted from diseased scentless chamomile tissues inoculated with scentless chamomile isolate 003B1. Lanes 8 and 9: DNA extracted from pure cultures of pea isolates Jul0403 and Jul0413.

4.3.2. Specificity of Primers for the Scentless Chamomile Strains

A total of 17 isolates including isolates from lentil, pea and soybean and several other *Colletotrichum* species were tested with ChF/ChR primers, under the same PCR conditions as used for detection of scentless chamomile isolates. Only the DNA from scentless chamomile isolates was amplified, with a single band around 420 bp (Lane 9-11, Figure 4.3); no amplicons was observed for other isolates or species. Thus, the 420 bp band was a strain-specific marker for *C. truncatum* isolates from scentless chamomile.

Figure 4.3. PCR amplification of genomic DNA from different *Colletotrichum* spp. with the primers ChF/ChR. Lanes1-5: DNA from *C. gloeosporiodes* isolate cga060, *C. acutatum* isolate CBS509-97, *C. orbiculare* isolate 5-6-33, *C. circinans* isolate 2-6-33 and *C. lini* isolate CBS172.51; Lanes 6-8: DNA from *C. truncatum* isolates of lentil Ct14-0202, Ct1-0202, and May9930; Lanes 9-11: DNA from *C. truncatum* isolates of scentless chamomile 01-G3A1-4, 00193C, 00248A1; Lanes 12-14: DNA from *C. truncatum* isolates of pea Jul0403, Jul0413, Jul0412; Lanes 15-17: DNA from *C. truncatum* isolates of soybean Jul0431, Jul0436, Jul0433. Lane M is the DNA ladder.

4.3.3. Detection of *Colletotrichum* spp Isolates from Lentil, Soybean and Pea

Four pairs of primers (Table 4.1) were designed for detection of *C. truncatum* originated from lentil, soybean and pea. Under PCR conditions designed for detecting

lentil isoaltes, the primers LenF/LenR amplified DNA only from lentil isolates, and no amplicons were produced from *C. truncatum* from other host isolates or other *Colletotrichum* spp. (Figure 4.4). Similarly, the primers for isolates from soybean only amplified the DNA of *C. truncatum* isolates from soybean (Figure 4.5).

Figure 4.4. PCR amplification of genomic DNA from different *Colletotrichum* isolates and species with the primers LenF1/LenR1. Lanes 1-5: DNA extracted from lentil isolates Ct11-0202, Ct12-0202, Ct1-0202, Apr0102 and May9991; Lanes 6-8: DNA extracted from scentless chamomile isolates 01-G3A1-4, 00193C, 00248A; Lanes 9-11: DNA extracted from pea isolates Jul0403, Jul0413 and Jul0414; Lanes 12-14: DNA extracted from soybean isolates Jul0433, Jul0436 and Jul0438; Lane 15: DNA extracted from C. *gloeosporioides* isolate cga060; Lane 16: DNA extracted from C. *lini* isolate CBS172.51; Lane 17: DNA extracted from *C. acutatum* CBS509-97. Lane M: DNA ladder.

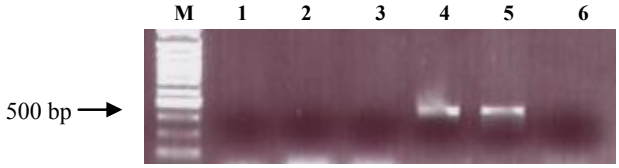

Figure 4. 5. PCR amplification of genomic DNA from different *Colletotrichum* isolates and species with the primers SoyF/SoyR. Lane 1: DNA extracted from scentless chamomile isolate 0214B; lane 2: DNA extracted from lentil isolate May9934; lane 3: DNA extracted from pea isolate Jul0414; Lanes 4 and 5: DNA extracted from soybean isolates Jul0438, and Jul0439; Lane 6: DNA extracted from *C. gloeosporiodes* isolate Cga060; Lane M, DNA ladder.

Previous results (chapter 3) showed that the isolates from pea may be divided into to two sub-groups that were most closely related to *C. circinans* and *C. coccodes*, based on sequence data. Based on ITS1 sequence differences, two pairs of primers were designed specifically for the *C. circinans* - and *C. coccodes* - like isolates from pea. The primers PeaF1/PeaR1 amplified a single band for the isolate falling into the *C. circinans* subgroup (Figure 4.6), while the primers PeaF2/PeaR2 only amplified the isolate more related to the *C. coccodes*. DNA of *C. truncatum* isolates from scentless chamomile, lentil or soybean was not amplified with these two pairs of primers.

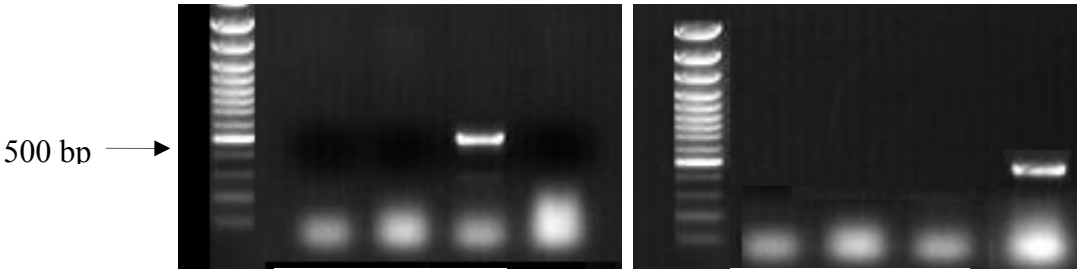

Figure 4.6. PCR amplification of genomic DNAs from pea isolates *C. circinans* – like and *C. coccodes* – like with primers PeaF1/ PeaR1 (A) and PeaF2/ PeaR2 (B). Lanes 1-3: DNA extracted from lentil isolate Ct12-0202, soybean isolate Jul0438 and pea isolate *C. circinans* - like Jul0414; Lane 4: DNA extracted from pea isolate *C. coccodes*- like Jul0403.

4.3.4. Specificity of Primers for the Lentil Isolates from Inoculated Plants

Using the primers LenF1/LenR1, a lentil isolate (Ct11-0202) was detected from diseased tissues of lentil and pea plants 1 week after inoculation, with amplification of a 454 bp DNA fragment (Figure 4.7). The genomic DNA used in this test was isolated directly from diseased plant tissues. No amplicons were observed from DNA prepared from plant tissues inoculated with other *C. truncatum* isolates or other *Colletotrichum* species (Figure 4.7).

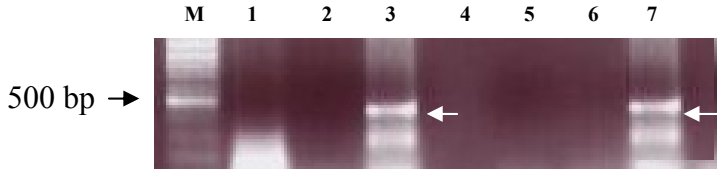

Figure 4.7. PCR amplification of DNA extracted from inoculated plant tissues with the primers LenF1/LenR1. Lane M: DNA marker; Lanes 1 and 2: DNA extracted from chamomile plants inoculated with lentil and chamomile isolates of *C. trunatum* Ct11-0202 and 003B1; Lanes 3 and 4: DNA extracted from pea and soybean plants inoculated with a lentil isolate Ct11-0202; and lanes 5-7: DNA extracted from lentil plants inoculated with a chamomile isolate 003B1, *C. gloeosporiodes* isolate Cga060, and a lentil isolate Ct11-0202. Arrow indicates specific band generated from lentil isolates.

4.4. Discussion

Primer selection for PCR identification of fungal pathogens based on sequence information of the rDNA-ITS region has been reported previously. Sreenivasaprasad et al. (1996) suggested the application of rDNA-ITS sequencing information as markers for species delineation in the genus *Colletotrichum*. In another study, specific primers generated from random amplified polymorphic DNA (RAPD) were developed to detect a single strain of the biocontrol fungus *C. coccodes* on the target weed species *Abutilon theophrasti* Medik. (velvetleaf) and from soil samples (Dauch et al. 2003). Results from

both of these studies indicated that PCR techniques based on fungal sequence information were useful for detection and diagnostic purposes. In the current study, five sets of primers were designed based on rDNA-ITS sequences of selected *Colletotrichum* spp, for differentiation of *C. truncatum* isolates from scentless chamomile, lentil, and soybean, as well as closely related species from pea. The PCR strain-specific primers were effective in differentiating *C. truncatum* from different hosts; this technique may be useful for separating morphologically similar strains of this pathogen.

PCR detection of the scentless chamomile strain of *C. truncatum* was affected by the concentration of fungal DNA. This demonstrated a threshold for detection, as well as a possibility for fungal quantification. If real-time PCR is used with these primers, strains of *C. truncatum* from diverse environmental samples may be specifically quantified (McCartney et al. 2003; Suarez et al. 2005). The amount of DNA template used for PCR amplification is an important factor for successful detection of *Colletotrichum* strains. The lowest amount of detectable DNA was between 5 ng and 50 ng using the current PCR conditions for these scentless chamomile isolates. Thresholds of DNA for detection of other *C. truncatum* strains were not determined.

The effectiveness of primers for monitoring target strains was further demonstrated by selective detection of these strains in inoculated plants. For example, the primers ChF/ChR detected DNA of the scentless chamomile strain from pure cultures and inoculated host plants, but did not amplify DNA from other *C. truncatum* isolates or the scentless chamomile strain applied to other non-host plants (data not shown). Similarly, the primers LenF/LenR were able to detect the lentil isolate Ct11-0202 in infected pea and lentil, but not on inoculated plants of scentless chamomile or soybean.

The latter two were not considered as hosts of Ct11-0202 because the fungus generally did not penetrate plant epidermal cells after forming appressoria (Chapter 5). In contrast, this strain successfully infected lentil and pea, resulting in extensive inter- and intracellular hyphal development after penetration and consequent increase in fungal biomass in the plant tissue. It is possible that the detection of Ct11-0202 in lentil and pea was due to higher amounts of hyphal growth that often coincides with the onset of disease symptoms caused by semibiotrophic *Colletotrichum* pathogens (Wei at al. 1997; Goodwin 2001). Although the PCR assay was conducted one week after plant inoculation, it is possible that the infection can be detected prior to symptom expression due to rapid growth of fungal hyphae in infected tissues (Chapter 5). Extensive development of primary phyphae in epidermal cells developed 24-48 h after inoculation, increasing the probability of early PCR detection.

Frequently, PCR primers are often developed based on unique sequences identified in rDNA-ITS fragments. Recently, a procedure was developed to amplify diagnostic fragments with random amplified polymorphic DNA (RAPD)-PCR, and cluster analysis of generated RAPD banding patterns was used to highlight intra-specific variations (Tullu et al. 2003). Also, RAPD profiles have been used as starting information to generate more specific markers such as primers for sequence characterized and amplified regions (SCAR). In this study, physiological races (Ct0 and Ct1) of C. *truncatum* from lentil could not be differentiated by primers LenF/LenR designed based on their ITS-rDNA sequences. Detection and differentiation of these races might be investigated with other molecular techniques.

Molecular detection or PCR amplification with strain - specific primers has other potential applications as well. Primers ChF/ChR, specific for *C. truncatum* isolates from scentless chamomile, can be used to track this biocontrol agent after release and may be useful for monitoring the biocontrol agent (e.g., persistence and dispersal) in different environments (Avis et al. 2001; Zhou et al. 2004). These two attributes are important for the understanding of the environmental impact of the biocontrol agent. In addition, this marker may help protect the commercial value of patented biocontrol strains by deterring duplication. Markers developed through this study for lentil, soybean or pea strains of *Colletotrichum* spp. may be used for early diagnosis of infection prior to disease appearance, assisting in early warning of disease epidemics.

In conclusion, the PCR method developed for detection and differentiation of strains of *C. truncatum* and closely related species can be a useful tool for studying the environmental fate of the biocontrol agent.

V. HOST SPECIFICITY OF *COLLETOTRICHUM TRUNCATUM*

5.1. Introduction

In previous studies, it has been demonstrated that distinguishable molecular characters exist among *C. truncatum* isolates from scentless chamomile, lentil, and soybean. Analysis of rDNA-ITS sequences indicates that lentil isolates are closely related to those of scentless chamomile, whereas soybean isolates are more different. Although the current study has produced evidence that discriminates the scentless chamomile strain (mycoherbicide) from other *C. truncatum* strains at a molecular level, plant inoculation offers a direct assessment of pathogenicity and crop safety. To be used in crop systems, this biocontrol agent (BA) must be highly host specific, because strains of the same fungal species also cause anthracnose on lentil in western Canada (Morrall 1988; Morrall and Pedersen 1991). The concern on host specificity of the BA may be further intensified by an earlier report that *C. trifolii* (a species closely related to *C. truncatum*) was highly pathogenic on several legume crops including lentil, pea, and alfalfa (Mortensen and Makowski 1994). As a result of this broad host range, *C. trifolii* was rejected as a mycoherbicide candidate for biocontrol of black medick (*Medicago lupulina* L.), a weedy legume in pasture and forage crops. In the literature, *C. truncatum* has also been reported to cause anthracnose on other legume crops including soybean (Roy 1982; Manandhar et al. 1985), alfalfa (Graham et al. 1976), mung bean (Han and Lee 1995), and urdbean (Kausaul and Sharna 1998). To ensure crop safety, pathogenicity

of this potential BA should be clearly determined on important prairie crops, especially lentil and pea.

Based on a preliminary study, this potential BA for scentless chamomile was almost completely non-pathogenic to crops grown on the Canadian prairies (Peng et al. 2005). Only 2 isolates out of 14 caused slight infections on lentil, but no infection was observed on pea or alfalfa. Due to the closeness of ITS-rDNA sequences, it is sensible to clarify if there is any potential for scentless chamomile and lentil isolates to cross infect, or infect other crops. Assessment of *C. truncatum* pathogenicity, however, can be complicated by several factors including: a) latent infection by the pathogen where symptomless colonization of host tissues may not be evident until a change occurs in the physiological state (such as senescence) of the host tissue (Cerkauskas and Sinclair 1980; Cerkauskas et al. 1988; Fernando et al. 1994); b) varying degree of resistance by crop cultivars (Manandhar et al. 1985; Manandhar et al. 1988; Boyette 1991); and c) variability in virulence depending on host origin of the isolate (Hartman et al. 1986). Two races of *C. truncatum* have been identified on lentil (Anderson 2003; Buchwaldt et al. 2004); some lentil cultivars are resistant to race Ct1 but none is resistant to Ct0. It is, therefore, prudent to evaluate the pathogenicity of BA on multiple lentil cultivars.

In parallel to pathogenicity assessments based on disease severity, studies on the infection process may offer further insights. Many *Colletotrichum* pathogens are hemibiotrophs that exhibit an initial biotrophic phase of host colonization (Goodwin 2001), followed by the development of secondary infection hyphae and destructive necrosis (Morin et al. 1996; Latunde-Dada et al. 1997; Wei et al. 1997). Some *Colletotrichum* spp. have shown intracellular or intercellular invasion, or a combination

of both strategies (Bailey and Jeger 1992) during the infection and colonization of host tissues. Detailed observations on infection behavior should aid in characterization of host specificity as illustrated with *C. truncatum* on pea, soybean and lentil (Manandhar et al. 1985; O'Connell et al. 1993; Chongo et al. 2002).

The objectives of this study were to: 1) determine infection potential of *C. truncatum* isolates from scentless chamomile and selected legume crop species; 2) evaluate potential pathogenicity of the BA on multiple lentil cultivars; and 3) characterize infection behavior of selected *C. truncatum* isolates on host and non-host species

5.2. Materials and Methods

5.2.1. Preparation of Plant Materials

Common crop cultivars (Table 5.1) were selected for the inoculation study. Scentless chamomile seeds, collected from field sites near Hafford, Saskatchewan, were sprinkled on 0.5-cm Redi Earth (W.R. Grace, Ajax, ON) overlaid on a soil-less mix in a commercial seeding tray (51 x 26 x 6 cm). The mix consisted of sand, peat moss, and vermiculite (1:4:8) amended with 1% (w/v) of 16-8-12 fertilizer (N-P_2O_5-K_2O). Seeded trays were covered with another 0.5-cm Redi Earth layer, watered, and maintained in the greenhouse at 20 ± 3°C with 14-h supplementary lighting. At the 2-leaf stage, seedlings were transplanted individually into 7.5-cm diameter plastic pots containing the same soil-less mix, and maintained in the greenhouse for about 2 weeks until they reached the 4- to 6-leaf stage. For crop species such as lentil, soybean, pea, and alfalfa, two seeds were planted in the soil-less mix in a 7.5-cm pot and kept under similar conditions in the greenhouse for 2 to 3 week until reaching the 4-leaf stage. Each pot was thinned to one plant after the first true leaf expanded fully.

Table 5.1. Crop cultivars used in plant inoculation with *Colletotrichum truncatum* isolates from different hosts

Plant	Species name	Cultivar	Source
Lentil	*Lens culinaris* Medik.	Eston	CDC, U of S [a]
Soybean	*Glycine max* L.	Primo RR	PRO Seeds of Canada
Pea	*Pisum sativum* L.	Carneval	Saskatchewan Wheat Pool
Alfalfa	*Medicago sativa* L.	Beaver	SW Seed Canada Ltd.
Scentless chamomile	*Metricaria perforata* Mérat	N/A	AAFC [b]

[a] Crop Development Center, University of Saskatchewan.
[b] Agriculture & Agri-Food, Saskatoon Research Centre.

5.2.2. Preparation of Fungal Inoculum

Isolates of *Colletotrichum* spp. (Table 5.2) were preserved at -80°C in 5% skim milk amended with 20% glycerol in 2 - ml vials. To recover the isolates, the content of a vial was transferred to potato dextrose agar (PDA) in a Petri dish and incubated at 22°C for 1 week. Resulting cultures were then transferred on V8-juice medium (200 ml V8 juice, 20 g agar, 1 L de-ionized water) in Petri dish and incubated at 23ºC for sporulation. After about 2 weeks, sporulating cultures were flooded with distilled water amended with 0.1% Tween® 80 surfactant and scraped with a bent glass rod to dislodge conidia. Conidial suspensions were filtered through two layers of cheesecloth and concentrations were estimated with a hemacytometer. Conidial suspensions were adjusted to about $5x10^4$ to $1x10^5$ spores/ml and used for inoculation of detached leaves or sprayed on the whole plants.

Table 5.2. Source of *Colletotrichum* species used for the inoculation study

Isolate	Species	Host origin	Source
003B1	*C. truncatum*	Scentless chamomile (*Matricaria perforata*)	AAFC [a]
CT110202	*C. truncatum*	Lentil (*Lens culinaris*)	CDC, U of S [b]
Jul0433	*C. truncatum*	Soybean (*Glycine max*)	AAFC
Jul0402	*Colletotrichum* sp.	Pea (*Pisum sativum*)	AAFC
94SD	*C. trifolli*	Alfalfa *(Medicago sativa*)	AAFC

[a] Agriculture & Agri-Food, Saskatoon Research Centre.
[b] Crop Development Center, University of Saskatchewan.

5.2.3. Plant Inoculation

Depending on the experiment, inoculation was carried out on either whole plants or detached leaves. Whole plants were inoculated at the 4-leaf stage with 5 ml of conidial suspensions using an airbrush sprayer, as previously described. Control plants were sprayed with sterilized water. Plants were placed immediately in a dew chamber (Percival Scientific Inc., Boone, IA) with 100% relative humidity at 20 ± 2°C for 24 h. After the dew period, plants were transferred to a greenhouse.

For inoculation of detached leaves, the second youngest leaves or leaflets from the top were severed from greenhouse-grown plants and placed on sterile moist filter paper in 9-cm Petri dishes with the adaxial side up. Each leaf/leaflet was inoculated with six 15-µl droplets of conidial suspension ($5x10^4$conidia/ml) and incubated at 20°C. The lower inoculum concentration was used on detached leaves to facilitate observations of the infection process. Leaflets that received sterilized water (Tween 80 were added) were used as controls.

5.2.4. Disease Assessment

Disease severity on whole plants was assessed 1 and 2 weeks after inoculation using a 0-6 scale (Table 5.3) based on percentage of necrotic tissues on a whole plant, where 0 indicated an absence of disease and 100% represented a dead plant. This scale, adapted from Little and Hills (1978), reflects the fact that the humans eye's ability to distinguish small differences in percentage of diseased areas is best near zero or 100% and poorest around 50%.

Table 5. 3. Disease severity scale corresponding to percent necrotic plant tissues

Disease scale	0	1	2	3	4	5	6
Diseased tissue (%) *	0	1-7	7-25	25-50	50-75	75-93	93-100

* From Little and Hills (1978).

5.2.5. Observation of Infection

At intervals after detached leaves were inoculated with conidia of *C. truncatum*, samples of inoculated leaflets were placed in a fixing buffer (Appendix B) for 12 h, then rehydrated in gradually decreased ethanol solutions of 100%, 70%, 50%, 25%, 10%, 0% for about 2 h at each concentration, and stained with trypan blue (0.05%) for 12 h. At the end of staining, samples were rinsed with tap water until leaf tissues became transparent. To determine conidial germination and penetration of leaf tissues, stained samples were examined initially under a light microscope (Lertz Diaplan, Germany). The percentage of germination on each leaflet was determined by counting 100 spores in random fields, and a spore with a germ tube that was at least as long as the spore width was considered to have germinated. About 50 germinated spores were examined to assess appressorial

formation. To examine infection hyphae within leaf tissues, a confocal laser-scanning microscope (Zeiss LSM 510 META, Jena, Germany) was used and images were produced under the following conditions: excitation wavelength was 543 nm. 25x water immersed objective; and the emission was collected from 603 nm to 625 nm by META channel. The pinhole size was 145 micrometer. Different emission images were sometimes combined for optimal visual effects.

5.2.6. Experiment I – Pathogenicity of Isolates *Colletotrichum* spp. from Different Plant Species

Whole plants of scentless chamomile, lentil, pea, soybean, and alfalfa **(**Table 5.1) were inoculated with C*olletotrichum* spp. from various hosts (Table 5.2). The plants were inoculated and maintained as previously described. Disease symptoms and severity were evaluated 1 and 2 weeks after inoculation using the 0-6 scale. The experiment used a completely randomized design (CRD) with four replicates for each treatment, and was conducted four times.

5.2.7. Experiment II – Infection Characteristics of *Colletotrichum* spp. Isolates

Five isolates of *Colletotrichum* spp. (Table 5.2) from different hosts (Table 5.1) were applied to detached leaflets of these same host species using the method described earlier. Samples were taken at 24 h intervals, after incubation up to 96 h. Samples were examined for spore germination, appressorium formation, primary and secondary hyphae, and disease symptoms after fixing and staining. The experiment was a CRD with four replicates and was conducted twice.

5.2.8. Experiment III – Pathogenicity and Potential Latent Infection on Selected Lentil Cultivars

Five common lentil cultivars were assessed; Eston, CDC Blaze, CDC Robin, CDC Redberry, and CDC Rouleau. Dr. Vandenberg at the Crop Development Center, University of Saskatchewan, provided seed of these cultivars. Eston and CDC Blaze are susceptible to both Ct1 and Ct0 races of *C. truncatum*, whereas the other three cultivars are resistant to race Ct1 only. Four isolates of *C. truncatum* were assessed, with two originating from lentil [CT11-0202 (Ct0), Jul9901 (Ct1)] and two from scentless chamomile (00-3B1 and 00-193C). Conidial suspensions were applied to whole plants as well as detached leaves of all five lentil cultivars using the method described earlier. To further examine the impact of cultivar resistance on infection process, detached leaves (2 wk old) of the cultivar CDC Robin were inoculated with Ct1 and Ct0 lentil isolates. Samples were stained and observed 48hpi and 72hpi, respectively, using the protocol described earlier.

Disease symptoms and severity were evaluated 2 wks after inoculation using the 0-6 scale. After rating, potential latent infection and colonization in symptomless plants were assessed as follows: 25 symptomless leaflets from each replicate were surface sterilized (3 s. in 70% ethanol, 60 s. in 0.6% NaOCl, rinsed twice in sterile water, blotted with sterile paper towel), placed on paraquat agar [20 mg paraquat, 200 mg chloramphenicol, and 10 g agar in 1 L of water, adapted from Peng and Sutton (1991)], and incubated for 7 d at 20°C. The herbicide was used to induce leaf senescence and facilitate sporulation and production of acervuli (Cerkauskas and Sinclair 1980). In a preliminary trial, fungal propagules on leaf surfaces were killed completely using the surface sterilization protocol (data not shown), but fungal structures inside leaf tissues

were alive (100% recovery when surface sterilization was carried out 24 h after inoculation of susceptable hosts). To verify the identity of recovered cultures, 10% of sporulating cultures were transferred to potato dextrose broth (PDB) and incubated at 20°C for a week. Fungal DNA was extracted and verified using the PCR markers described in the previous chapter.

5.2.9. Data Analysis

Statistical Analysis System (SAS, version 8.2, SAS Institute Inc., Box 8000, Cary, NC) was used for data analyses. Based on Bartlett's test, the variance for data from repeated trials was homogeneous. Therefore, all data were pooled prior to analysis. PROC UNIVARIATE was used to determine data normality. Percentage data were transformed with arcsine square root prior to analysis to test the distribution, but non-transformed means were presented for ease of interpretation. All quantitative data were subjected to ANOVA and treatment means were separated using Fisher's protected LSD at $P = 0.05$.

5.3. Results

5.3.1. Disease Symptoms Caused by Different Isolates of *Colletotrichum* spp.

Colletotrichum isolates varied in pathogenicity on the plant species examined based on the host origin. Isolates of *C. truncatum* from scentless chamomile caused disease only on scentless chamomile. Symptoms were first visible 7 days post inoculation (dpi) on whole plants, but 4 dpi on detached leaves. The early symptom was a dark brown leaf spot (Figure 5.1a) that enlarged and coalesced, often killing lower

leaves (Figure 5.1b) by 14 dpi. This symptom, however, rarely developed on newly produced leaves.

The lentil isolate Ct11-0202 (Ct0) caused disease on whole plants and detached leaves of lentil and pea, as well as detached leaves of alfalfa, but not on scentless chamomile or soybean. The latent period was relatively short; symptoms were visible 3 dpi with initial tan-coloured and water-soaked leaf lesions (Figure 5.2). Inoculated leaves often died 7 dpi. Stem lesions were dark brown and frequently coalesced as the disease progressed, girdling the stem and causing the plant to collapse. Distinctive dark acervuli and setae sometimes formed on stem lesions. The pea isolate of *Colletotrichum* sp. produced symptoms on whole plants and detached leaves of pea, lentil, and soybean (Figure 5.3), but not on scentless chamomile or alfalfa. Symptoms were similar to those caused by the lentil isolate of *C. truncatum* on lentil and pea. On soybean plants however, lesions were noticeably smaller and disease onset was several days later than on lentil and pea. Unlike the lentil isolate, this pea isolate rarely killed any plants and the disease was generally limited to leaves.

The soybean isolate of *C. truncatum* caused disease on whole plants and detached leaves of soybean, lentil, and pea (Figure 5.4), but disease development was much slower than that observed for other isolates, especially on soybean plants where symptoms were not visible until 14 dpi. Inoculation with *C. trifolii* from alfalfa resulted in disease only on alfalfa and lentil. Inoculation of alfalfa produced a leaf spot symptom that became visible at 7 dpi; lesions were generally less than 3 mm in diameter and had not expanded substantially by 14 dpi. When observed under a dissecting microscope, lesions caused by

the soybean isolate of *C. truncatum* appeared slightly sunken with a dark brown edge on soybean leaves, but light brown on lentil (Figure 5.4).

5.3.2. Disease Severity Caused by Different Isolates of *Colletotrichum* spp.

The scentless chamomile isolate of *C. truncatum* caused moderate disease (47%) on its original host, but no other plant species showed any symptom of infection (Table 5.4). None of the *Colletotrichum* isolates from other plants caused disease on scentless chamomile, except one isolate from soybean that resulted in a few leaf spots in one trial only. The lentil isolate caused severe disease on both lentil and pea, and infected plants generally died by 14 dpi. In contrast, the pea isolate resulted in only light disease on test plants except scentless chamomile. Overall lentil is the most sensitive host species compared to others tested.

5.3.3. Observations of Plant Infection

Regardless of the isolate, there was no difference in the frequency of conidial germination on leaves of different plants ($P > 0.05$) 24-h post inoculation (hpi). In general, more than 90% of the conidia had germinated at 24 hpi with germ tubes of variable lengths and formation of appressoria (Table 5.5). Several isolates produced infection vesicles in host epidermal cells (Figure 5.5), from which thick primary hyphae (approx. 4 to 5 μm in diameter) originated 24 to 96 hpi (Figure 5.5; 5.6). In some cases, thinner secondary hyphae (approx. 2 μm in diameter) were observed 48 to 96 hpi (Figure 5.6C, D), which occurred just prior to the onset of disease symptoms.

For the scentless chamomile isolate of *C. truncatum*, appressoria were produced by 45-78% of germinated spores on leaves of different plants at 24 hpi, and these percentages increased to 64-88% by 48 hpi (Table 5.5). On scentless chamomile, infection vesicles (Figure 5.5) were observed 24 hpi. Primary and secondary hyphae developed by 48 and 72 hpi, respectively. Disease symptoms on detached scentless chamomile leaves were visible one day after the formation of secondary hypha. Conidia of this isolate germinated well and produced a large number of appressoria on leaves of other host plants. However, the development of infection vesicles, secondary hyphae, or disease symptoms was not observed on lentil, pea, soybean, or alfalfa up to 7 dpi (Table 5.5).

Several lentil isolates exhibited a similar infection pattern to that of the scentless chamomile isolates. The selected isolate (race Ct0) produced appressoria more frequently on lentil, pea, and alfalfa than on soybean and scentless chamomile. Infection vesicles and secondary hyphae were formed most rapidly on lentil (Table 5.5), but the time required for the onset of disease symptoms was the same on lentil, pea, and alfalfa (96 hpi). Secondary hypha and disease symptoms were not observed on scentless chamomile or soybean, although infection vesicles were observed occasionally in epidermal cells at 48-96 hpi. The isolates from pea and alfalfa showed poor appressorial formation on scentless chamomile (Table 5.5), with no infection vesicle, secondary hypha, or disease observed at 96 hpi. Both of these isolates produced infection vesicles or secondary hyphae in lentil, alfalfa and pea, but only the alfalfa isolate caused lesions on detached leaves of these plants (72 hpi). The pea isolate caused a hypersensitive

reaction on soybean leaves, which resulted in restricted cell death adjacent to penetration sites.

For the soybean isolate, there was noticeably less appressorial formation on scentless chamomile and pea than on soybean (Table 5.5). Development of infection vesicles and secondary hypha was observed only in soybean leaves.

Table 5.4. Disease severity caused by inoculation with isolates of *Colletotrichum* spp. assessed 2 wk after inoculation [a]

Isolate	Host of origin	Scentless chamomile	Mean disease severity on whole plant (%) [b]			
			Lentil	Pea	Soybean	Alfalfa
003B1	Scentless chamomile	46.9 a [c]	0	0	0	0
Ct11-0202	Lentil	0	90.6 a	72.8 a	0.4 b	0.8 b
Jul0402	Pea	0	1.6 c	0.5 b	0.8 b	0.3 b
Jul0433	Soybean	0.1 b	3.6 b	0.1 b	1.5 a	1.2 b
94-SD	Alfalfa	0	6.6 b	1.0 b	0.9 b	2.5 a

[a] Converted from data collected using a 0 – 6 scale, and the severity 0 and 100% indicated no disease and a dead plant, respectively.
[b] Results were averaged over four trials.
[c] Means with the same letter in a column are not significantly different (LSD, P=0.05)

Table 5.5. Formation of appressoria, infection vesicles, infection hyphae and symptoms by *Colletotrichum* spp. on detached leaves of selected host species.

Fungal species (host origin)	Plant inoculated	Appressoria (%) 24h		48h		Infection vesicle	Secondary hyphae	Initial symptom
C. truncatum	S. chamomile	78	a [a]	88	a	24 h [b]	72 h	96 h
(00-3B1)	Lentil	50	cd	79	ab	-	-	-
(S. chamomile)	Pea	45	d	64	c	-	-	-
	Soybean	72	ab	88	a	-	-	-
	Alfalfa	57	c	66	c	-	-	-
C. truncatum	S. chamomile	60	b	70	c	96 h	-	-
(Ct110202)	Lentil	78	a	95	a	24 h	48 h	96 h
(Lentil)	Pea	71	ab	83	b	72 h	96 h	96 h
	Soybean	49	c	61	d	48 h	-	-
	Alfalfa	79	a	94	a	72 h	96 h	96 h
Colletotricum sp.	S. chamomile	30	c	35	c	-	-	-
(Jul0402)	Lentil	69	b	80	b	96h	-	-
(Pea)	Pea	76	a	90	a	48 h	96 h	-
	Soybean	75	a	84	b	-	-	HR [c]
	Alfalfa	69	b	87	b	72 h	-	-
C. truncatum	S. chamomile	41	d	44	d	-	-	-
(Jul0433)	Lentil	69	b	80	ab	-	-	-
(Soybean)	Pea	44	d	52	c	-	-	-
	Soybean	77	a	87	a	24 h	48 h	96 h
	Alfalfa	61	c	72	bc	-	-	-
C. trifolii	S. chamomile	25	c	33	d	-	-	-
(94SD)	Lentil	69	a	79	b	24 h	48 h	72 h
(Alfalfa)	Pea	51	b	67	c	24 h	48 h	72 h
	Soybean	54	b	68	c	-	-	-
	Alfalfa	71	a	89	a	24 h	48 h	72 h

[a] Means with the same letters are not significantly different based on LSD, at $P = 0.05$.
[b] Qualitative assessment on the presence (h post inoculation) or absence (-) of the structure in plant samples examined.
[c] HR-Hypersensitive reaction.

5.3.4. Infection of Lentil Cultivars by Isolates of *C. truncatum* from Scentless Chamomile and Lentil

Inoculation of lentil plants with the scentless chamomile isolates 003B1 and 00193C caused no disease symptoms on the five cultivars tested, while the two lentil isolates caused 5-100% disease on different cultivars (Table 5.6, Figures 5.7, 5.8). On detached lentil leaves, the cultivar CDC Blaze exhibited a hypersensitive response to both scentless chamomile isolates (Figure 5.7), but this reaction was not observed on the other cultivars.

Both scentless chamomile isolates caused latent infection of lentil (Table 5.6), based on recovery of the fungus from inoculated leaves that were surface sterilized 24 h after inoculation. The incidence varied depending on isolate and cultivar. Isolate 003-B1 was recovered from symptomless leaflets of all cultivars at low to moderate frequency (2-23%) but 00-193C was reisolated only from the cultivars CDC Redberry and CDC Rouleau and only at moderate frequency (10-18%).

The cultivar CDC Robin had different reactions to races Ct0 and Ct1 (Figure 5.8). When leaves were inoculated with Ct0, appressoria developed to form strong primary hyphae (Figure 5.8A, B), secondary hyphae, and disease symptoms. When CDC Robin leaves were inoculated with Ct1, appressoria formed but primary hyphae were thin and long, there was no branching, and epidermal cells produced a hypersensitive response under each appressorium.

Table 5.6. Disease severity (DS) and latent infection (LI) caused by scentless chamomile (00-3B1, 00-193C) and lentil (Jul9901, Ct11-0202) isolates of *Colletotrichum truncatum* on five lentil cultivars (n=12)

	Isolates from lentil				Isolates from chamomile			
	Jul9901 (Ct1)		Ct11-0202 (Ct0)		00-3B1		00-193C	
Lentil Cultivar	DS [a]	LI	DS	LI	DS	LI	DS	LI
Eston	100 [b]	NT [c]	100	NT	0	23 [b]	0	0
CDC Blaze	80	NT	100	NT	0	9	0	0
CDC Robin	5	NT	100	NT	0	18	0	0
CDC Redberry	80	NT	100	NT	0	2	0	18
CDC Rouleau	100	NT	100	NT	0	12	0	10

[a] DS - disease severity was based on %diseased area of each plant. LI - latent infection was based on fungal incidence from symptomless, surface sterilized leaves on paraquat agar.
[b] Means were calculated over three trials.
[c] NT - Not tested.

5.4. Discussion

Symptoms and disease severity on selected leguminous species caused by closely related *Colletotrichum* isolates (based on rDNA-ITS sequences) provide a direct assessment of the host specificity for these pathogens. Isolates of *C. truncatum* from scentless chamomile exhibited the highest level of host specialization, causing disease only on plants of scentless chamomile. This result supports the conclusion of a previous study that this biocontrol agent likely is not pathogenic to plants outside the genus *Matericaria* (Peng et al. 2005). Previous reports have identified *C. truncatum* as the causal agent of anthracnose on lentil, pea and soybean (Manandhar et al. 1985; Morrall 1988; Anderson et al. 2000). The current study demonstrated that isolates of *C. truncatum* differ in aggressiveness and host specialization, depending on their origin. For

example, lentil isolates caused severe disease on both lentil and pea, girdling the stems and killing the plants. The same isolates caused only minor symptoms on soybean and alfalfa. In contrast, soybean isolates produced very light infection on all crop species tested. These results, in combination with rDNA characteristics reported in Chapter 3 and 4, reinforce the assertion that *C. truncatum* isolates from scentless chamomile are different from those that caused anthracnose on lentil, pea, soybean, or alfalfa. Caution should be exercised when extrapolating the data because, for most crop species, only one cultivar was used in the study. As shown by the current study as well as others on lentil (Chongo 1998; Buckwaldt et al. 2004), pathogenicity of *C. truncatum* can vary substantially depending on the crop cultivar involved. Studies including many crop cultivars would provide a more robust demonstration of crop safety for this biocontrol agent. None of the isolates from crop species caused disease on scentless chamomile, except one from soybean that produced a few leaf spots on one scentless chamomile plant only. The incidence did not reoccur in three replicated trials conducted later. This may indicate that the disease was caused by contamination from a scentless chamomile isolate of *C. truncatum*. Should the infection happen again in any of the following trials, specific primers designed for scentless chamomile and soybean strains could be used to definitively confirm the host origin of the pathogen.

In a previous report, *C. truncatum* from lentil was only weakly pathogenic on pea and nonpathogenic on soybean (Anderson et al. 2000). In the current study, the lentil isolate Ct11-0202 (race Ct0) was highly virulent on both pea and lentil, and an isolate of *Colletotrichum* sp. from pea caused much less disease on both plant species. The variation in aggressiveness among lentil isolates on pea may be attributed to the different

pathogen isolates and crop cultivars used in these studies. This observation of lentil isolates capable of causing severe disease on pea is of importance because pea and lentil crops often are grown in the same areas in Saskatchewan. Epidemiology and management of the disease on both crops should probably take this potential for cross - infection into consideration. The general low virulence of the pea isolate is also of interest. All pea isolates used in the current study were collected in the same year from limited field sites and they were found to be similar to *C. coccodes* and *C. circinans*, respectively, based on rDNA-ITS sequences. These two species have not been reported as pathogens on pea, but results of this study indicate that they may be associated with pea. A conclusion on the primary causal agent of anthracnose on pea, however, appears premature at this time because too few pathogen isolates and crop cultivars were examined in the study. Nevertheless, it may be hypothesized, based on the sequence and plant-inoculation data, that *C. truncatum, C. coccodes* and *C. circinans* are all capable of infecting pea, and that some lentil isolates of *C. truncatum* may cause severe disease on pea plants.

Conidial germination on leaves of the plant species assessed did not differ for the isolates of *Colletotrichum* spp. tested. This probably occurred because the majority of resistant or non-host interactions between plants and fungal pathogens do not take place until the pathogen has penetrated the cuticle layer of the plant (Kolattukudy et al. 1995; Yates et al. 1996). This observation is similar to those reported previously for *C. gloeosporioides* f. sp. *aeschynomene*, in which conidial germination was initiated indiscriminately on its host (*Aeschynomene virginica*) and a non-host (*Pisum sativum*) as long as a hydrophilic surface was present (Sharon and Barhoom 2004). Spore

germination normally responds to chemo-physical characteristics of the plant surface or chemicals such as cuticular waxes or exudates (Tucker and Talbot 2001). Once a spore has germinated, a hydrophobic surface can often induce appressorial formation even on non-host plants. Although studies on other fungal pathogens indicate that spore germination and appressorial formation are linked and coordinated with each other through several signal pathways (Lee 2003), data from the current study indicated that appressorial formation was generally highest on the host of origin. For example, germinated spores of pea, soybean, or alfalfa isolates produced significantly fewer appressoria on scentless chamomile leaves than on their respective hosts. This is important because a higher proportion of appressorial formation often contributes to more successful penetration and infection of a host by the pathogen (Sharon and Barhoom 2004).

After penetration, isolates from scentless chamomile and lentil produced similar infection structures in their respective hosts. Initially, a balloon-like infection vesicle was formed in an epidermal cell, and primary infection hyphae developed from the vesicle shortly after. These thick primary hyphae grow strictly inside epidermal cells and can be distinguished readily from secondary infection hyphae that are much thinner and grow intercellularly throughout the leaf tissues. It appears that both pathogens infect their hosts using an intracellular strategy; they establish themselves initially in host epidermal cells by producing primary hyphae (PH) shortly after host penetration. This process was demonstrated clearly with confocal imaging where PH were frequently observed 24 h after inoculation. Epidermal cells colonized by PH remained alive (biotrophic colonization) until a necrotrophic phase of infection was triggered. For both pathogens,

the latter phase was associated with rapid spread of secondary hyphae. This process generally coincided with the loss of host-tissue integrity and onset of disease symptoms. These are typical hemibiotrophic infections that have been revealed with several *Colletotrichum* spp. (Goodwin 2001). Metabolic interactions during the biotrophic phase are considered important to pathogen-host recognition (Kolattukudy et al. 1995; Wei et al. 1997), which influences the outcome of infection profoundly. O'Connel et al. (1993) suggested that *Colletotrichum* spp. with initial intracellular, hemibiotrophic colonization might have a narrower host range than those with intercellular development of infection hyphae after penetration. It is possible that the infection mechanism of scentless chamomile and lentil isolates of *C. truncatum* contribute to their host specificity, especially for scentless chamomile isolates that cause disease only on scentless chamomile.

It was noteworthy that scentless chamomile isolates could be isolated from surface-sterilized lentil leaves that had been inoculated with the pathogen, although the incidence was low and disease symptoms never occurred on these lentil plants. Additionally, infection vesicles and primary infection hypha were not observed in lentil inoculated with scentless chamomile isolates of *C. truncatum*. It appears that these scentless chamomile isolates can penetrate lentil leaves, but establishment and colonization of epidermal cells failed, possibly due to host resistance responses. On detached lentil leaves, scentless chamomile isolates were able to produce a large number of appressoria on each cultivar, but progression of infection beyond this stage did not occur. The hypersensitive response observed on the cultivar CDC Blaze likely contributed directly to the termination of the infection process. On other cultivars, the

hyphae that penetrated the cell wall may become dormant, as indicated by results from other studies (Singh 1988; Viswanathan et al. 1998). It is not clear how long these 'dormant hyphae' will survive in the infected lentil leaves, or whether they would eventually be able to cause disease if these lentil plants were left for a longer period of time. In latent infections associated with other *Colletotrichum* pathogens, disease expression is often associated with a change in physiological state of host tissue (Cercauskas 1988; Fernando et al. 1994). In the current study, onset of disease symptoms caused by scentless chamomile isolates of *C. truncatum* generally coincided with development of secondary hyphae in scentless chamomile, but there is no evidence that this destructive phase of colonization (Manandhar et al. 1985; O'Connell et al. 1993) occurred in lentil tissues. Therefore, the low incidence of scentless chamomile isolates re-isolated from lentil may be proof of pathogenicity on lentil. To answer this question unequivocally, a study with longer observation times and more frequent sampling would be required.

The current study demonstrated that *C. truncatum* isolates from scentless chamomile did not cause disease or any substantially negative effect on lentil, pea, soybean, or alfalfa. This strict host specificity, in combination with the rDNA sequencing data reported earlier, imply that these isolates of *C. truncatum* differ from those that cause anthracnose diseases on the crop species. There would be a low risk to pulse cops grown on the prairies if using biocontrol agent against scentless chamomile.

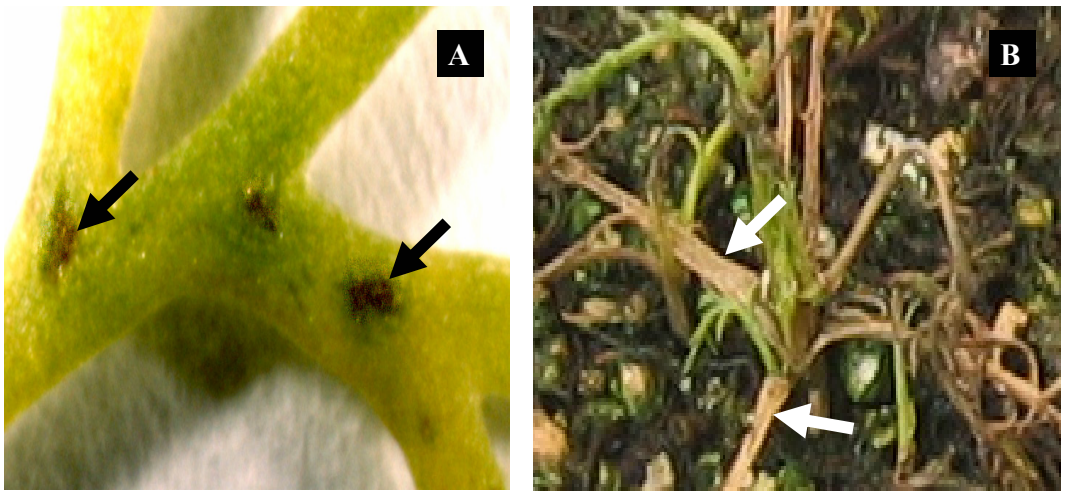

Figure 5.1. Disease symptoms on leaflets (A, 4 dpi) and lower leaves (B, 14 dpi) of scentless chamomile inoculated with an isolate of *C. truncatum* (00-3B1) from scentless chamomile.

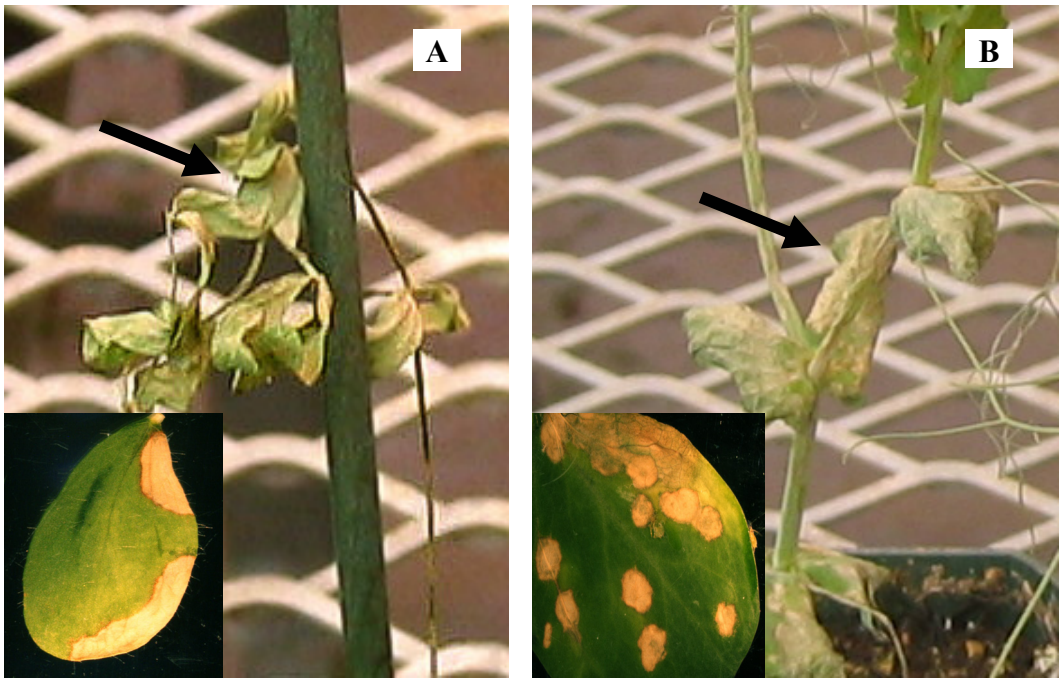

Figure 5.2. Disease symptoms on whole plant of lentil (A) and pea (B) inoculated with a lentil isolate of *C. truncatum* (CT11-0202) at 7 dpi. The inserts show lesions on individual leaflet.

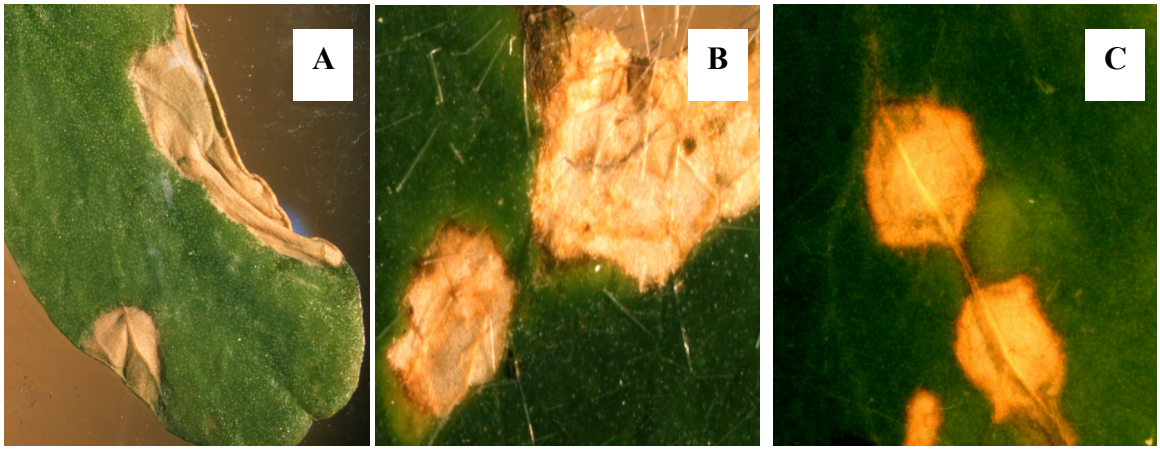

Figure 5.3. Disease symptoms of plant leaves inoculated with isolate Jul0402 of *Colletotrichum* sp. from pea. On lentil (A), pea (B), and soybean (C) leaves at 14 dpi.

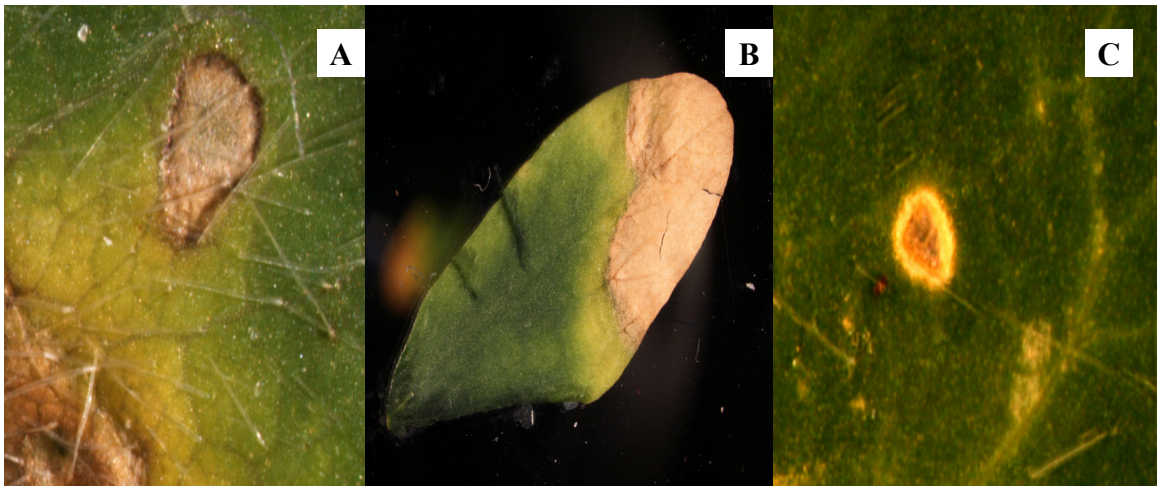

Figure 5.4. Lesions of plant leaves caused by a soybean isolate of *C. truncatum* . On soybean (A), lentil (B), and pea (C) leaves at 14 dpi.

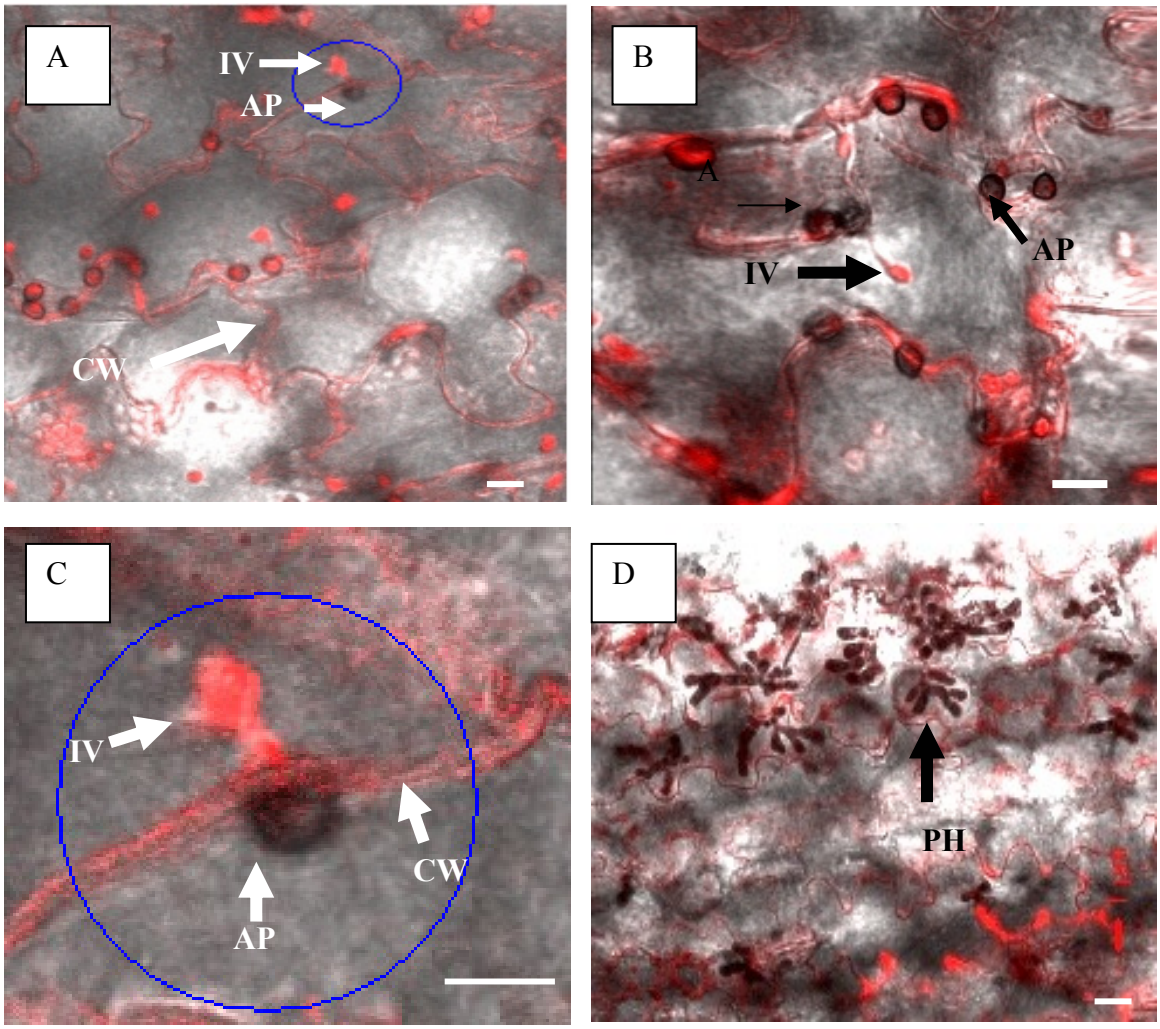

Figure 5.5. Early stages of infection process on plants inoculated with isolate 003-B1 of *C. truncatum* from scentless chamomile. A and B: Appressoria (AP) on leaf surfaces and infection vesicles (IV) within epidermal cells; C: an enlarged IV from A (blue circle); D: primary hyphae (PH) produced within epidermal cells. Bar = 10 µm.

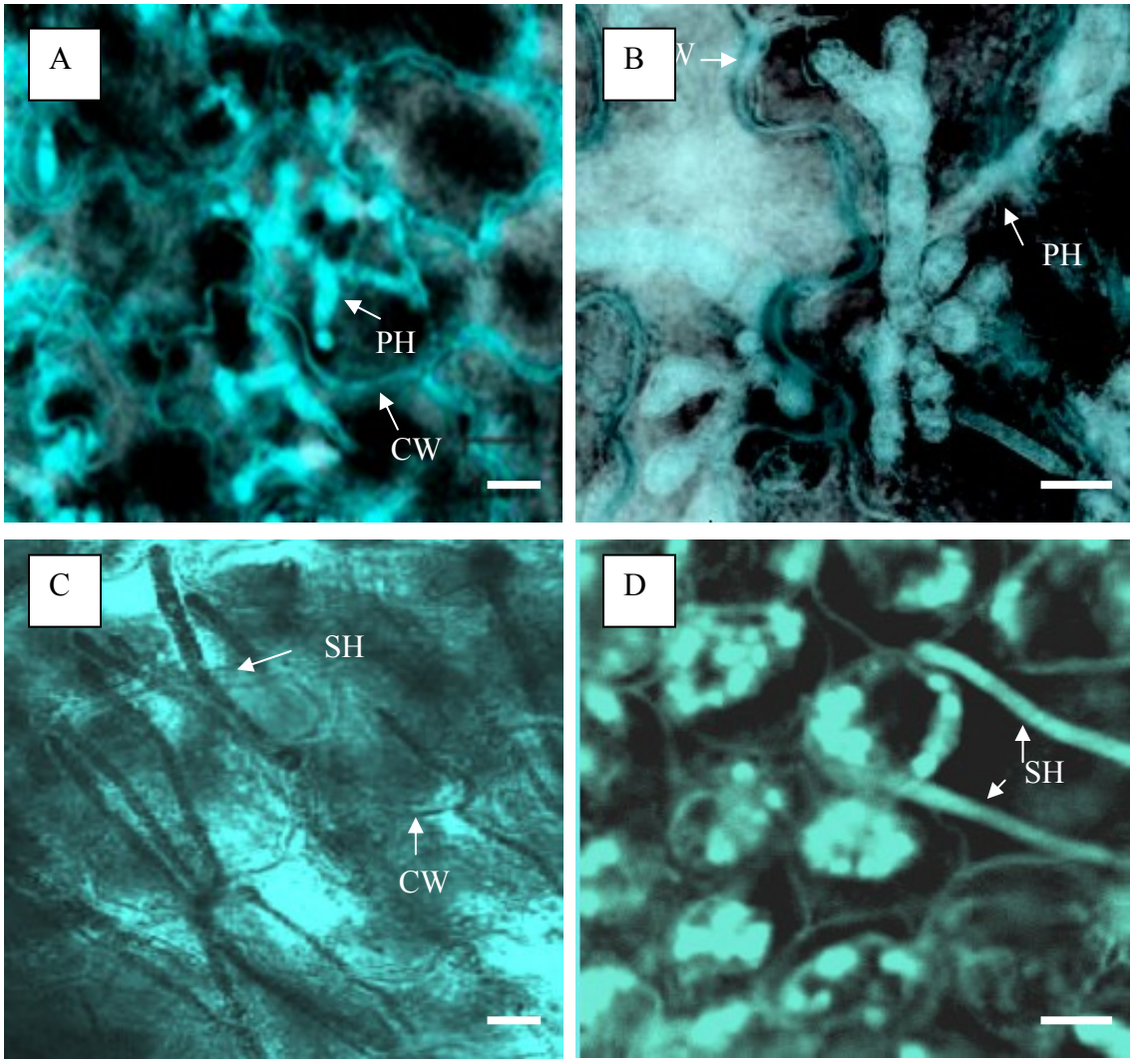

Figure 5.6. Infection hyphae of 003-B1 *C. truncatum* isolate development on scentless chamomile. Intracellular primary hyphae (PH) produced in epidermal cells (A and B) and secondary hyphae (SH) under epidermal cells (C) and mesophyll tissues (D). Bar = 10µm

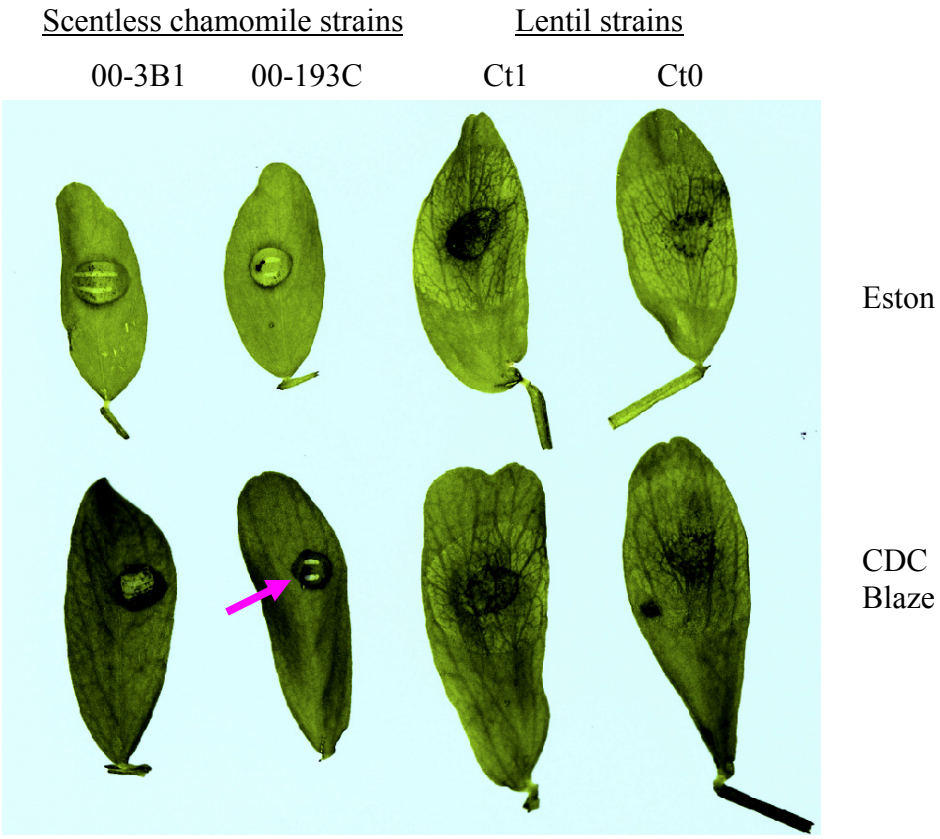

Figure 5.7. Symptoms caused by isolates of *Colletotrichum truncatum* from scentless chamomile (00-3B1, 00-193C) and lentil (Ct1, Ct0) on detached leaves of the lentil cultivars Eston and CDC Blaze at 5 dpi. Arrows indicate the hypersensitive response (HR) area under water drop caused by scentless chamomile isolates on the cultivar CDC Blaze.

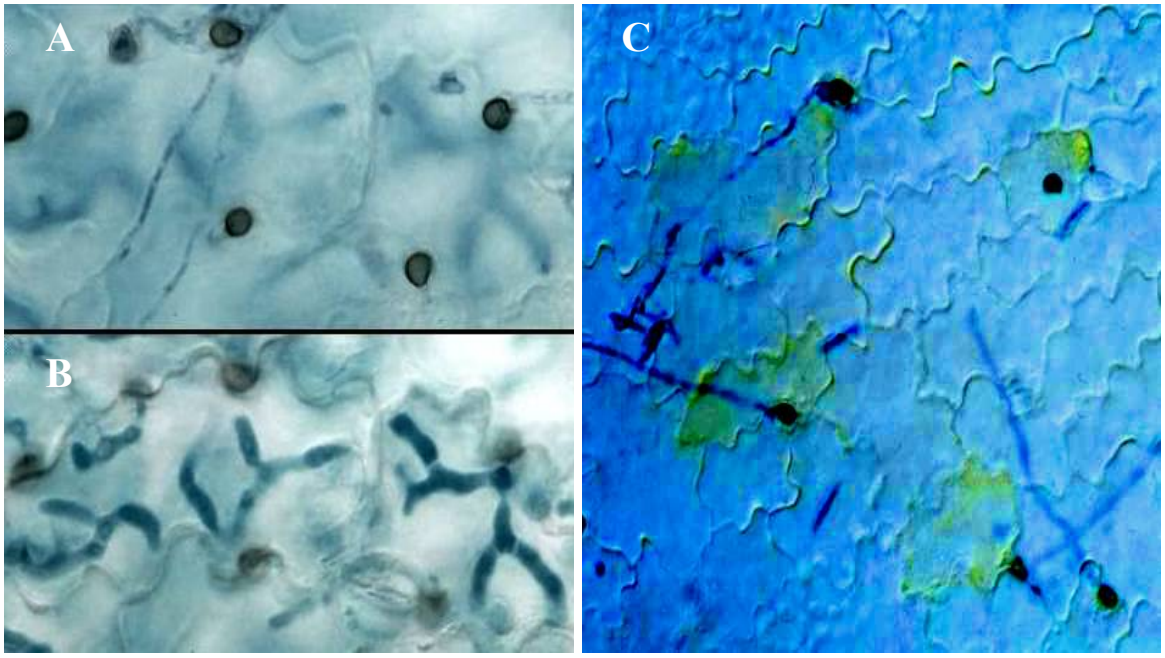

Figure 5.8. Infection of CDC Robin leaves by Ct0 (A and B, 48 hpi) and Ct1 (C, 72 hpi) races of *Colletotrichum truncatum*. In a compatible interaction (A and B), thick primary hyphae were visible in epidermal cells (400 x) underneath appressoria. In an incompatible interaction (C), death of epidermal cells (hypersensitive response-HR, yellow-green colour) was observed after fungal penetration. A and B are the same sample with varying focus on appressoria (A) and primary hyphae in epidermal cells, respectively.

VI. GENERAL DISCUSSION

The main objective of this study was to investigate the relatedness of *C. truncatum* isolates from scentless chamomile to *C. truncatum* pathogens on lentil and pea, and to assess the risk of using the former for biocontrol of scentless chamomile in western Canada.

Based on ITS-rDNA sequences, *C. truncatum* isolates from scentless chamomile can be discriminated from anthracnose pathogens of lentil or pea. Isolates from the same host exhibited high sequence similarities, whereas those from different hosts showed recognizable divergences. The molecular information provides criterion that permits differentiation of strains with similar conidial morphology. It is generally agreed that the concept of a species in the genus *Colletotrichum* is not well defined due to 1) insufficient variation in classical descriptive criteria and, 2) difficulties in dealing with pathogens of similar morphology but different host specificity (Sutton 1992). The examination of highly conserved rDNA, along with other criteria represents a useful approach to addressing taxonomic uncertainties (Sheriff et al. 1994; Bailey et al. 1996). For example, *C. truncatum* isolates from scentless chamomile and lentil exhibited similar conidial size and shape, which made it impossible to differentiate them based on morphological characteristics. With sequence data, however, they were separated consistently into two distinct clusters according to the host of origin. Similar results have been reported by Ford et al. (2004) from a study on Canadian isolates of *C. truncatum* from lentil

compared with Australian isolates of *C. truncatum* from hosts other than lentil; RAPD markers and 18-25S rDNA sequences demonstrated genetic divergences among isolates from lentil and other host species. Observations in this study support the inference by Ford et al. (2004) that rDNA sequencing is useful for discriminating different strains of *C. truncatum* at sub-specific levels. The current study also supports the suggestion that *C. truncatum* from scentless chamomile may be different from the anthracnose pathogens on lentil and pea (Peng et al. 2005). It is important to differentiate these isolates because, in the region where the biocontrol of scentless chamomile is targeted, the economic impact of anthracnose on lentil crops is increasing (Chongo et al. 2002; Anderson 2004).

Highly specific PCR primers designed based on the sequence information effectively differentiated among *Colletotrichum* isolates from scentless chamomile and selected crop species. These primers may be used for rapid detection or differentiation of the pathogens that cause diseases on scentless chamomile, lentil, pea or soybean crops. For diagnostic purposes, this technique may be useful for early detection of the pathogens prior to the occurrence of typical disease symptoms. In comparison to conventional diagnostic methods based on fungal isolation and culturing, PCR-based techniques are more rapid and accurate (Mahuku et al. 1999; Dauch et al. 2003; Ward et al. 2004). In addition, these techniques can potentially provide both qualitative and quantitative information on the pathogen, a feature that can be useful for tracking the biocontrol agent after being released into different environments. Zhou et al. (2004) used strain-specific primers as molecular markers to assess the movement and persistence of *Phoma macrostoma*, a biocontrol agent for broadleaf weeds in turf and lawn, and provided valuable data on potential environmental impact by the biocontrol agent. It is likely that

similar tracking strategy and methodology can be used when conducting risk assessment for the *C. truncatum* isolates from scentless chamomile.

In the inoculation study, observations that *C. truncatum* isolates from scentless chamomile caused no disease on the crop species tested, demonstrated the strict host specificity of this biocontrol agent. This host specificity may be related to a distinctive biotrophic phase of the fungus exemplified by intracellular colonization of host epidermal cells after penetration. This intimate association with living host cells may be critical to host recognition by *C. truncatum* (O'Connell et al. 1993). On the crop species tested, the scentless chamomile isolates generally did not develop beyond the appressorial stage and intracellular fungal hyphae or infection vesicles were not observed. This validation of host specificity is critical to development of this biocontrol agent because the same fungal species causes anthracnose diseases on lentil and pea (Morral 1988, Anderson et al. 2000). The test of pathogenicity using multiple lentil cultivars with different susceptibility to the pathogen race Ct1 and Ct0 demonstrated the general incompatibility of the biocontrol agent with lentil of varying genetic background. Although a low level of latent infection was indicated on some lentil cultivars, there was no clear negative impact of endophytic colonization of lentil plants by the biocontrol agent. This study also demonstrates that *C. truncatum* from lentil can be highly virulent on both lentil and pea, which is slightly different from observations in a previous study in which *C. truncatum* isolates from lentil were only weakly pathogenic on pea (Anderson et al. 2000). It is possible that different isolates of *C. truncatum* from lentil or pea vary in aggressiveness. Assessment of a larger number of isolates and crop cultivars is required to elucidate virulence spectrum of the pathogen on the two crop species more clearly,

especially due to the possibility of potential infection of pea by *C. circinans* and *C. coccodes* as indicated in this study.

It is concluded that, based on ITS-rDNA sequences, *C. truncatum* isolates from scentless chamomile can be distinguished from anthracnose pathogens on lentil and pea. The high host specificity of these isolates, coupled with the distinct rDNA-ITS characteristics, reduce concerns about potential negative impact of these isolates on pulse crops if used for biocontrol of scentless chamomile in western Canada.

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5.8 rDNA-ITS segment of *Colletotrichum* spp.

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D3                TCTCCGTTGGTGAACCAGCGGAGGGATCATTACTGAGTTACCG--CTCATCAACCCTTTG 58
G08               ------------------------------------------------------CCCTTTG 7
D22               TCTCCGTTGGTGAACCAGCGGAGGGATCATTACTGAGTTACCG--CTCATCAACCCTTTG 58
D14               TCTCCGTTGGTGAACCAGCGGAGGGATCATTACTGAGTTACCG--CTCTATAACCCTTTG 58
C18               TCTCCGTTGGTGAACCAGCGGAGGGATCATTACTGAGTTACCG--CTCTATAACCCTTTG 58
D21               TCTCCGTTGGTGAACCAGCGGAGGGATCATTACTGAGTTACCG--CTCTATAACCCTTTG 58
E8                TCTCCGTTGGTGAACCAGCGGAGGGATCATTACTGAGTTACCG--CTCTATAACCCTTTG 58
E11               TCTCCGTTGGTGAACCAGCGGAGGGATCATTACTGAGTTACCG--CTCTATAACCCTTTG 58
C4                TCTCCGTTGGTGAACCAGCGGAGGGATCATTACTGAGTTACCG--CTCTATAACCCTTTG 58
C30               TCTCCGTTGGTGAACCAGCGGAGGGATCATTACTGAGTTACCG--CTCTATAACCCTTTG 58
G03               TCTCCGTTGGTGAACCAGCGGAGGGATCATTACTGAGTTACCG--CTCTATAACCCTTTG 58
F14               TCTCCGTTGGTGAACCAGCGGAGGGATCATTACTGAGTTACCG--CTCTATAACCCTTTG 58
c38               TCTCCGTTGGTGAACCAGCGGAGGGATCATTACTGAGTTACCG--CTCTATAACCCTTTG 58
C6                TCTCCGTTGGTGAACCAGCGGAGGGATCATTACTGAGTTACCG--CTCTATAACCCTTTG 58
D12               TCTCCGTTGGTGAACCAGCGGCGGGATCATTACTGAGTTACCG--CTCTATAACCCTTTG 58
C12               TCTCCGTTGGTGAACCAGCGGAGGGATCATTACTGAGTTACCG--CTCTATAACCCTTTG 58
D16               TCTCCGTTGGTGAACCAGCGGAGGGATCATTACTGAGTTACCG--CTCTACAACCCTTTG 58
E12               TCTCCGTTGGTGAACCAGCGGAGGGATCATTACTGAGTTACCG--CTCTACAACCCTTTG 58
F19               TCTCCGTTGGTGAACCAGCGGAGGGA-CATTACTGAGTTACCG--CTCTACAACCCTTTG 57
C25               TCTCCGTTGGTGAACCAGCGGAGGGATCATTACTGAGTTACCG--CTCTACAACCCTTTG 58
E9                TCTCCGTTGGTGAACCAGCGGAGGGATCATTACTGAGTTACCG--CTCTACAACCCTTTG 58
F9                TCTCCGTTGGTGAACCAGCGGAGGGATCATTACTGAGTTACCG--CTCTACAACCCTTTG 58
G02_AY539806_     TCTCCGTTGGTGAACCAGCGGAGGGATCATTACTGAGTTACCG--CTCTACAACCCTTTG 58
C31               TCTCCGTTGGTGAACCAGCGGAGGGATCATTACTGAGTTACCG--CTCTACAACCCTTTG 58
C3                TCTCCGTTGGTGAACCAGCGGAGGGATCATTATCGAGTTACCG--CTCTTCAACCCTTTG 58
C7                TCTCCGTTGGTGAACCAGCGGAGGGATCATTATCGAGTTACCG--CTCTTCAACCCTTTG 58
C8                TCTCCGTTGGTGAACCAGCGGAGGGATCATTATCGAGTTACCG--CTCTTCAACCCTTTG 58
C26               TCTCCGTTGGTGAACCAGCGGAGGGATCATTATCGAGTTACCG--CTCTTCAACCCTTTG 58
C27               TCTCCGTTGGTGAACCAGCGGAGGGATCATTATCGAGTTACCG--CTCTTCAACCCTTTG 58
E2                TCTCCGTTGGTGAACCAGCGGAGGGATCATTATCGAGTTACCG--CTCTTCAACCCTTTG 58
E4                TCTCCGTTGGTGAACCAGCGGAGGGATCATTATCGAGTTACCG--CTCTTCAACCCTTTG 58
E5                TCTCCGTTGGTGAACCAGCGGAGGGATCATTATCGAGTTACCG--CTCTTCAACCCTTTG 58
F8                TCTCCGTTGGTGAACCAGCGGAGGGATCATTATCGAGTTACCG--CTCTTCAACCCTTTG 58
F20               TCTCCGTTGGTGAACCAGCGGAGGGATCATTATCGAGTTACCG--CTCTTCAACCCTTTG 58
F21               TCTCCGTTGGTGAACCAGCGGAGGGATCATTATCGAGTTACCG--CTCTTCAACCCTTTG 58
C28               TCTCCGTTGGTGAACCAGCGGACGGATCATTATCGAGTTACCG--CTCTTCAACCCTTTG 58
C21               TCTCCGTTAGTGAACCAGCAGAGGGATTATTATCGAGTTACTG--CTCTTTAACCCTTTG 58
G01_DQ195708_     ------------------------------------------------------CCCTTTG 7
F7                TCTCCGTTGGTGAACCAGCGGAGGGATCATTATCGAGTTACCG--CTCTACAACCCTTTG 58
G09_DQ195690_     ------------------------------------------------------CCCTTTG 7
G05_AJ301985_     ---------GTGAACCAGCGGAGGGATCATTACTGAGTTACCG--CTCTACAACCCTTTG 49
G06_AJ301976_     ----------------------------TTACTGAGTTACCG--CTCTACAACCCTTTG 29
C17               TCTCCGTTGGTGAACCAGCGGAGGGATCATTACTGAGTTACCG--CTCTACAACCCTTTG 58
c33               TCTCCGTTGGTGAACCAGCGGAGGGATCATTACTGAGTTACCG--CTCTACAACCCTTTG 58
C34               TCTCCGTTGGTGAACCAGCGGAGGGATCATTACTGAGTTACCG--CTCTACAACCCTTTG 58
F2                TCTCCGTTGGTGAACCAGCGGAGGGATCATTACTGAGTTACCG--CTCTACAACCCTTTG 58
F3                TCTCCGTTGGTGAACCAGCGGAGGGATCATTACTGAGTTACCG--CTCTACAACCCTTTG 58
F1                TCTCCGTTGGTGAACCAGCGGAGGGATCATTACTGAGTTACCG--CTCTACAACCCTTTG 58
F4                TCTCCGTTGGTGAACCAGCGGAGGGATCATTACTGAGTTACCG--CTCTACAACCCTTTG 58
F5                TCTCCGTTGGTGAACCAGCGGAGGGATCATTACTGAGTTACCG--CTCTACAACCCTTTG 58
F6                TCTCCGTTGGTGAACCAGCGGAGGGATCATTACTGAGTTACCG--CTCTACAACCCTTTG 58
E7                TCTCCGTTGGTGAACCAGCGGAGGGATCATTACTGAGTTACCG--CTCTATAACCCTTTG 58
G07               ---------------------------CATTACTGAGTTACCG--CTCTATAACCCTTTG 31
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E7                TGAACATACCT--AACTGTTGCTTCGGCGGGCAGGGGGTCCC--CTCG----------G 103
G07               TGAACATACCT--AACTGTTGCTTCGGCGGGCAGGGGGTCCC--CTCG----------G 76
C24               TGAACATACCT--AACTGTTGCTTCGGCGGGCAGGGGGTCCC--CTCG----------G 103
F11               TGAACATACCT--AACTGTTGCTTCGGCGGGCAGGGGGTCCC--CTCG----------G 103
D13               TGAACATACCT--AACTGTTGCTTCGGCGGGCAGGGGGTGCCG-CCTGC----------G 105
G04_AB233340_     TGAACATACCT--AACTGTTGCTTCGGCGGGCAGGGGGTGCCG-CCTGC----------G 103
C19               TGAACATACCT--AACTGTTGCTTCGGCGGGCAGGGGGTGCCG-CCTGC----------G 105
D1                TGAACATACCT--AACTGTTGCTTCGGCGGGCAGGGGGTGCCG-CCTGC----------G 105
c23               TGAACATACCT--AACTGTTGCTTCGGCGGGCAGGGGGTGCCG-CCTGC----------G 104
D6                TGAACATACCT--AACTGTTGCTTCGGCGGGCAGGGGGTGCCG-CCTGC----------G 105
C5                TGAACATACCT--TACTGTTGCTTCGGCGGGCAGAGGTCCCCTC--------------GG 102
F17               TGAACGTACCT--AAAGGAAGCTTCACAGGGCAGGGGAAGCCTCTCGCG----------G 105
F18               TGAACGTACCT--AACCGTTGCTTCGGCGGGCAGGGGAAGCCTCTCGCG----------G 105
c36               TGAACGTACCT--AACCGTTGCTTCGGCGGGCAGGGGAAGCCTCTCGCG----------G 106
C16               TGAACGTACCT--AACCGTTGCTTCGGCGGGCAGGGGAAGCCTCTCGCG----------G 106
D7                TGAACATACCT--AACCGTTGCTTCGGCGGGCGG--GAGGTCC----------------G 98
E6                TGAACATACCTC-AAACGTTGCCTCGGCGGGCAGCCGGAGCCC----------------A 101
C1                TGAACATACCTATAACTGTTGCTTCGGCGGGTAGGGTCTCCGCGACCCT----------- 107
F16               TGAACGTTACCTTTATCGTTGCTTCGGCGGGTGG--------------------------C 95
C29               TTCATAACCCTT----TGTTGTCCGACTCTGTTG--------------------------- 90
                     *     *       *            *

D3                AGGACGTCTCCCGGCCCTCTCCCGTCCGC--------GGGTGGGGCGCCCGCCGGAGGAT 156
G08               AGGACGTCTCCCGGCCCTCTCCCGTCCGC--------GGGTGGGGCGCCCGCCGGAGGAT 105
D22               AGGACGTCTCCCGGCCCTCTCCCGTCCGC--------GGGTGGGGCGCCCGCCGGAGGAT 156
D14               GGTCCCCCTCCCGGCCGCGCCCT-CGC----------GGGCGTGGCGCCCGCCGGAGGAT 161
C18               GGTCCCCCTCCCGGCCGCGCCCT-CGC----------GGGCGTGGCGCCCGCCGGAGGAT 161
D21               GGTCCCCCTCCCGGCCGCGCCCT-CGC----------GGGCGTGGCGCCCGCCGGAGGAT 161
E8                GGTCCCCCTCCCGGCCGCGCCCT-CGC----------GGGCGTGGCGCCCGCCGGAGGAT 161
E11               GGTCCCCCTCCCGGCCGCGCCCT-CGC----------GGGCGTGGCGCCCGCCGGAGGAT 161
C4                GGTCCCCCTCCCGGCCGCGCCCT-CGC----------GGGCGTGGCGCCCGCCGGAGGAT 161
C30               GGTCCCCCTCCCGGCCGCGCCCT-CGC----------GGGCGTGGCGCCCGCCGGAGGAT 161
G03               GGTCCCCCTCCCGGCCGCGCCCT-CGC----------GGGCGTGGCGCCCGCCGGAGGAT 161
F14               GGTCCCCCTCCCGGCCGCGCCCT-CGC----------GGGCGTGGCGCCCGCCGGAGGAT 161
c38               GGTCCCCCTCCCGGCCGCGCCCT-CGC----------GGGCGTGGCGCCCGCCGGAGGAT 161
C6                GGTCCCCCTCCCGGCCGCGCCCT-CGC----------GGGCGTGGCGCCCGCCGGAGGAT 161
D12               GGTCCCCCTCCCGGCCGCGCCCT-CGC----------GGGCGTGGCGCCCGCCGGAGGAT 161
C12               GGTCCCCCTCCCGGCCGCGCCCT-CGC----------GGGCGTGGCGCCCGCCGGAGGAT 161
D16               GGTCCCCCTCCCGGCCGCGCCCT-CAC----------GGGCGTGGCGCCCGCCGGAGGAT 165
E12               GGTCCCCCTCCCGGCCGCGCCCT-CAC----------GGGCGTGGCGCCCGCCGGAGGAT 165
F19               GGTCCCCCTCCCGGCCGCGCCCT-CAC----------GGGCGTGGCGCCCGCCGGAGGAT 164
C25               GGTCCCCCTCCCGGCCGCGCCCT-CAC----------GGGCGTGGCGCCCGCCGGAGGAT 165
E9                GGTCCCCCTCCCGGCCGCGCCCT-CAC----------GGGCGTGGCGCCCGCCGGAGGAT 165
F9                GGTCCCCCTCCCGGCCGCGCCCT-CAC----------GGGCGTGGCGCCCGCCGGAGGAT 165
G02_AY539806_     GGTCCCCCTCCCGGCCGCGCCCT-CAC----------GGGCGTGGCGCCCGCCGGAGGAT 163
C31               GGTCCCCCTCCCGGCCGCGCCCT-CAC----------GGGCGTGGCGCCCGCCGGAGGAT 161
C3                GGTCCGCCTCCCGGCCGCGCCCCACACC---------GGGCGTGGCGCCCGCCGGAGGAT 163
C7                GGTCCGCCTCCCGGCCGCGCCCCACACC---------GGGCGTGGCGCCCGCCGGAGGAT 163
C8                GGTCCGCCTCCCGGCCGCGCCCCACACC---------GGGCGTGGCGCCCGCCGGAGGAT 163
C26               GGTCCGCCTCCCGGCCGCGCCCCACACC---------GGGCGTGGCGCCCGCCGGAGGAT 163
C27               GGTCCGCCTCCCGGCCGCGCCCCACACC---------GGGCGTGGCGCCCGCCGGAGGAT 163
E2                GGTCCGCCTCCCGGCCGCGCCCCACACC---------GGGCGTGGCGCCCGCCGGAGGAT 163
E4                GGTCCGCCTCCCGGCCGCGCCCCACACC---------GGGCGTGGCGCCCGCCGGAGGAT 163
E5                GGTCCGCCTCCCGGCCGCGCCCCACACC---------GGGCGTGGCGCCCGCCGGAGGAT 163
F8                GGTCCGCCTCCCGGCCGCGCCCCACACC---------GGGCGTGGCGCCCGCCGGAGGAT 163
F20               GGTCCGCCTCCCGGCCGCGCCCCACACC---------GGGCGTGGCGCCCGCCGGAGGAT 163
F21               GGTCCGCCTCCCGGCCGCGCCCCACACC---------GGGCGTGGCGCCCGCCGGAGGAT 163
C28               GGTCCGCCTCCCGGCCGCGCCCCACACC---------GGGCGTGGCGCCCGCCGGAGGAT 163
C21               GGTCTGCCTCCTAGCTGCGCCCCACACT---------AGGTGTAGCGCCTGCTGGAGGAT 163
G01_DQ195708_     GGACGCCCTCCCGGCCGCGCCCTCCTCCTCCGGGAGGGGTCGCGGCGCCCGCCGGAGGAT 113
F7                GGACGCCCTCCCGGCCCCGCCCCCCTACC-----AGGGGACGTGGCGCCCGCCGGAGGAT 158
G09_DQ195690_     --GTCCCCCCCCGGCCGCGCCCCTCGC---------GGGGCGTGGCGCCCGCCGGAGGAT 98
G05_AJ301985_     GAAC-CCCTCCCGGTGACGCCCT-CACG---------GG-CGTCGCGCCCGCCGGAGGAT 142
G06_AJ301976_     GAAC-CCCTCCCGGTGACGCCCT-CACG---------GG-CGTCGCGCCCGCCGGAGGAT 122
C17               GAAC-CCCTCCCGGTGACGCCCT-CACG---------GG-CGTCGCGCCCGCCGGAGGAT 151
c33               GAAC-CCCTCCCGGTGACGCCCT-CACG---------GG-CGTCGCGCCCGCCGGAGGAT 151
C34               GAAC-CCCTCCCGGTGACGCCCT-CACG---------GG-CGTCGCGCCCGCCGGAGGAT 151
F2                GAAC-CCCTCCCGGTGACGCCCT-CACG---------GG-CGTCGCGCCCGCCGGAGGAT 151
F3                GAAC-CCCTCCCGGTGACGCCCT-CACG---------GG-CGTCGCGCCCGCCGGAGGAT 151
F1                GAAC-CCCTCCCGGTGACGCCCT-CACG---------GG-CGTCGCGCCCGCCGGAGGAT 151
F4                GAAC-CCCTCCCGGTGACGCCCT-CACG---------GG-CGTCGCGCCCGCCGGAGGAT 151
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F5                GAAC-CCCTCCCGGTGACGCCCT-CACG---------GG-CGTCGCGCCCGCCGGAGGAT 151
F6                GAAC-CCCTCCCGGTGACGCCCT-CACG---------GG-CGTCGCGCCCGCCGGAGGAT 151
E7                GGACGCCCTCCCGGCCACGCCCTTCACG---------GGGCGAGGCGCCCGCCGGAGGAT 154
G07               GGACGCCCTCCCGGCCACGCCCTTCACG---------GGGCGAGGCGCCCGCCGGAGGAT 127
C24               GGACGCCCTCCCGGCCACGCCCTTCACG---------GGGCGAGGCGCCCGCCGGAGGAT 154
F11               GGACGCCCTCCCGGCCACGCCCTTCACG---------GGGCGAGGCGCCCGCCGGAGGAT 154
D13               GACCCCCCTCCCGGCCCTGCCCT-CACG---------GG-CGGAGCGCCCGCCGGAGGAT 154
G04_AB233340_     GACCCCCCTCCCGGCCCTGCCCT-CACG---------GG-CGGAGCGCCCGCCGGAGGAT 152
C19               GACCCCCCTCCCGGCCCTGCCCT-CACG---------GG-CGGAGCGCCCGCCGGAGGAT 154
D1                GACCCCCCTCCCGGCCCTGCCCT-CACG---------GG-CGGAGCGCCCGCCGGAGGAT 154
c23               GACCCCCCTCCCGGCCCTGCCCT-CACG---------GG-CGGAGCGCCCGCCGGAGGAT 153
D6                GACCCCCCTCCCGGCCCTGCCCT-CACG---------GG-CGGAGCGCCCGCCGGAGGAT 154
C5                GGAC-CCCTCCCGGCCCCGTCCCTCGC----------GGACGGAGCGCCCGCCGGAGGAT 151
F17               GCCTCCCCTTCCGGCGCCGGCCCCCAGCAC-----GGGGACGGGGCGCCCGCCGGAGGAA 160
F18               GCCTCCCCTCCCGGCGCCGGCCCCCACCAC-----GGGGACGGGGCGCCCGCCGGAGGAA 160
c36               GCCTCCCCTCCCGGCGCCGGCCCCCACCAC-----GGGGACGGGGCGCCCGCCGGAGGAA 161
C16               GCCTCCCCTCCCGGCGCCGGCCCCCACCAC-----GGGGACGGGGCGCCCGCCGGAGGAA 161
D7                CCTCC--CCCCCGGCCCCG----C--TCGC-----GGGG-CGCCC-GCCGGAGGAAAAAC 143
E6                GCTCCGGCGCCCGGAGCCG----CCTTCTC-----GGCG-CGCCCCACCCGCCGGCGGAC 151
C1                -CCCGGCCTCCCGCCTCCGGGC----------------GGGTCGGCGCCCGCCGGAGGAT 150
F16               GCCCAGCGCCCCCAAGGCCCCCC---------------CGCGGGGCGCCCGCCGGAGGAA 140
C29               ------CCTCCGGGGGCGACCCTGCCTTCG--------GGCGGGGGCTCCGGGTGGACACT 136
                        *                                    ** *  *

D3                AACCAAACTCTGATTTAACGACGTTTCTTCTGAGTGACACAAGCAAATAA-TCAAAACTT 215
G08               AACCAAACTCTGATTTAACGACGTTTCTTCTGAGTGACACAAGCAAATAA-TCAAAACTT 164
D22               AACCAAACTCTGATTTAACGACGTTTCTTCTGAGTGACACAAGCAAATAA-TCAAAACTT 215
D14               ACCAAA-CTCTATTTTAACGACGTTTCTTCTGAGTGGCACAAGCAAATAA-TTAAAACTT 219
C18               ACCAAA-CTCTATTTTAACGACGTTTCTTCTGAGTGGCACAAGCAAATAA-TTAAAACTT 219
D21               ACCAAA-CTCTATTTTAACGACGTTTCTTCTGAGTGGCACAAGCAAATAA-TTAAAACTT 219
E8                ACCAAA-CTCTATTTTAACGACGTTTCTTCTGAGTGGCACAAGCAAATAA-TTAAAACTT 219
E11               ACCAAA-CTCTATTTTAACGACGTTTCTTCTGAGTGGCACAAGCAAATAA-TTAAAACTT 219
C4                ACCAAA-CTCTATTTTAACGACGTTTCTTCTGAGTGGCACAAGCAAATAA-TTAAAACTT 219
C30               ACCAAA-CTCTATTTTAACGACGTTTCTTCTGAGTGGCACAAGCAAATAA-TTAAAACTT 219
G03               ACCAAA-CTCTATTTTAACGACGTTTCTTCTGAGTGGCACAAGCAAATAA-TTAAAACTT 219
F14               ACCAAA-CTCTATTTTAACGACGTTTCTTCTGAGTGGCACAAGCAAATAA-TTAAAACTT 219
c38               ACCAAA-CTCTATTTTAACGACGTTTCTTCTGAGTGGCACAAGCAAATAA-TTAAAACTT 219
C6                ACCAAA-CTCTATTTTAACGACGTTTCTTCTGAGTGGCACAAGCAAATAA-TTAAAACTT 219
D12               ACCAAA-CTCTATTTTAACGACGTTTCTTCTGAGTGGCACAAGCAAATAA-TTAAAACTT 219
C12               ACCAAA-CTCTATTTTAACGACGTTTCTTCTGAGTGGCACAAGCAAATAA-TTAAAACTT 219
D16               ACCAAA-CTCTATTTTAACGACGTTTCTTCTGAGTGGCACAAGCAAATAA-TTAAAACTT 223
E12               ACCAAA-CTCTATTTTAACGACGTTTCTTCTGAGTGGCACAAGCAAATAA-TTAAAACTT 223
F19               ACCAAA-CTCTATTTTAACGACGTTTCTTCTGAGTGGCACAAGCAAATAA-TTAAAACTT 222
C25               ACCAAA-CTCTATTTTAACGACGTTTCTTCTGAGTGGCACAAGCAAATAA-TTAAAACTT 223
E9                ACCAAA-CTCTATTTTAACGACGTTTCTTCTGAGTGGCACAAGCAAATAA-TTAAAACTT 223
F9                ACCAAA-CTCTATTTTAACGACGTTTCTTCTGAGTGGCACAAGCAAATAA-TTAAAACTT 223
G02_AY539806_     ACCAAAACTCTATTTTAACGACGTTTCTTCTGAGTGGCACAAGCAAATAA-TTAAAACTT 222
C31               ACCAAA-CTCTATTTTAACGACGTTTCTTCTGAGTGGCACAAGCAAATAA-TTAAAACTT 219
C3                ACCCAAACTCTATTGCAACGACGTTTCTTCTGAGTGGCACAAGCAAATAA-TTAAAACTT 222
C7                ACCCAAACTCTATTGCAACGACGTTTCTTCTGAGTGGCACAAGCAAATAA-TTAAAACTT 222
C8                ACCCAAACTCTATTGCAACGACGTTTCTTCTGAGTGGCACAAGCAAATAA-TTAAAACTT 222
C26               ACCCAAACTCTATTGCAACGACGTTTCTTCTGAGTGGCACAAGCAAATAA-TTAAAACTT 222
C27               ACCCAAACTCTATTGCAACGACGTTTCTTCTGAGTGGCACAAGCAAATAA-TTAAAACTT 222
E2                ACCCAAACTCTATTGCAACGACGTTTCTTCTGAGTGGCACAAGCAAATAA-TTAAAACTT 222
E4                ACCCAAACTCTATTGCAACGACGTTTCTTCTGAGTGGCACAAGCAAATAA-TTAAAACTT 222
E5                ACCCAAACTCTATTGCAACGACGTTTCTTCTGAGTGGCACAAGCAAATAA-TTAAAACTT 222
F8                ACCCAAACTCTATTGCAACGACGTTTCTTCTGAGTGGCACAAGCAAATAA-TTAAAACTT 222
F20               ACCCAAACTCTATTGCAACGACGTTTCTTCTGAGTGGCACAAGCAAATAA-TTAAAACTT 222
F21               ACCCAAACTCTATTGCAACGACGTTTCTTCTGAGTGGCACAAGCAAATAA-TTAAAACTT 222
C28               ACCCAAACTCTATTGCAACGACGTTTCTTCTGAGTGGCACAAGCAAATAA-TTAAAACTT 222
C21               ACCTAAACTCTATTGCAACGACGTTTCTTCTGAGTAGCACAAGCAAATAA-TTAAAACTT 222
G01_DQ195708_     AACCAAACTCTGATTTAACGACGTTTCTTCTGAGTGGCACAAGCAAATAA-TTAAAACTT 172
F7                A-CCTAACTCTATTTTAACGACGTTTCTTCTGAGTGGCACAAGCAAATAA-TTAAAACTT 216
G09_DQ195690_     ACCAAAACTCTATTTTAACGACGTCTCTTCTGAGTGGCACAAGCAAATAA-TTAAAACTT 157
G05_AJ301985_     ACCAAA-CTCTATTTTAACGACGTTTCTTCTGAGTGGCACAAGCAAATAA-TTAAAACTT 200
G06_AJ301976_     ACCAAA-CTCTATTTTAACGACGTTTCTTCTGAGTGGCACAAGCAAATAA-TTAAAACTT 180
C17               ACCAAA-CTCTATTTTAACGACGTTTCTTCTGAGTGGCACAAGCAAATAA-TTAAAACTT 209
c33               ACCAAA-CTCTATTTTAACGACGTTTCTTCTGAGTGGCACAAGCAAATAA-TTAAAACTT 209
C34               ACCAAA-CTCTATTTTAACGACGTTTCTTCTGAGTGGCACAAGCAAATAA-TTAAAACTT 209
F2                ACCAAA-CTCTATTTTAACGACGTTTCTTCTGAGTGGCACAAGCAAATAA-TTAAAACTT 209
F3                ACCAAA-CTCTATTTTAACGACGTTTCTTCTGAGTGGCACAAGCAAATAA-TTAAAACTT 209
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F1                ACCAAA-CTCTATTTTGACGACGTTTCTTCTGAGTGGCACAAGCAAATAA-TTAAAACTT 209
F4                ACCAAA-CTCTATTTTAACGACGTTTCTTCTGAGTGGCACAAGCAAATAA-TTAAAACTT 209
F5                ACCAAA-CTCTATTTTAACGACGTTTCTTCTGAGTGGCACAAGCAAATAA-TTAAAACTT 209
F6                ACCAAA-CTCTATTTTAACGACGTTTCTTCTGAGTGGCACAAGCAAATAA-TTAAAACTT 209
E7                ACCAAA-CTCTATTTTAACGACGTTTCTTCTGAGTGGCACAAGCAAATAA-TTAAAACTT 212
G07               ACCAAA-CTCTATTTTAACGACGTTTCTTCTGAGTGGCACAAGCAAATAA-TTAAAACTT 185
C24               ACCAAA-CTCTATTTTAACGACGTTTCTTCTGAGTGGCACAAGCAAATAA-TTAAAACTT 212
F11               ACCAAA-CTCTATTTTAACGACGTTTCTTCTGAGTGGCACAAGCAAATAA-TTAAAACTT 212
D13               ACCAAA-CTCTATTTTAACGACGTTTCTTCTGAGTGGCACAAGCAAATAA-TTAAAACTT 212
G04_AB233340_     ACCAAA-CTCTATTTTAACGACGTTTCTTCTGAGTGGCACAAGCAAATAA-TTAAAACTT 210
C19               ACCAAA-CTCTATTTTAACGACGTTTCTTCTGAGTGGCACAAGCAAATAA-TTAAAACTT 212
D1                ACCAAA-CTCTATTTTAACGACGTTTCTTCTGAGTGGCACAAGCAAATAA-TTAAAACTT 212
c23               ACCAAA-CTCTATTTTAACGACGTTTCTTCTGAGTGGCACAAGCAAATAA-TTAAAACTT 211
D6                ACCAAA-CTCTATTTTAACGACGTTTCTTCTGAGTGGCACAAGCAAATAA-TTAAAACTT 212
C5                ACCAAA-CTCTATTTTAACGACGTTTCTTCTGAGTGGCACAAGCAAATAA-TTAAAACTT 209
F17               -ACCAAACTCTATTTACACGACGTCTCTTCTGAGTGGCACAAACAAATAA-TTAAAACTT 218
F18               -ACCAAACTCTATTTACACGACGTCTCTTCTGAGTGGCACAAGCAAATAA-TTAAAACTT 218
c36               -ACCAAACTCTATTTACACGACGTCTCTTCTGAGTGGCACAAGCAAATAA-TTAAAACTT 219
C16               -ACCAAACTCTATTTACACGACGTCTCTTCTGAGTGGCACAAGCAAATAA-TTAAAACTT 219
D7                C--CAACTCTTATTTTAACGACGTCTCTTCTGAGTGGCACAAGCAAATAA-TCAAAACTT 200
E6                CACTAAACTCTATTGCAACGACGTCTCTTCTGAGTGGTACAAGCAAATAA-TCAAAACTT 210
C1                AACCAAACTCTGATTTAACGACGTTTCTTCTGAGTGGTACAAGCAAATAA-TCAAAACTT 209
F16               ACCTAACTCTTGACAACTGTATGGCCTCTCTGAGTAACTATACTTAATAAGTTAAAACTT 200
C29               --TCAAACTCTTGCGTAACTTTG--CAGTCTGAGTAAACTTAATTAATAAATTAAAACTT 192
                       *    *           *     *******      *   ***** * *******

D3                TTAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAA 275
G08               TTAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAA 224
D22               TTAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAA 275
D14               TTAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAA 279
C18               TTAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAA 279
D21               TTAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAA 279
E8                TTAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAA 279
E11               TTAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAA 279
C4                TTAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAA 279
C30               TTAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAA 279
G03               TTAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAA 279
F14               TTAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAA 279
c38               TTAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAA 279
C6                TTAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAA 279
D12               TTAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAA 279
C12               TTAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAA 279
D16               TTAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAA 283
E12               TTAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAA 283
F19               TTAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAA 282
C25               TTAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAA 283
E9                TTAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAA 283
F9                TTAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAA 283
G02_AY539806_     TTAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAA 282
C31               TTAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAA 279
C3                TTAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAA 282
C7                TTAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAA 282
C8                TTAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAA 282
C26               TTAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAA 282
C27               TTAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAA 282
E2                TTAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAA 282
E4                TTAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAA 282
E5                TTAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAA 282
F8                TTAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAA 282
F20               TTAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAA 282
F21               TTAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAA 282
C28               TTAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAA 282
C21               TTAACAATAGATCTCTTAGTTCTAGCATCAATAAAGAACGCAGCGAAATGCAATAAGTAA 282
G01_DQ195708_     TTAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAA 232
F7                TTAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAA 276
G09_DQ195690_     TTAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAA 217
G05_AJ301985_     TTAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAA 260
G06_AJ301976_     TTAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAA 240
C17               TTAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAA 269
c33               TTAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAA 269
C34               TTAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAA 269
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F2                TTAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAA 269
F3                TTAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAA 269
F1                TTAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAA 269
F4                TTAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAA 269
F5                TTAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAA 269
F6                TTAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAA 269
E7                TTAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAA 272
G07               TTAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAA 245
C24               TTAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAA 272
F11               TTAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAA 272
D13               TCAACAACGGATCTCTTGGTTCTGGCATCGATGAAAAACGCAGCGAAATGCGATAAGTAA 272
G04_AB233340_     TCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAA 270
C19               TCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAA 272
D1                TCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAA 272
c23               TCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAA 271
D6                TCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAA 272
C5                TCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAA 269
F17               TTAACAACGGATCTCTTGGTTCTGGCATCGATGAAAAACGCAGCGAAATGCGATAAGTAA 278
F18               TTAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAA 278
c36               TTAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAA 279
C16               TTAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAA 279
D7                TTAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAA 260
E6                TTAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAA 270
C1                TTAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAA 269
F16               TCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAA 260
C29               TTAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAA 252
                  * *****  ******** ***** ***** ** ** *************** ********

D3                TGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGCA 335
G08               TGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGCA 284
D22               TGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGCA 335
D14               TGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGCA 339
C18               TGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGCA 339
D21               TGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGCA 339
E8                TGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGCA 339
E11               TGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGCA 339
C4                TGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGCA 339
C30               TGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGCA 339
G03               TGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGCA 339
F14               TGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGCA 339
c38               TGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGCA 339
C6                TGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGCA 339
D12               TGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGCA 339
C12               TGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGCA 339
D16               TGTGAATTGCANAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGCA 343
E12               TGTGAATTGCANAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGCA 343
F19               TGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGCA 342
C25               TGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGCA 343
E9                TGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGCA 343
F9                TGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGCA 343
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C31               TGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGCA 339
C3                TGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGCA 342
C7                TGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGCA 342
C8                TGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGCA 342
C26               TGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGCA 342
C27               TGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGCA 342
E2                TGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGCA 342
E4                TGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGCA 342
E5                TGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGCA 342
F8                TGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGCA 342
F20               TGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGCA 342
F21               TGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGCA 342
C28               TGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGCA 342
C21               TGTGAATTGCAGAATTCAGTGAATTATTAAATCTTTAAACGCATATTACGCCTGCTAGCA 342
G01_DQ195708_     TGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGCA 292
F7                TGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGCA 336
G09_DQ195690_     TGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGCA 277
G05_AJ301985_     TGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGCA 320
G06_AJ301976_     TGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGCA 300
C17               TGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGCA 329
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c33                 TGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGCA 329
C34                 TGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGCA 329
F2                  TGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGCA 329
F3                  TGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGCA 329
F1                  TGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGCA 329
F4                  TGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGCA 329
F5                  TGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGCA 329
F6                  TGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGCA 329
E7                  TGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGCA 332
G07                 TGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGCA 305
C24                 TGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGCA 332
F11                 TGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGCA 332
D13                 TGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGCA 332
G04_AB233340_       TGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGCA 330
C19                 TGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGCA 332
D1                  TGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGCA 332
c23                 TGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGCA 331
D6                  TGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGCA 332
C5                  TGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGCA 329
F17                 TGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCTCGCCAGCA 338
F18                 TGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCTCGCCAGCA 338
c36                 TGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCTCGCCAGCA 339
C16                 TGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCTCGCCAGCA 339
D7                  TGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGCA 320
E6                  TGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGCA 330
C1                  TGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGCA 329
F16                 TGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGTA 320
C29                 TGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCCCTGGTA 312
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D3                  TTCTGGCGGGCATGCCTGTTCGAGCGTCATTTCAACCCTCAAGCTCT-GCTTGGTGTTGG 394
G08                 TTCTGGCGGGCATGCCTGTTCGAGCGTCATTTCAACCCTCAAGCTCT-GCTTGGTGTTGG 343
D22                 TTCTGGCGGGCATGCCTGTTCGAGCGTCATTTCAACCCTCAAGCTCT-GCTTGGTGTTGG 394
D14                 TTCTGGCGGGCATGCCTGTTCGAGCGTCATTTCAACCCTCAAGCCCG-GCTTGGTGTTGG 398
C18                 TTCTGGCGGGCATGCCTGTTCGAGCGTCATTTCAACCCTCAAGCCCG-GCTTGGTGTTGG 398
D21                 TTCTGGCGGGCATGCCTGTTCGAGCGTCATTTCAACCCTCAAGCCCG-GCTTGGTGTTGG 398
E8                  TTCTGGCGGGCATGCCTGTTCGAGCGTCATTTCAACCCTCAAGCCCG-GCTTGGTGTTGG 398
E11                 TTCTGGCGGGCATGCCTGTTCGAGCGTCATTTCAACCCTCAAGCCCG-GCTTGGTGTTGG 398
C4                  TTCTGGCGGGCATGCCTGTTCGAGCGTCATTTCAACCCTCAAGCCCG-GCTTGGTGTTGG 398
C30                 TTCTGGCGGGCATGCCTGTTCGAGCGTCATTTCAACCCTCAAGCCCG-GCTTGGTGTTGG 398
G03                 TTCTGGCGGGCATGCCTGTTCGAGCGTCATTTCAACCCTCAAGCCCG-GCTTGGTGTTGG 398
F14                 TTCTGGCGGGCATGCCTGTTCGAGCGTCATTTCAACCCTCAAGCCCG-GCTTGGTGTTGG 398
c38                 TTCTGGCGGGCATGCCTGTTCGAGCGTCATTTCAACCCTCAAGCCCG-GCTTGGTGTTGG 398
C6                  TTCTGGCGGGCATGCCTGTTCGAGCGTCATTTCAACCCTCAAGCCCG-GCTTGGTGTTGG 398
D12                 TTCTGGCGGGCATGCCTGTTCGAGCGTCATTTCAACCCTCAAGCCCG-GCTTGGTGTTGG 398
C12                 TTCTGGCGGGCATGCCTGTTCGAGCGTCATTTCAACCCTCAAGCCCG-GCTTGGTGTTGG 398
D16                 TTCTGGCGGGCATGCCTGTTCGAGCGTCATTTCAACCCTCAAGCCCG-GCTTGGTGTTGG 402
E12                 TTCTGGCGGGCATGCCTGTTCGAGCGTCATTTCAACCCTCAAGCCCG-GCTTGGTGTTGG 402
F19                 TTCTGGCGGGCATGCCTGTTCGAGCGTCATTTCAACCCTCAAGCCCG-GCTTGGTGTTGG 401
C25                 TTCTGGCGGGCATGCCTGTTCGAGCGTCATTTCAACCCTCAAGCCCG-GCTTGGTGTTGG 402
E9                  TTCTGGCGGGCATGCCTGTTCGAGCGTCATTTCAACCCTCAAGCCCG-GCTTGGTGTTGG 402
F9                  TTCTGGCGGGCATGCCTGTTCGAGCGTCATTTCAACCCTCAAGCCCG-GCTTGGTGTTGG 402
G02_AY539806_       TTCTGGCGGGCATGCCTGTTCGAGCGTCATTTCAACCCTCAAGCCCA-GCTTGGTGTTGG 401
C31                 TTCTGGCGGGCATGCCTGTTCGAGCGTCATTTCAACCCTCAAGCCCT-GCTTGGTGTTGG 398
C3                  TTCTGGCGGGCATGCCTGTTCGAGCGTCATTTCAACCCTCAAGCCCG-GCTTGGTGTTGG 401
C7                  TTCTGGCGGGCATGCCTGTTCGAGCGTCATTTCAACCCTCAAGCCCG-GCTTGGTGTTGG 401
C8                  TTCTGGCGGGCATGCCTGTTCGAGCGTCATTTCAACCCTCAAGCCCG-GCTTGGTGTTGG 401
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C27                 TTCTGGCGGGCATGCCTGTTCGAGCGTCATTTCAACCCTCAAGCCCG-GCTTGGTGTTGG 401
E2                  TTCTGGCGGGCATGCCTGTTCGAGCGTCATTTCAACCCTCAAGCCCG-GCTTGGTGTTGG 401
E4                  TTCTGGCGGGCATGCCTGTTCGAGCGTCATTTCAACCCTCAAGCCCG-GCTTGGTGTTGG 401
E5                  TTCTGGCGGGCATGCCTGTTCGAGCGTCATTTCAACCCTCAAGCCCG-GCTTGGTGTTGG 401
F8                  TTCTGGCGGGCATGCCTGTTCGAGCGTCATTTCAACCCTCAAGCCCG-GCTTGGTGTTGG 401
F20                 TTCTGGCGGGCATGCCTGTTCGAGCGTCATTTCAACCCTCAAGCCCG-GCTTGGTGTTGG 401
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C28                 TTCTGGCGGGCATGCCTGTTCGAGCGTCATTTCAACCCTCAAGCCCG-GCTTGGTGTTGG 401
C21                 TTCTAGCGGGTATGCCTGTTCAAGCGTTATTTTAACCCTTAAGCCTA-GCTTAGTGTTAG 401
G01_DQ195708_       TTCTGGCGGGCATGCCTGTTCGAGCGTCATTTCAACCCTCAAGCTTC-GCTTGGTGTTGG 351
F7                  TTCTGGCGGGCATGCCTGTTCGAGCGTCATTTCAACCCTCAAGCTCT-GCTTGGTGTTGG 395
G09_DQ195690_       TTCTGGCGGGCATGCCTGTTCGAGCGTCATTTCAACCCTCAAGCTCC-GCTTGGTGTTGG 336
G05_AJ301985_       TTCTGGCGGGCATGCCTGTTCGAGCGTCATTTCAACCCTCAAGCTCT-GCTTGGTGTTGG 379
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G06_AJ301976_      TTCTGGCGGGCATGCCTGTTCGAGCGTCATTTCAACCCTCAAGCTCT-GCTTGGTGTTGG 359
C17                TTCTGGCGGGCATGCCTGTTCGAGCGTCATTTCAACCCTCAAGCTCT-GCTTGGTGTTGG 388
c33                TTCTGGCGGGCATGCCTGTTCGAGCGTCATTTCAACCCTCAAGCTCT-GCTTGGTGTTGG 388
C34                TTCTGGCGGGCATGCCTGTTCGAGCGTCATTTCAACCCTCAAGCTCT-GCTTGGTGTTGG 388
F2                 TTCTGGCGGGCATGCCTGTTCGAGCGTCATTTCAACCCTCAAGCTCT-GCTTGGTGTTGG 388
F3                 TTCTGGCGGGCATGCCTGTTCGAGCGTCATTTCAACCCTCAAGCTCT-GCTTGGTGTTGG 388
F1                 TTCTGGCGGGCATGCCTGTTCGAGCGTCATTTCAACCCTCAAGCTCT-GCTTGGTGTTGG 388
F4                 TTCTGGCGGGCATGCCTGTTCGAGCGTCATTTCAACCCTCAAGCTCT-GCTTGGTGTTGG 388
F5                 TTCTGGCGGGCATGCCTGTTCGAGCGTCATTTCAACCCTCAAGCTCT-GCTTGGTGTTGG 388
F6                 TTCTGGCGGGCATGCCTGTTCGAGCGTCATTTCAACCCTCAAGCTCT-GCTTGGTGTTGG 388
E7                 TTCTGGCGGGCATGCCTGTTCGAGCGTCATTTCAACCCTCAAGCTCT-GCTTGGTGTTGG 391
G07                TTCTGGCGGGCATGCCTGYTCGAGCGTCATTTCAACCCTCAAGCTCT-GCTTGGTGTTGG 364
C24                TTCTGGCGGGCATGCCTGTTCGAGCGTCATTTCAACCCTCAAGCTCT-GCTTGGTGTTGG 391
F11                TTCTGGCGGGCATGCCTGTTCGAGCGTCATTTCAACCCTCAAGCTCT-GCTTGGTGTTGG 391
D13                TTCTGGCGGGTATGCCTGTTCGAGCGTCATTTCTACCCTCAAGCTCT-GCTTGGTGTTGG 391
G04_AB233340_      TTCTGGCGGGCATGCCTGTTCGAGCGTCATTTCAACCCTCAAGCTCT-GCTTGGTGTTGG 389
C19                TTCTGGCGGGCATGCCTGTTCGAGCGTCATTTCAACCCTCAAGCTCT-GCTTGGTGTTGG 391
D1                 TTCTGGCGGGCATGCCTGTTCGAGCGTCATTTCAACCCTCAAGCTCT-GCTTGGTGTTGG 391
c23                TTCTGGCGGGCATGCCTGTTCGAGCGTCATTTCAACCCTCAAGCTCT-GCTTGGTGTTGG 390
D6                 TTCTGGCGGGCATGCCTGTTCGAGCGTCATTTCAACCCTCAAGCTCT-GCTTGGTGTTGG 391
C5                 TTCTGGCGGGCATGCCTGTTCGAGCGTCATTTCAACCCTCAAGCTCT-GCTTGGTGTTGG 388
F17                TTCTGGCGAGCATGCCTGTTCGAGCGTCATTTCAACCCTCAAGCACC-GCTTGGTTTTGG 397
F18                TTCTGGCGAGCATGCCTGTTCGAGCGTCATTTCAACCCTCAAGCACC-GCTTGGTTTTGG 397
c36                TTCTGGCGAGCATGCCTGTTCGAGCGTCATTTCAACCCTCAAGCACC-GCTTGGTTTTGG 398
C16                TTCTGGCGAGCATGCCTGTTCGAGCGTCATTTCAACCCTCAAGCACC-GCTTGGTTTTGG 398
D7                 TTCTGGCGGGCATGCCTGTTCGAGCGTCATTTCAACCCTCAAGCACC-GCTTGGCGTTGG 379
E6                 TTCTGGCGGGCATGCCTGTTCGAGCGTCATTTCAACCCTCAAGCACC-GCTTGGCGTTGG 389
C1                 TTCTGGCGGGCATGCCTGTTCGAGCGTCATTTCAACCCTCAAGCTCT-GCTTGGTGTTGG 388
F16                TTCTGGCGGGCATGCCTGTTCGAGCGTCATTTCAACCATCAAGCCCCAGGCTTGTGTTGG 380
C29                TTCCGGGGGGCATGCCTGTTCGAGCGTCATTTCACCACTCAAGCCTC-GCTTGGTATTGG 371
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D3                 GGCT-CTACGGTTGA-CGTAGGCCCTTAAAGGTAGTGGCGGACCC-TCTCGGAGCCTCCT 451
G08                GGCT-CTACGGTTGA-CGTAGGCCCTTAAAGGTAGTGGCGGACCC-TCTCGGAGCCTCCT 400
D22                GGCT-CTACGGTTGA-CGTAGGCCCTTAAAGGTAGTGGCGGACC--TCTCGGAGCCTCCT 450
D14                GGCC-CTACGGTAGA-CGTAGGCCCTTAAAGGTAGTGGCGGACCC-TCCCGGAGCCTCCT 455
C18                GGCC-CTACGGTAGA-CGTAGGCCCTTAAAGGTAGTGGCGGACCC-TCCCGGAGCCTCCT 455
D21                GGCC-CTACGGTAGA-CGTAGGCCCTTAAAGGTAGTGGCGGACCC-TCCCGGAGCCTCCT 455
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E11                GGCC-CTACGGTAGA-CGTAGGCCCTTAAAGGTAGTGGCGGACCC-TCCCGGAGCCTCCT 455
C4                 GGCC-CTACGGTAGA-CGTAGGCCCTTAAAGGTAGTGGCGGACCC-TCCCGGAGCCTCCT 455
C30                GGCC-CTACGGTAGA-CGTAGGCCCTTAAAGGTAGTGGCGGACCC-TCCCGGAGCCTCCT 455
G03                GGCC-CTACGGTAGA-CGTAGGCCCTTAAAGGTAGTGGCGGACCC-TCCCGGAGCCTCCT 455
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D12                GGCC-CTACGGTAGA-CGTAGGCCCTTAAAGGTAGTGGCGGACCC-TCCCGGAGCCTCCT 455
C12                GGCC-CTACGGTAGA-CGTAGGCCCTTAAAGGTAGTGGCGGACCC-TCCCGGAGCCTCCT 455
D16                GGCC-CTACGGTCGA-CGTAGGCCCTTAAAGGTAGTGGCGGACCC-TCCCGGAGCCTCCT 459
E12                GGCC-CTACGGTCGA-CGTAGGCCCTTAAAGGTAGTGGCGGACCC-TCCCGGAGCCTCCT 459
F19                GGCC-CTACGGTCGA-CGTAGGCCCTTAAAGGTAGTGGCGGACCC-TCCCGGAGCCTCCT 458
C25                GGCC-CTACGGTCGA-CGTAGGCCCTTAAAGGTAGTGGCGGACCC-TCCCGGAGCCTCCT 459
E9                 GGCC-CTACGGTCGA-CGTAGGCCCTTAAAGGTAGTGGCGGACCC-TCCCGGAGCCTCCT 459
F9                 GGCC-CTACGGTCGA-CGTAGGCCCTTAAAGGTAGTGGCGGACCC-TCCCGGAGCCTCCT 459
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C31                GGCC-CTACGGTCGA-CGTAGGCCCTTAAAGGTAGTGGCGGACCC-TCCCGGAGCCTCCT 455
C3                 GGCC-CCACGGTCGA-CGTGGGCCCTTAAAGGTAGTGGCGGACCC-TCCCGGAGCCTCCT 458
C7                 GGCC-CCACGGTCGA-CGTGGGCCCTTAAAGGTAGTGGCGGACCC-TCCCGGAGCCTCCT 458
C8                 GGCC-CCACGGTCGA-CGTGGGCCCTTAAAGGTAGTGGCGGACCC-TCCCGGAGCCTCCT 458
C26                GGCC-CCACGGTCGA-CGTGGGCCCTTAAAGGTAGTGGCGGACCC-TCCCGGAGCCTCCT 458
C27                GGCC-CCACGGTCGA-CGTGGGCCCTTAAAGGTAGTGGCGGACCC-TCCCGGAGCCTCCT 458
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E4                 GGCC-CCACGGTCGA-CGTGGGCCCTTAAAGGTAGTGGCGGACCC-TCCCGGAGCCTCCT 458
E5                 GGCC-CCACGGTCGA-CGTGGGCCCTTAAAGGTAGTGGCGGACCC-TCCCGGAGCCTCCT 458
F8                 GGCC-CCACGGTCGA-CGTGGGCCCTTAAAGGTAGTGGCGGACCC-TCCCGGAGCCTCCT 458
F20                GGCC-CCACGGTCGA-CGTGGGCCCTTAAAGGTAGTGGCGGACCC-TCCCGGAGCCTCCT 458
F21                GGCC-CCACGGTCGA-CGTGGGCCCTTAAAGGTAGTGGCGGACCC-TCCCGGAGCCTCCT 458
C28                GGCC-CCACGGTCGA-CGTGGGCCCTTAAAGGTAGTGGCGGACCC-TCCCGGAGCCTCCT 458
C21                GGCC-TTACAGTTAA-CGTAGGCCCTTAAAGGTAGTGGCAGACCC-TCCTAGAGCCTCCT 458
G01_DQ195708_      GGCA-CTACGGTTGA-CGTAGGCCCTCAAAAGTAGTGGCGGACCC-TCCCGGAGCCTCCT 408
F7                 GGCC-CTACGGTTGA-CGTAGGCCCTCAAAGGTAGTGGCGGACCC-TCCCGGAGCCTCCT 452
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G09_DQ195690_      GGCC-CTACGGTTGA-CGTAGGCCCTTAAAGGTAGTGGCGGACCC-TCCCGGAGCCTCCT 393
G05_AJ301985_      GGCC-CTACGGTTAAACGTAGGCCCTTAAAGGTAGTGGCGGACCC-TCTCGGAGCCTCCT 437
G06_AJ301976_      GGCC-CTACGGTTAAACGTAGGCCCTTAAAGGTAGTGGCGGACCC-TCTCGGAGCCTCCT 417
C17                GGCC-CTACGGTTAAACGTAGGCCCTTAAAGGTAGTGGCGGACCC-TCTCGGAGCCTCCT 446
c33                GGCC-CTACGGTTAAACGTAGGCCCTTAAAGGTAGTGGCGGACCC-TCTCGGAGCCTCCT 446
C34                GGCC-CTACGGTTAAACGTAGGCCCTTAAAGGTAGTGGCGGACCC-TCTCGGAGCCTCCT 446
F2                 GGCC-CTACGGTTAAACGTAGGCCCTTAAAGGTAGTGGCGGACCC-TCTCGGAGCCTCCT 446
F3                 GGCC-CTACGGTTAAACGTAGGCCCTTAAAGGTAGTGGCGGACCC-TCTCGGAGCCTCCT 446
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G07                GGCC-CTACGGTTGA-CGTAGGCCCTTAAAGGTAGTGGCGGACCC-TCTCGGAGCCTCCT 421
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F17                GGCC-CCACGGCACA-CGTGGGCCCTTGAAGGTAGTGGCGGACCC-TCCCGGAGCCTCCT 454
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D7                 GGCTTCCACGGCTGA-CGTGGGCCCCCAAAGACAGTGGCGGACCC-TCGCGGAGCCTCCT 437
E6                 GGCC-CTACGGCTTC-CGTAGGCCCCGAAATACAGTGGCGGACCC-TCCCGGAGCCTCCT 446
C1                 GGCC-CTACAGCTGA-TGTAGGCCCTCAAAGGTAGTGGCGGACCC-TCCCGGAGCCTCCT 445
F16                GGAC-CTGCGGCTGC-CGCAGGCCCTGAAAAACAGTGGCGGGCTC---GCTAGTCACACC 435
C29                GCAA--CGCGGTCCGCCGCGTGCCTCAAATCGTCCGGCTGGGTCT-----TCTGTCCCCT 424
                   *       *        *   ***    *       *   *               *

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D22                TTGCGTAGTAACATTTCGT-CTCGCATTGGGATTCGGAGGG-ACTCTAGCCGTAAAACCC 508
D14                TTGCGTAGTAACTTAACGT-CTCGCACTGGGATCCGGAGGG-ACTCTTGCCGTAAAACCC 513
C18                TTGCGTAGTAACTTAACGT-CTCGCACTGGGATCCGGAGGG-ACTCTTGCCGTAAAACCC 513
D21                TTGCGTAGTAACTTAACGT-CTCGCACTGGGATCCGGAGGG-ACTCTTGCCGTAAAACCC 513
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F1                  TTGCGTAGTAACT-AACGT-CTCGCACTGGGATTCGGAGGG-ATTCTAGCCGTAAAACCC 503
F4                  TTGCGTAGTAACT-AACGT-CTCGCACTGGGATTCGGAGGG-ATTCTAGCCGTAAAACCC 503
F5                  TTGCGTAGTAACT-AACGT-CTCGCACTGGGATTCGGAGGG-ATTCTAGCCGTAAAACCC 503
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G07                 TTGCGTAGTAAC---------------------------------------------- 433
C24                 TTGCGTAGTAACT-AACGT-CTCGCATTGGGATTCGGAGGG-ACTCTTGCCGTAAAACCC 505
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E6                  TTGCGTAGTAACATACCAC-CTCGCA------------------------------------ 471
C1                  TTGCGTAGTAACTTTACGT-CTCGCACTGGGATCCGGAGGG-ACTCTTGCCGTAAAACCC 503
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C29                 AAGCGTTGTGGAA-ACTAT--TCGCTAAAGGGTGTTCGGGAGGCTAC-GCCGTAAAACAA 480
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G08                 CC------------------------------------------------------ 460
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F8                  CCCAAACTTTTACCAGGTTGACCTCGGATCAGGTAGGAATACCCGC-TGAACTTAA- 571
F20                 CCCAAACTTTTACCAGGTTGACCTCGGATCAGGTAGGAATACCCGC-TGAACTTAA- 571
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F7                  CCCAATTTTTCAA-TGGTTGACCTCGGATCAGGTAGGAATACCCGC-TGAACTTAA- 563
G09_DQ195690_       CC---------------------------------------------------------- 452
G05_AJ301985_       CCAATTTTT--TA-AGGTT------------------------------------- 510
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G07                 ---------------------------------------------------------- 
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D13                 CCAAATTTT-TAA-AGGTTGACCTCGGATCAGGTAGGAATACCCGC-TGAACTTAA- 560
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E6                  ---------------------------------------------------------- 
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F16                 CCCACTATATCAA--GGTTGACCTCGGATCAGGTAGGAAGACCCGC-TGAACTTAA- 545
C29                 CCCCATTTCTAAG---GTTGACCTCGGATCAGGTAGGGATACCCGC-TGAACTTAA- 532
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[a] Highlighted areas are specific sequence sections that differentiate *C. truncatum* isolates from other hosts. Isolates highlighted in yellow, light blue, purple and grey colors are from lentil; scentless chamomile, soybean and pea, respectively. Based on these sequences, specific primers were designed to differentiate host specific isolates.

APPENDIX B: MEDIA AND BUFFER RECIPES

V8 agar recipe

200 ml V8 juice
800 ml distilled water
4.0 g $CaCO_3$
Mixed and autoclave at 121ºC for 25-30 minutes.

Oatmeal agar recipe

15 g oat meal flour
10 g agar
1L distilled water
Mixed and autoclave at 121ºC for 20-25 minutes.

Cryofreezer Solution

Solution 1
10 g skim milk power
100 ml distilled water
Solution 2
40 ml glycerol
60 ml distilled water

Autoclave solution 1 and solution 2 in separate containers at 121ºC for 20-25 minutes. Remove immediately from the autoclave. Mix solution 2 into solution 1. Mix well and store at 4ºC.

Genomic DNA Extraction Buffer

500 ml 2% CTAB buffer:
Hexadecyltrimethyl ammonium bromide (CTAB), 10 g (Sigma H-5882)
100 mM Tris, 6.06 g
10 mM EDTA, 1.46 g
0.7 M NaCl, 20.5 g
Mix up to 500 ml with ddH_2O and store at room temperature.

Plant Tissue Fixing Buffer

Methanol: chloroform: acetate acid at 60:30:10
Mix well and store at room temperature.