INVESTIGATION INTO THE MATING SYSTEM AND POPULATION STRUCTURE OF *COLLETOTRICHUM TRUNCATUM* FROM LENTIL

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ABSTRACT

Colletotrichum truncatum is the pathogenic agent of lentil anthracnose, a disease responsible for severe yield losses in Western Canada. This ascomycetous fungus is believed to reproduce asexually under field conditions, but the sexual stage, *Glomerella truncata*, has been obtained under laboratory conditions. Preliminary studies on a limited number of isolates suggested that *C. truncatum* exhibits the typical bipolar mating system of heterothallic ascomycetous fungi; however, this result had to be confirmed on a larger number of isolates, especially considering that all other *Colletotrichum* species studied to date do not seem to conform to this system. The aim of this study was to increase understanding of the mating system of *C. truncatum*. Based on preliminary information, it was hypothesized: 1) that *C. truncatum* is a heterothallic fungus with a unilocus, biallelic mating system; and 2) that sexual reproduction occurs in the field. These hypotheses were tested by i) performing classical mating studies to confirm heterothallism and bipolarity of *C. truncatum*; ii) analyzing the genetic basis for cross fertility with the use of molecular markers; iii) probing for the presence of genes responsible for mating types; iv) determining if different mating types coexist on a small geographical scale; and v) assessing the genetic diversity of field isolates of *C. truncatum* by conducting a molecular population study, and determine the extent of linkage disequilibrium. Twenty-one isolates were crossed in all possible combinations on lentil material. When each isolate was used alone, no sexual structures were detected. However, when two different isolates were put in contact on lentil stems, perithecia were produced in 22% of the cases. The 21 field isolates fell into two mating incompatibility groups (IG), supporting the bipolar nature of the mating system of *C. truncatum*. Molecular markers differing between two parental isolates were found in their progeny, confirming heterothallism. The results are consistent with the typical mating system of ascomycete, in which mating types are controlled by the mating type (MAT) locus with two alleles, MAT1-1 and MAT1-2, each controlling a mating type. However, in *C. truncatum*, degenerate primers targeting conserved regions of MAT1-2 revealed that all isolates tested carried a copy of MAT1-2, independently of their mating incompatibility group. This is not consistent with the typical mating system of heterothallic ascomycetes, suggesting that, as proposed for other *Colletotrichum* species, the mating system of *C. truncatum* is likely controlled at least partially by another system. A molecular population study undertaken on *C. truncatum*

isolates from different Saskatchewan locations and crop years showed that genetic diversity was low and suggested a high level of clonality. However, a moderate level of linkage disequilibrium existed in the population, indicating the possibility of at least some level of sexual reproduction. This study also showed that isolates of different mating types were spatially coexisting in the same field, sometimes on the same plant, suggesting that geographical isolation is probably not a factor restricting sexual reproduction.

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FAO	Food and Agriculture Organization of the United Nations
AFLP	Amplified Fragment Length Polymorphism
ITS	Internal transcribed spacer
PFGE	Pulsed-field gel electrophoresis
RAPD	Random Amplified Polymorphic DNA
CDC	Crop Development Centre
HMG	High mobility group
CP	Clumped-perithecial
SP	Scattered-perithecial
CC	Clumped-conidial
RFLP	Restriction fragment length polymorphism
OMA	Oatmeal-agar medium
IG	Incompatibility group
AAFC	Agriculture and Agri-food Canada
PDA	Potato-dextrose-agar medium
UPGMA	Unweighted pair-group method with arithmetical averages

CHAPTER 1

INTRODUCTION

The cultivated lentil, *Lens culinaris* subsp. *culinaris* L., is an important pulse crop in Western Canada. Nearly all (99%) Canadian lentils are grown in Saskatchewan, where production began in 1969, with only 600 ha grown. Forty years later, the area has increased to 963,200 ha harvested in 2009 (FAO, 2011). Canada is currently the world's leading lentil exporter, and the main producer of green lentils (Anonymous, 2010).

Fungal diseases are the major biological constraint to lentil seed productivity (Taylor *et al.*, 2007). Lentil anthracnose, caused by *Colletotrichum truncatum* (Schwein) Andrus and W.D. Moore, is one of the most severe diseases of lentil on the Prairies. This disease was first identified in Manitoba in 1987 (Morrall, 1988), and reached Saskatchewan three years later (Morrall and Pedersen, 1991). In the late 1980s, a study around Portage la Prairie in Manitoba gave a first estimate of yield losses ranging from 40 to 60% due to anthracnose (Morrall *et al.*, 1990). In 1990, seed yield reductions of up to 70% were estimated in Saskatchewan (Morrall and Pedersen, 1991) and the disease still causes significant losses nowadays. Control can be achieved by using resistant cultivars, applying fungicides and maintaining crop rotations of 4 years (Chongo and Bernier, 2000). The use of resistant cultivars is preferred for both environmental and economic reasons but is complicated in the species by the existence of two pathogenic races, Ct0 and Ct1, which have different levels of aggressiveness (Buchwaldt *et al.*, 2004). Resistance to race Ct1 has been identified in cultivated lentil (Buchwaldt *et al.*, 2004) and has been used to develop partially resistant cultivars. Sources of partial resistance to the more aggressive race Ct0 are now also available (Tullu *et al.*, 2006).

Lentil anthracnose is induced by the anamorphic (asexual) stage of the pathogen. The teleomorphic stage (*Glomerella truncata* Armstrong-Cho and Banniza, 2006) can be induced under laboratory conditions (Armstrong-Cho and Banniza, 2006) but has not been observed under natural conditions, and the mating system remains largely unknown. Mating studies in other *Colletotrichum* species are scarce, but those available suggest that this genus displays atypical mating system(s) (Vaillancourt *et al.*, 2000a).

Typical heterothallic ascomycetes have a bipolar mating system, with mating types

determined by a mating type locus with at least 2 idiomorphs, i.e. alleles with no sequence identity (Turgeon *et al.* 1993), called MAT1-1 and MAT1-2. Compatibility is heterogenic, so partners of a fertile cross carry different idiomorphs at the MAT locus. The presence of MAT genes in *Colletotrichum* has been investigated in a few species, but associated with classical mating studies in only two species, *C. graminicola* (Vaillancourt *et al.*, 2000b) and *C. lindemuthianum* (Rodriguez-Guerra *et al.,* 2005). In both species, a typical MAT1-2 idiomorph was found in all isolates, independently of their mating compatibility, which contradicts the typical heterogenic compatibility system of ascomycetous fungi. This suggests that another system plays a part in mating compatibility in those species.

To date, it is unknown if sexual reproduction of *C. truncatum* plays a role in the disease under natural conditions. The fact that the teleomorph has not been observed under natural conditions does not indicate that sexual reproduction does not occur. Some fungal species are asexual most of the time, but occasionally undergo a sexual phase (Burnett, 2003). In this case, detection of sexual structures in the field can be difficult. When direct evidence of sexual reproduction is not available, indirect evidence may be deducted from population structure studies. Understanding the mating system of the fungus and knowing if matings can occur in natural conditions is important because sexual reproduction results in genetic recombination that could, in the case of *C. truncatum*, lead to the appearance of new races other than races Ct0 and Ct1. Those races could potentially be able to infect lentil that carry resistance against the two currently known races. Development of new races by recombination in the progeny has been reported under laboratory conditions for *C. lindemuthianum* (Bastia and Chaves, 1982).
The focus of this study was to increase understanding of the mating system of *C. truncatum*. Based on the mating system typically described in ascomycetes, as well as the information obtained on a limited number of laboratory crossings between *C. truncatum* isolates from lentil (Armstrong-Cho and Banniza, 2006), it was hypothesized that *C. truncatum* is a heterothallic fungus with a unilocus, biallelic mating system. Additionally, because the teleomorph *G. truncata* was obtained under laboratory conditions, it was hypothesized that sexual reproduction may occur in the field. This thesis describes work conducted to 1) determine the nature of the mating system of *C. truncatum* by performing crosses between a range of selected isolates, and explore the inheritance of the mating types by performing crosses and backcrosses and using molecular markers; 2) determine if sexually compatible isolates cohabit on a small scale in the

field; and 3) conduct a molecular population study with AFLP markers to determine whether outbreeding occurs in the field.

CHAPTER 2

LITERATURE REVIEW

2.1. Lentil anthracnose

2.1.1. History of lentil anthracnose in Canada

The first symptoms of a new lentil disease were reported in 1986 and 1987 on a farm in southern Manitoba. Local growers had been noticing a new disease problem for several years, but there was initially some confusion over the nature of the disease, as the 1980s also saw the establishment of another major lentil disease, ascochyta blight due to *Ascochyta lentis* (Morrall, 1997). In 1986, samples of diseased seeds from the infected crop were subjected to an agar plate test that showed very low levels of *Ascochyta lentis* infection, but higher occurrence of an orange-coloured fungus resembling *Colletotrichum*. Tissue samples from the same farm in 1987 showed symptoms similar to those of lentils seedlings artificially inoculated with the *Colletotrichum* isolated the previous year (Morrall, 1997). The pathogen was identified by several sources as *C. truncatum* and described as a new lentil disease (Morrall, 1988, 1997). A survey in southern Manitoba in 1988 showed that 18 fields out of 65 sampled were infected, albeit mostly at low levels (Morrall *et al*., 1989). The disease was discovered in Saskatchewan in July 1990 near Zealandia (Morrall and Pederson, 1991), and a subsequent survey during the same year lead to the detection of anthracnose in several parts of Saskatchewan, suggesting that the disease had probably been present for several years (Morrall and Pederson, 1991). In the following years, the disease spread throughout the whole lentil growing zone in Canada, and was detected for the first time in the USA in 1992, in an area of North Dakota within 220km of the Manitoba border (Venette *et al*., 1994).

Before the Canadian epidemic, little was known about *Colletotrichum* as a lentil pathogen, although it was not totally unheard of. Anthracnose was reported on lentil in Ethiopia, Syria and Brazil (Stewart and Yirgou, 1967; Bellar and Kebabeh, 1983; Baldanzi *et al.*, 1988 *in* Buchwaldt *et al.*, 2004). Interestingly, the isolate from Ethiopia was described as *C. destructivum*. Morrall (1997) observed severe anthracnose on lentil in Pakistan in 1990, just as

the epidemic was unfurling in Canada. He reported that the local pathologists knew the disease existed but that the exact species responsible for it was not identified. A few years later, lentil anthracnose was discovered in Europe (Bulgaria) and the pathogenic agent was described as *C. truncatum* (Kaiser *et al*., 1998).

2.1.2. Systematics

Colletotrichum comprises a variety of plant pathogens especially, but not exclusively, of tropical regions (Sutton, 1992) that cause diseases known as anthracnoses on a wide range of crop plants and weeds (Bailey and Jeger, 1992). *Colletotrichum* is the name of the anamorphic state. The teleomorphic state, known only for a few *Colletotrichum* spp. and rarely seen in nature (Sutton, 1992), is classified in the genus *Glomerella*, class Sordariomycetes, subclass Hypocreomycetida**.** The genus *Glomerella* belongs to the phylum Ascomycota, which is characterized by the production of sexual spores, called ascospores, inside an ascus. In most cases, as in the *Glomerella* genus, the number of ascospores per ascus is eight, indicating the occurrence of a single mitosis after meiosis (Worrall, 1999).

2.1.2.1. The genus *Colletotrichum*: a taxonomic imbroglio

The genus *Colletotrichum* is characterized by significant taxonomic uncertainty. Traditional morphological features often fail to elucidate relationships within and amongst taxa (Correll and Gordon, 1999). The concept of species in *Colletotrichum* lacks definition firstly because the genus shows little variation in morphological features and secondly because it encompasses pathogens that are morphologically similar, but show different host specificity (Sutton, 1992). Historically, a fungus discovered on a new host was often interpreted as a new species, even if a similar fungus was known on another host. As a consequence, there are approximatively 900 descriptions of *Colletotrichum* species (Sutton, 1992). This led some later authors to separate *Colletotrichum* not only into species, but sometimes into less defined taxons such as ‘group species’ or ‘species aggregates’. Von Arx (1957) accomplished a significant reduction in the number of accepted species to 11 by giving a very broad definition to some of the species. This simplification was necessary, but was too drastic, and probably inaccurate because the author did not have access to all species and was partially working from descriptions, which limited the number of morphological characters available for classification. Sutton (1980, 1992) proposed 39 species, some divided into formae speciales. This list of species

is often considered as the starting point for modern taxonomical studies (eg. Hyde *et al*., 2009), but numerous low rank relationships are still controversial.

In order to solve this problem, various methods have been used to complement morphological and pathogenicity studies. They encompass physiological and phylogenetic studies, as well as the study of secondary metabolites (Cai *et al.,* 2009). An additional problem is that the type specimens, some of which are dating back from the 19th century, are often missing, or are too degraded to be used for molecular studies (Cai *et al.*, 2009). Consequently, even with the help of modern taxonomical methods, species misidentification is a common and lingering problem because there is no proper epitype to compare unknown isolates to. Additionally, some molecular methods commonly used to elucidate relationship between species in fungi were shown to be insufficient in *Colletotrichum* if not coupled with other methods. The noteworthy example is the rDNA internal transcribed spacer (ITS) sequences that were widely used for over ten years to characterize *Colletotrichum* species. Crouch *et al*. (2009) showed in a study of 14 *Colletotrichum* species with falcate-spores that the use of ITS data alone was inadequate and suggested that as much as 86% of the ITS sequences deposited on GenBank may be misidentified. Multilocus molecular phylogenetic analysis (Damm *et al*., 2009) and polyphasic approaches using genetic distinctness as well morphology and biology data (Cai *et al*., 2009) will hopefully help resolving some of the relationships within *Colletotrichum*.

2.1.2.2. The nature and classification of *Colletotrichum* causing anthracnose on lentil

Colletotrichum truncatum was first described under this name in 1935 as a pathogen of *Phaseolus lunatus* (Lima bean) and anecdotally of the common bean, *P. vulgaris* (Andrus and Moore, 1935). At this early date, this fungus had already been described under several different names: *Vermicularia truncata*, *V. polytricha*, *C. caulivorum* and *C. glycines.* (Tiffany and Gilman, 1954). *Colletotrichum viciae*, described on *Vicia villosa*, was shown to be a synonym of *C. truncatum* on the basis of a culture slide (compiled in Tiffany and Gilman, 1954). The species was then downgraded to the rank of *forma specialis* of *C. dematium* (von Arx, 1957 *in* Sutton, 1992), but was later considered a distinct species (Sutton, 1992).

Colonies are usually salmon or mouse grey, with conidia formed in orange masses (Fig. 2.1). Morphological features traditionally used to distinguish *C. truncatum* from other *Colletotrichum* species encompasse the size of the conidia (12-16 µm of length, 4-6 µm of width), their slightly falcate shape, (Sutton, 1992), the size (8-11 x 5-9 µm) and form (ovate to

clavate) of appressoria, as well as the production of sterile scleriotia (Khan and Sainclair, 1992).

The species causing anthracnose on lentil in Canada was determined to be *C. truncatum* before molecular phylogenetic methods of species determination were available. Recently, it has been suggested that the *Colletotrichum* species responsible for lentil anthracnose is not *C. truncatum*. A multilocus molecular phylogenetic analysis showed that isolates of *C. truncatum* from Lima bean, common bean, soybean (*Glycine max*), alfalfa (*Medicago sativa*) and other species formed a well-defined clade (Damm *et al*., 2009). In contrast, isolates from lentil, pea (*Pisum sativum*) and faba bean (*Vicia faba*) cluster tightly with isolates of *C. destructivum* on the basis of rDNA data, and show close similarity to that species in term of morphology and infection strategy (Latunde-Dada and Lucas, 2007). These results are consistent with a host specificity study that showed that isolates of *C. truncatum* from lentil were pathogenic to lentil, faba bean and field pea, but not to common bean, lupine and alfalfa (Gossen *et al.*, 2009). On the basis of this new information, isolates from lentil, as well as these from pea and faba bean, would belong to the *C. destructivum* aggregate, and not be related to the species *C. truncatum* as described by Andrus and Moore (1935) on Lima bean (Damm *et al.*, 2009). Interestingly, in the study by Latunde-Dada and Lucas (2007), *C. higginsianum* clusters also relatively closely to *C. destructivum*. Sun and Zhang (2009) proposed that *C. higginsianum* is a synonym of *C. destructivum.*

Considering that recent studies strongly suggest that the causal agent of anthracnose on lentil is not *C. truncatum*, the exact species is still unknown. In this thesis, the name *C. truncatum* will be used in the broad sense and referred to as *C. truncatum senso lato*, including isolates from lentil, pea and faba bean, but precision on the host will be given whenever necessary. The teleomorphic stage of *C. truncatum* isolated from lentil, *G. truncata*, produces perithecia usually obpyriform, brown-black in colour, with average dimensions of 350 x 200 μm. Asci contain eight ascospores in line (Armstrong-Cho and Banniza, 2006). Perithecia develop on top of the surface of substratum, solitary or arranged in small groups (Fig. 2.1).

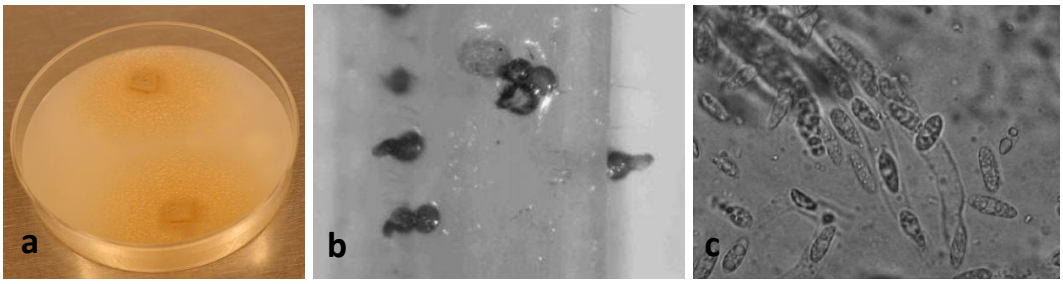

Figure 2.1: Typical culture of *Colletotrichum truncatum* isolated from lentil material (CT-28) growing on oatmeal agar medium (a); *C. truncatum* perithecia (b) and ascospores (c). *b and c from Armstrong-Cho and Banniza, 2006.*

Two pathogenic races of *C. truncatum* from lentil, Ct0 and Ct1, were identified based on the reaction of 7 differential lines inoculated with 50 isolates (Buchwaldt *et al*., 1999, 2004). These races do not show differences in host range (Anderson, 2003). They are present at similar frequencies in fields planted with susceptible cultivars (Buchwaldt *et al.*, 2004).

2.1.3. Cytogenetics of *Colletotrichum*

The genus *Colletotrichum* comprises species that are haploid during the asexual phase, which is the prominent phase of their life cycle. Diploidy is restricted to the sexual phase (Skipp *et al*., 1995).

Several limitations hamper the cytogenetic studies in *Colletotrichum.* As most species are either not sexual at all, or rarely so, microscopy-based cytogenetic studies, usually undertaken during meiosis when the chromosomes are best visible, are mostly not feasible. Additionally, the size of the chromosomes in this genus is distributed within a wide range, and no single technique has a resolution adequate to detect the smallest as well as the largest of them. Several techniques have been used alone or combined to determine the number and size of chromosomes in several *Colletotrichum* species. They encompass microscopic observation, pulsed-field gel electrophoresis (PFGE) (Mills and Cluskey 1990), flow cytometry (O'Sullivan *et al.*, 1998) and telomere fingerprinting (O'Sullivan *et al.*, 1998).

Two classes of chromosomes are described in *Colletotrichum*. Macro chromosomes are those over 7Mb in size, while chromosomes of a size smaller than 1.2Mb are designated as minichromosomes (Masel *et al.*, 1990). The haploid number of macro chromosomes of *G. cingulata* f. sp *phaseoli* (= *G. lindemuthiana*) is 4 (Roca *et al*., 2003), and apparently no

minichromosomes are present, but in the anamorph *C. lindemuthianum*, the total number of chromosomes varies between 4 and 16, with numerous very small ones (Rocas *et al*., 2003). Minichromosomes were observed in several species of *Colletotrichum*, including *C. truncatum* isolated from *Medicago sativa*, and were shown on several occasions, but not always, to be linked to pathogenicity (Masel *et al.*, 1990, 1993). In a study on isolates of *C. gloeosporioides* from *Stylosanthes*, karyotypes were shown to be very variable, especially with regard to varying numbers of minichromosomes. Isolates belonging to biotype A, which are generalist pathogens attacking most species of *Stylosanthes*, were shown to carry 8 to 10 minichromosomes, whereas isolates of biotype B, specialists of *S. guianensis,* were containing only 2 to 5 of them (Masel *et al.*, 1990). However, in a study published in 1993, Masel *et al*. showed that isolates of different collection of *C. gloeosporioides* from *Stylosanthes* could be classified into two groups based on the number of minichromosomes, but that these groups were not correlated to biotypes, host species or origin. In the same study, the authors showed that an isolate of *C. truncatum* isolated from *Medicago sativa* in Australia was carrying 5 minichromosomes ranging from 0.35 to 1Mb.

The genomes of two *Colletotrichum* species have recently been sequenced. The size of the genome of isolate M1001 of *C. graminicola* was determined to be 51.6Mb (*Colletotrichum* Sequencing Project, Broad Institute of Harvard and MIT (http://www.broadinstitute.org/)) and the genome of isolate IMI 349063 of *C. higginsianum* was established to be 49.08Mb (*Colletotrichum higginsianum* Genome Project, Max Planck Institute (http://www.mpiz-koeln.mpg.de)).

2.1.4. Life cycle of *Colletotrichum truncatum*

2.1.4.1. Inoculum

Buchwaldt *et al.* (1996) described the existence of several sources of inoculum. Acervuli form on diseased tissues on the plant, as well as on fallen abscised leaflets in rainy conditions. Conidia are rain-dispersed by being splashed both to other parts of the diseased plant and to surrounding plants. However, wind-dispersal also occurs through dispersion of infected lentil debris generated during harvest. Dispersal of stubble during harvest could reach 240 meters from the source with a wind above 9km/h. Winds above 39km/h were also shown to be able to transport lentil debris as pod walls, stem segment, as well as soil. This wind dispersion is thought to have had a role in the spread of anthracnose in Western Canada (Buchwaldt *et al.*, 1996).

Lentil anthracnose is not considered likely to be seedborne, as seeds from severely infected lentil plants were shown to be infected in only 2% of the cases, and the transmission of the pathogen from seed to seedling is almost non-existent (Gibson, 1994).

2.1.4.2. Infection process

Histological studies showed that in lentil inoculated with isolates of *C. truncatum* isolated from lentil, host invasion is occurring through penetration with the help of appressoria. Conidia germinated between 3 and 6 hours postinoculation (hpi) and appressoria were formed between 6 and 12hpi (Chongo *et al.*, 2002). Infection vesicles appear in epidermal cells underneath the penetration sites at 20hpi and develop into large intracellular primary hyphae only within the initially infected cells (Armstrong-Cho *et al*., 2012). The primary hyphae are either unbranched or display two or more lobes. During this symptomless phase that lasts 52 to 56 hours, the fungus displays a biotrophic behaviour. It is followed by the production of secondary hyphae that invade surrounding tissue signalling the start of a destructive phase where the fungus acts as a necrotrophic organism. (Armstrong-Cho *et al*., 2012). This hemibiotrophy is a characteristic feature of many *Colletotrichum* species (Luttrell, 1974). Two different infection strategies were described for *Colletotrichum* species. In the first step, common to both strategies, the fungus penetrates into the host directly or by utilizing melanised appressoria. The second step differentiates two strategies: In intracellular hemitrophic infection, large primary hyphae are produced within epidermal cells, either only in the cell where penetration first occurred, or also in surrounding cells. A biotrophic phase exists for a varying period. In the second type of infection strategy, subcuticular intramural infection, the hyphae initially spread below the cuticle and around the host epidermal cell walls, which leads to the disintegration of the cells walls. The biotrophic phase is almost absent (reviewed in Bailey *et al.,* 1992). *C. truncatum* of lentil belongs to the first category, with a significant biotrophic period (Armstrong-Cho *et al*., 2012).

Bailey (1991) suggested that the fact the host cells are initially invaded without being killed is an important contribution to successful pathogenesis by avoiding induction of the defense responses associated with hypersensitive cell death.

Armstrong-Cho *et al.* (2012) studied the infection process of *C. truncatum* isolates of both, races Ct0 and Ct1, on the anthracnose-susceptible lentil cultivar Eston and of the partially Ct1-resistant cultivar CDC Robin. The results suggested that the difference of infection success between races may depend in part on differences in conidial germination, the formation of

appressoria and the length of the biotrophic period.

2.1.4.3. Symptoms

Anthracnose affects all aerial parts of the plant. Wet conditions permit rapid development of the disease (Buchwaldt *et al.*, 1994). Symptoms start with the appearance of superficial lesions on leaves and stems of young lentil plants (Buchwaldt *et al.* 1996). Anthracnose is often observed about 6 weeks after seeding or at early flowering (Chongo *et al.*, 1999), but plants may be infected, although symptomless, as soon as 3 weeks after seeding (Gibson, 1994). Diseased stems and lower leaves first show greenish water-soaked lesions, which evolve into necrosis, finally resulting in premature leaf drop. On stems, lesions first appear at the base and then move upwards. A severe attack on stems can cause the plant to wilt and subsequently to die (Buchwaldt *et al.*, 1996; Chongo and Bernier, 2000). Acervuli with conidia appear on necrotic spots, followed by microsclerotia on all the infected parts of the plant (Fig. 2.2).

Figure 2.2: Infection focus of anthracnose caused by *Colletotrichum truncatum* in a lentil field (a, b), typical lesion on a lentil pod (c), leaves (d) and stem (e).

2.1.4.4. Survival

Colletotrichum truncatum can survive up to 44 months in buried lentil debris (Buchwaldt *et al.,* 1996). On the soil surface, viability decreases after only 12 months. The fungus survives in the form of microsclerotia attached to lentil debris.

2.1.5. Host range

Colletotrichum truncatum sensu lato is pathogenic to several plant species in several families. Amongst others, *C. truncatum* has been reported to infect the following crop species: lentil *(Lens culinaris*), soybean (*Glycine max*), pea (*Pisum sativum,* Morrall, 1989), fababean (*Vicia faba,* Sutton, 1980 ; Morrall, 1989), bean (*Phaseolus vulgaris*, Andrus & Moore, 1935), peanut (*Arachis hypogaea*), as well as the weed noogoora burr (*Xanthium occidentale*), several wild *Vicia* species (Morrall *et al*., 1989) and scentless chamomile (*Matricaria perforata*). It was also incidentally found on subterranean clover (*Trifolium subterraneum* L.) (Barbetti, 1985).

Isolates obtained from a given species are more or less specific to that species. For example, isolates of *C. truncatum* from lentil were shown to be pathogenic to several cultivars of faba bean and pea (Morrall *et al.*, 1989, Anderson *et al.*, 2000) and chickpea (Anderson, 2003). Single host preference was noted with isolates from hemp sesbania (*Sesbania exaltata*) (Boyette, 1991). In contrast, isolates from soybean are pathogenic to numerous species (Sinclair, 1988). Mating between isolates from different hosts seems to be rare in the *Colletotrichum* genus, but it was shown to happen in *G. cingulata* (Cisar *et al.*, 1994). Whereas most isolates from the same host were cross-fertile in that study, very few combinations of isolates from different hosts were fertile.

2.1.5.1. *Lens culinaris*, the cultivated lentil

The cultivated lentil (Fabaceae) is a self-pollinating annual food legume of the cool season (Bayaa and Erskine, 1998). The principal interest of lentil as a crop is the mature seed with interesting dietetic characteristics and fast cooking proprieties (Muehlbauer *et al.*, 1985). Lentil is a valuable protein source, hence this pulse is considered to be a good substitute for meat. Lentil cultivation in Canada started in 1969 on a small area (Slinkard *et al*., 1990) and expanded rapidly to 963,200 ha harvested at the present time.

Foliar diseases have an important impact on economic yield of lentil (Nyggard and Hawtin, 1981). Fungal diseases are the most problematic (Bayaa and Erskine, 1998). Ascochyta

blight, caused by *Ascochyta lentis,* and anthracnose, are the major biotic causes of yield reduction in Canada. Ascochyta blight was first reported in Canada in 1978 and has been responsible for significant yield losses of up to 70% (Morrall, 1997).

2.1.5.2. *Matricaria perforata* Merat, the scentless chamomile

Scentless chamomile was introduced into Canada from Europe at the end of the 19th century and has developed into an invasive species because it is very competitive with cultivated crops (Douglas *et al.*, 1992). *Colletotrichum truncatum* naturally occurs on scentless chamomile (Peng and Bailey, 2002) and has been studied for use as a biocontrol agent against this weed (Peng *et al.*, 2005). Because of the possible risk to crop species, pathogenicity tests have been conducted on a range of crop species and the relatedness of *C. truncatum* isolates from lentil and from chamomile has been investigated. Almost all of the isolates from chamomile tested were found to be specific to that species and another species in the genus *Matricaria*. Two isolates were able to infect lentil at a low level (Peng *et al.*, 2005; Forseille, 2009). Additionally, molecular studies using RAPD markers and rDNA sequences showed that *C. truncatum* isolates from lentil formed a monophyletic, well defined cluster, distinct from isolates from other hosts (Ford *et al.*, 2004; Forseille, 2011). However, the discovery of the teleomorphic stage of *C. truncatum* opens the possibility of sexual reproduction between isolates from different hosts, provided they are genetically close enough to be sexually compatible.

2.1.6. Disease control

Several diseases caused by *Colletotrichum* spp. on various hosts are controlled by a combination of chemical and cultural measures, and the use of resistant cultivars (Waller, 1992).

In lentil anthracnose, fungicides proved to have a certain efficacy (Chongo and Bernier, 2000). Under natural epidemic conditions, plots of cultivar Eston with very high levels of susceptibility to anthracnose were moderately diseased when treated with chlorothalonil, while non-sprayed plots showed severe disease. Yield of non-sprayed plots was 44% lower than that of sprayed plots (Morrall *et al.,* 1990).

Use of resistant cultivars is a major strategy in controlling plant disease and has been promoted as it also has a lesser impact on the environment. However, it is complicated due to the existence of two races in *C. truncatum*, Ct0 and Ct1. Sources of partial resistance to race Ct1 was found in the forage lentil cultivar 'Indianhead', which has some undesirable features but has been

exploited for the development of partially resistant cultivars such as CDC Robin (Vandenberg *et al.*, 2002). A source of partial resistance to race Ct0 has been discovered in the landrace VIR421 (Buchwaldt and Diederichsen, 2004), as well as in the wild lentil species *L. ervoides* and *L. lamottei* (Tullu *et al.* 2006) and is being introgressed into *L. culinaris*.

2.2. Sexual reproduction in ascomycetes

2.2.1. Role of mating systems

Reproduction strategies in fungi are diverse, and sexual reproduction is not necessarily one of them. Sex is a costly choice with no evident benefits for the individual fungus in a given generation (Brown, 1999). However, it is a powerful tool to create genetic recombination in the progeny and will have benefits for the evolution and success of the population. Additionally, sexual structures often have a role in survival and/or dispersal in fungi (Chamberlin and Ingram, 1997). For sexual reproduction to be possible, the parental cytoplasms must be genetically close enough to be able to mix and function properly. However, to benefit from genetic recombination, the parental nuclei should be sufficiently different. Therefore, there is the need for a system to regulate and enable two fungal individuals with different genetic information to mate but restrict mating between individuals genetically too different (Chiu and Moore, 1999). This is achieved by mating systems, also sometimes called breeding systems, which are controlled by nuclear genes. When such a system is present, the isolate is self-sterile, cross fertile and is said to be heterothallic. In the absence of such a system, the isolate is homothallic (Chiu and Moore, 1999).

2.2.2. Sexual differentiation and development of sexual structures in ascomycetes

In ascomycetes, the sexual cycle begins with the differentiation of the female sexual organ, the ascogonium. In many species, the ascogonium comprises a structure called trichogyne that acts as the receptive female element and can fuse with a male element (Fig. 2.3.). In the case of a heterothallic fungus, the male parent has to be of the opposite mating type. Fertilization starts when the male differentiated cell, either an antheridium or a spermatium, encounters the ascogonium. Two major steps are involved: plasmogamy (the fusion of parental cytoplasms), and karyogamy (the fusion of parental nuclei**).** The principal role of mating types is to control the formation of the dicaryotic hyphae after plasmogamy that is required for zygote formation

(Kronstad and Staben, 1997). Early experiments on *Ascobolus stercorarius* showed that the fertilizing element, called an oidium in this species, was producing a substance capable of controlling the growth of the trichogyne, but also to spatially orientate its growth (Bitis, 1956). In *Neurospora crassa*, it was shown that a pheromone-like substance secreted by the fertilizing element was able to attract trichogynes, but only when the fertilizing element and the trichogynes were of opposite mating types (Bistis, 1981).

After plasmogamy, ascogenous hyphae develop from the ascogonium and form a characteristic binucleate hook-shape structure called crozier. In this structure, a mitotic division occurs, followed by the formation of septa which segregate one of each parental nuclei in the top cell of the crozier (Fig. 2.3). The young ascus formed this way has two hapoid nuclei (Casselton, 2002). At this point, karyogamy occurs. The parental nuclei fuse into a single diploid nucleus, which immediately undergoes meiosis (Coppin *et al.*, 1997). After karyogamy, the diploid nucleus formed is heterozygous at the mating type factor locus. Therefore, the meiosis produces progeny of both mating type, each in equal numbers (Chiu and Moore, 1999).

In *Colletotrichum,* as in most of the ascomycetes, the four haploid nuclei formed during meiosis divide mitotically to produce eight nuclei, from which the ascospores evolve.

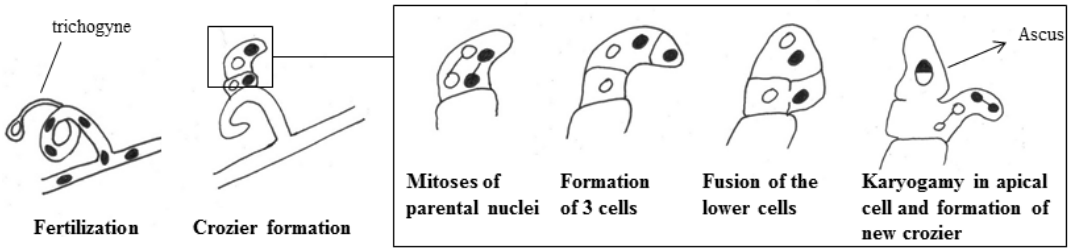

Figure 2.3: The formation on the ascogenous hyphae in a typical ascomycete.

2.2.3. Genetic control of mating types

In most ascomycetes, a species is either homothallic or heterothallic. Nearly all heterothallic species whose mating systems have been described have a single locus/two alleles control, determining a 2 mating-type polarity. The mating types are determined by a mating type locus with two idiomorphs, i.e. alleles with no sequence identity (Metzenberg and Glass, 1990).

The same locus has been given different names depending on the species (Kronstad and Staben, 1997). In *Colletotrichum*, it has been called MAT, with two idiomorphs called MAT1-1 and MAT1-2. The MAT1-1 idiomorph was shown to include the alpha domain of a DNA-binding protein that is highly conserved amongst species, whereas the MAT1-2 idiomorph comprises another highly conserved DNA binding protein known as the high mobility group (HMG) (Zhong and Steffenson, 2001). This particularity has been exploited to develop PCR-based methods to isolate and characterize the mating type idiomorphs (Arie *et al.*, 1997, 2000). Sexual incompatibility is homogenic, i.e. occurs between individuals carrying different idiomorphs at the mating locus (Korf, 1952). It has been suggested that the recognition of compatibility could be due to the formation of a heterodimer between the products of the two different mating alleles (Metzenberg, 1990). Mating type genes permit to determine nuclear identity and thus, make recognition possible between two nuclei of opposite mating types. These nuclei then migrate together in the ascogenous hyphae (Zickler *et al.*, 1995).

Homothallic species usually possess both idiomorphs in the same nucleus (Yun *et al*., 1999), but other mechanisms exist that can lead to a homothallic phenotype (reviewed in Billard *et al.*, 2010). When both idiomorphs are present, the existence of a mechanism permitting the alternate expression of the mating types has been suggested (Coppin, 1997). This mechanism would allow recognition of both parental nuclei expressing opposite mating types, making the cross functionally 'heterothallic'.

Asexual species also carry mating type genes. By comparing the sequences of *Aspergillus fumigatus* (asexual) and *A. nidulans* (homothallic), Varga (2003) demonstrated the existence of sequences in *A. fumigatus* similar to the MAT1-1 and MAT1-2 idiomorphs in *A. nidulans*. In some cases, the mating type genes in asexual species were shown to be potentially functional. Recent studies on *Aspergillus* showed that the *A. fumigatus* mat1-2 open reading frame introduced into the genome of the sexual *A. nidulans* was functional when coupled with the mat1-2 promoter from *A. nidulans*, but not when coupled with the promoter from *A. fumigatus* (Pyrzak *et al*., 2008). This suggests that the reason for asexuality in this species is not due to the absence of a functional mating type gene but to a defect in expression regulation. In a similar study on *Bipolaris sacchari*, which is also an asexual species, a MAT1-2 idiomorph-like sequence was found, which was 97 to 98% identical to the MAT1-2 sequence of the related heterothallic species *Cochliobolus heterotrophus* (Sharon *et al*., 1996). The introduction of the

MAT1-2 sequence of *B. sacchari* in a MAT1-1 isolate of *C. heterotrophus* made the isolate capable of self-fertility, indicating that the sterility of *B. sacchari* is not due to the absence of a functional MAT1-2. *Alternaria alternata*, an asexual species, carries the idiomorphs MAT1-1 and MAT1-2, and these sequences were shown to be functional in *C. heterotrophus* by heterologous expression (Arie *et al.*, 2000).

2.2.3.1. Organization of the mating type loci

Mating type loci can each contain one or more genes, depending on the species (Fig. 2.4). The MAT1-2 locus usually, but not always, carries a single gene, while the MAT1-1 locus carries one to three genes (Coppin *et al.*, 1997). For example, *Magnaporthe oryza* carries three genes at the MAT1-1 locus and two at the MAT1-2 locus (Kanamori *et al.*, 2007), but *Giberella fujikuroi* and *G. zeae*, while also carrying three genes at the former, carry only one at the latter (Yun *et al.*, 2000).

Functional analysis was performed on several species of the Sordariomycetidae in order to determine the functions of those different genes. The Sordariomycetidae is the sister subclass to the Hypocreomycetidae within the Sordariomycetes, containing the model organism *N. crassa*, as well as other well-studied species. In *Podospora anserina*, the MAT1-1 locus carries three genes called FMR1, SMR1 and SMR2 (Debuchy *et al.*, 1993). FMR1 and its functional analogue to the MAT1-2 locus, FPR1 are the master regulators responsible for mating specificity (Debuchy *et al.*, 1992). In order to determine the involvement of SMR1 and SMR2 in the mating process, Coppin *et al*. (1993) performed deletions of mating type sequences in the MAT1-1 locus and determined that FMR1 alone is sufficient to induce fertilization, whereas SMR1 and SMR2 are involved in perithecial development. This demonstrated that the involvement of mating types continues beyond the fertilization step. The same three gene organization at the MAT1-1 locus has been reported from *N. crassa* (called MAT A in this species), with mat A-1 being the major mating regulator. The two others genes, matA-2 and matA-3 are both constitutively expressed during the sexual and vegetative phases (Ferreira *et al.*, 1996). In this species however, the MAT1-2 (referred to as MAT a in *N. crassa*) locus contain two genes. The mat a-1 gene is the major mating regulator responsible for sexual identity, perithecial development as well as vegetative incompatibility (Staben and Yanofski, 1990) and regulates a pheromone precursor (Kim *et al*., 2002). The mat a-2 gene, however, does not seem to be transcribed (Pöggeler and

Kück, 2000).

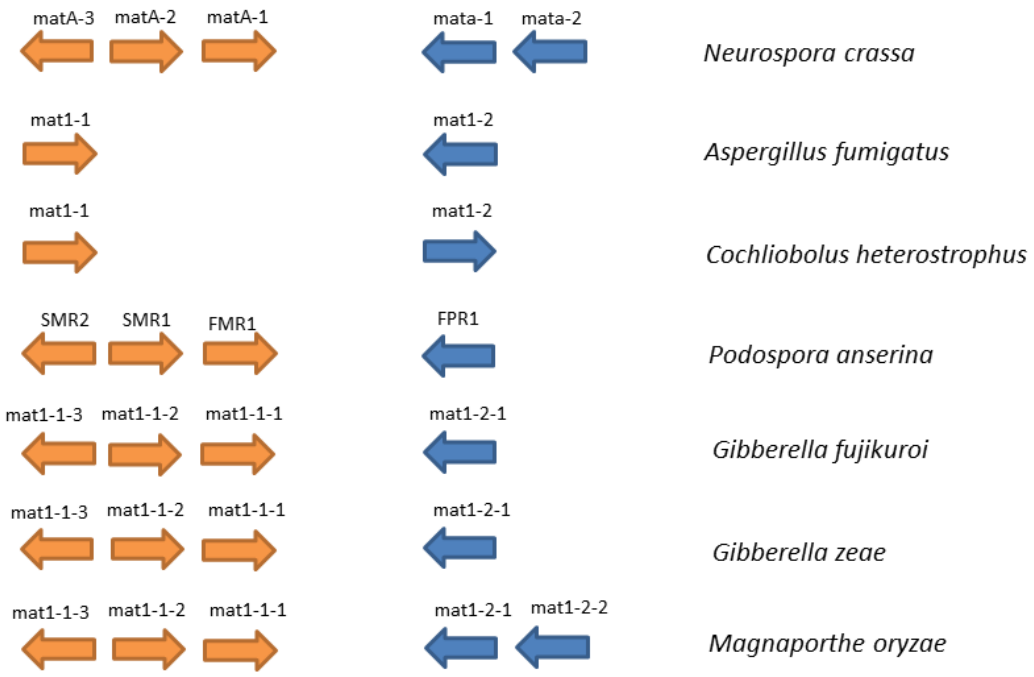

Figure 2.4: Comparison of the organization of the MAT loci in different ascomycetous species. Schemas on the left correspond to the MAT1-1 locus, those on the right correspond to MAT1-2. Each arrow corresponds to a gene.

2.2.3.2. Mating type switching

Mating types in filamentous ascomycetes are generally very stable. It is thought that this situation is due to the absence of silent copies of mating type sequences in the genome outside of the mating type loci (Coppin *et al.*, 1997). Silent copies, however, can occur in several yeast species, which confers to them a higher plasticity and enables them to switch mating types. This occurs when a silent copy of a mating type sequence becomes active through transposition in a heterothallic fungus of the opposite mating type. While rarer, a few filamentous ascomycetes are also capable of unidirectional reversal of mating type. In the Leotiomycetes class, *Botryotinia fuckeliana*, a heterothallic fungus with a bipolar mating system, is suspected to have a silent copy of a MAT1-1 sequence based on fact that some of the isolates that were predicted to carry the MAT1-2 idiomorph after meiosis were sexually compatible with both mating type testers (Faretra

and Pollastro, 1996). Within the Hypocreomycetidae, *Ceratocystis coerulescens* was shown to display unilateral mating type switching (Harrington and McNew, 1997). This is interesting because the genus *Ceratocystis* is taxonomically very close to the genus *Glomerella* (Zhang *et al.*, 2006).

2.2.4. Mating system in the genus *Glomerella*

The atypical sexuality in the genus *Glomerella* was well worded by J. Raper (1951) who described *G. cingulata* as a "sexually ambiguous species without peer". In *Glomerella*, the typical ascomycete sexual compatibility pattern does not seem to apply. *Glomerella cingulata* and *G. graminicola* were found to be both homothallic and heterothallic (Wheeler, 1954; Vaillancourt and Hanau, 1991). The number of mating types in the genus *Glomerella* is not limited to two, and fertile interactions are not always consistent with the typical single locus bipolar system (Vaillancourt *et al*., 2000a). Because most *Colletotrichum* species have no sexual state, genetic analyses have only been performed with very few *Glomerella* spp., and the genetic control of sexual compatibility remains mostly unresolved. Information obtained for three species, *G. cingulata* (Wheeler, 1956; Cisar and Tebeest, 1999), *G. graminicola* (Vaillancourt *et al.*, 2000b) and *G. lindemuthiana* (Rodriguez-Guerra *et al.*, 2005), suggests that those species have unusual mating systems that do not conform to the typical pattern seen in most ascomycetes.

2.2.4.1. The case of *Glomerella cingulata*

The mating system of *G. cingulata* has been extensively examined at the beginning and middle of the 20th century, starting with Edgerton's studies in 1914. He described two types of fertile cultures, one type which produced perithecia in clumps, and another type, obtained from the progeny of isolates of the first type that produced scattered perithecia bearing only a small proportion of fertile ascospores. Those two types were at the time understood as two opposite mating types, the wild-type corresponding to the 'plus' type, and the variant to the 'minus' type, following the nomenclature at that time (Blakeslee, 1904). However, the self-fertility of the 'plus' type distinguishes the two types of isolates from true mating types. For this reason, the types were later renamed as clumped-perithecial, (CP) and scattered-perithecial (SP) (Wheeler and McGahen, 1952).

Another characteristic was also shown to differ from a simple bipolar mating system:

Whereas the CP isolates gave a mixed progeny of CP and SP, the progeny obtained from ascospores of SP isolates were all of the SP type (Lucas *et al.*, 1944). Wheeler and McGahen (1952) suggested that the difference between the two types were due to a single gene with allele B^+ for the CP phenotype and allele B^1 controlling the SP phenotype. In this hypothesis, the B^+ nucleus is essential for fertility and allows isolates carrying it to self-fertilize, or outcross with a B^1 isolate, whereas the B^1 nucleus, lacking the fertility factor, is self-sterile and needs to be combined with a B^+ nucleus to produce sexual structures.

The discovery of another type of isolate, self-sterile and producing large numbers of conidia (CC, for clumped-conidial type), permitted to reveal the possible existence of another gene 'A' with three alleles, involved in the switch from conidial to perithecial development (Chilton *et al.*, 1945; Wheeler and McGahen, 1952).

Individually, two types can be described in terms of homo- or heterothallism, with the CP type behaving like a typical homothallic fungus, and the CC type being heterothallic, but at the species level, this classification is inapplicable.

True heterothallism is controlled by mating-type loci (Raju, 1992), but a heterothallic phenotype can be obtained through alternative genetic control. Wheeler (1954) proposed that *G. cingulata* shows what he called 'unbalanced heterothalism', which means that each partner of a fertile cross carries mutated fertility gene(s) that can either complement each other (in that case, they display cross-fertility) or not (cross-sterility). In this case, it has been suggested that heterothallism is the result of a mutation in the developmental pathway for homothallism (Vaillancourt *et al.*, 2000a). The progeny resulting from a cross between unbalanced heterothallic strains contain recombinant individuals, whereas a cross between true heterothallic strains will not result in recombinant progeny (Wheeler, 1954). Unbalanced heterothallism is also suspected in the case of *G. graminicola* (Vaillancourt *et al.*, 2000b).

2.2.4.2. Mating type genes in the *Glomerella* genus

The presence of MAT genes in *Glomerella* has been investigated in a few species, but has been associated with classical mating studies by crossing in only two species, *G. graminicola* (Vaillancourt *et al.*, 2000b) and *G. lindemuthiana* (Rodriguez-Guerra *et al*., 2005). In both species, a typical MAT1-2 was found in all a different system is involved in mating compatibility in *Glomerella* compared to most ascomycetes.

2.3. Population structure and outbreeding

2.3.1. Reproduction strategies and population structure

Genetic structure is defined by the amount and the distribution of variation within and between populations (McDonald and Mc Dermott, 1993). Population genetic structure is influenced by different parameters, including the pattern of food availability, the existence of resistant genes in the host population, the movement of plant products and the reproductive strategies (Correll and Gordon, 1999).

Fungal populations can display different reproductive strategies: some are exclusively asexual, and consequently clonal; some are most of the time asexual, but with a sexual phase on a regular cycle; some are sexual, either exclusively outbreeding, or exclusively inbreeding, or outbreeding and inbreeding (Burnett, 2003). It can be difficult to assess whether a population is clonal or whether recombination occurs. If a single recombinant period occurs between many asexual generations, an important proportion of individuals in the population is issued from asexual reproduction and the genetic constitution of the population may appear unchanged (Burnett, 2003).

These reproductive strategies have different impact on population structure. In the long term, an asexual population may be reduced to a single genotype. In contrast, sex leads to the recombination of alleles and the creation of new genotypes. At a given locus, one cycle of sexual reproduction restores the Hardy-Weinberg equilibrium in a population (Brown, 1999).

2.3.2. Measure of genetic diversity and linkage disequilibrium

Different properties can be used to determine whether a population is clonal, recombinant or both (Milgroom, 1996). It encompasses direct observation of reproductive structures, determination of genotypic diversity and the extent of linkage disequilibrium.

Genetic diversity is made up of two components: gene diversity (number and frequencies of alleles at individual loci) is not affected by the mating strategy, in contrast to genotype diversity (number and frequencies of genotypically distinct individuals), which is affected by the reproduction strategy of a population (Burnett, 2003).

Linkage disequilibrium (also called gametic disequilibrium) is defined as “non-random associations between alleles at different loci, whether or not they arise from linkage” (Weir, 1996). Estimates of linkage disequilibrium provide information about the reproduction strategy

in a population. Indeed, in a population where sexual reproduction occurs, alleles are recombined and the linkage disequilibrium is expected to be low. In contrast, high values of linkage disequilibrium are expected in mostly clonal populations where recombination is rare. A single cycle of sexual reproduction is enough to half the values of linkage disequilibrium (Brown, 1999). The simplest and most commonly used way to test linkage disequilibrium in a population is to calculate its deviation from Hardy-Weinberg equilibrium at each individual locus. Since this technique is based on the calculation of heterozygosis, it can be applied to diploid organisms only, including fungi such as the oomycete *Phytophthora* and many basidiomycetes (Tooley *et al*., 1985). However, in the more common case of haploid fungi, such as *C. truncatum*, other methods must be employed. One commonly used measure of linkage disequilibrium in such organisms is the index of association I_A (Brown, 1980, Maynard Smith *et al*, 1993), which assesses the multilocus gametic disequilibrium, *i.e.* the statistical associations between alleles at several loci simultaneously.

Genetic diversity and linkage disequilibrium can be assessed using data obtained with various DNA-based molecular marker techniques. Among those, amplified fragment length polymorphism (AFLP) (Vos *et al.,* 1995) analysis is often used in population studies because this method permits rapid data collection for the large number of individuals such studies require (Majer *et al.*, 1996). It involves the application of two restriction enzymes to the DNA to produce a large number of DNA fragments, followed by the ligation of those fragments with oligonucleotides adapters (preamplification step). Primers with some homology with the oligonucleotides are then used to amplify the fragments (Burnett, 2003). To reduce the number of fragments amplified – too large of a number would complicate the scoring – a selective amplification is performed by adding one to three nucleotides to the 3' end of the primer. The number of nucleotides to add at the 3' end depends on the size of the genome – the larger the genome, the more nucleotides are added.

2.3.3. Population structure in *Colletotrichum* spp.

Population structure studies were performed on several *Colletotrichum* species using various molecular methods. Results of these studies revealed that population structures vary widely between and within *Colletotrichum* species.

Rosewich *et al.* (1998) developed RFLP markers to study a collection of 411 *C. graminicola* isolates from sorghum sampled over 3 consecutive years in a disease nursery in

Georgia, USA. They showed that gene diversity was moderately high, which is matching the high variability of pathogenicity traits reported in this species. They noted that the gene diversity obtained for *C. graminicola* from sorghum, a species supposedly asexual, is similar to that of *Mycosphaerella graminicola*, a sexual species, suggesting that the global genetic variation in a fungal species does not depend on its reproductive strategy. However, they showed that genotypic diversity was extremely low for this species (9 haplotypes were found in the collection of 411 isolates), and suggested that asexual reproduction, not genetic drift and gene flow, had a significant effect on the population structure.

For the same species, *C. graminicola*, but isolated from another host, the turfgrass *Agrostis palustris*, Chen *et al.* (2002) showed a very different structure. In this population, genotypic diversity based on RAPD markers was very high (67 different haplotypes in the 87 isolates tested), but significant linkage disequilibrium existed. Clonal reproduction was observed, but the authors suggested some sort of recombination, either sexual or asexual, must have occurred to explain the extent of the genetic variation in this population.

A study by Urena-Padilla *et al*. (2002) on *C. acutatum*, *C. gloeosporioides* and *C. fragaria* is interesting in that it characterized populations of species that are pathogens of the same host (the strawberry), all of which were sampled in Florida, USA (the same years for the first two species) and submitted to RAPD analysis. Each of the species seemed to have different reproductive strategies. *Colletotrichum gloeosporioides* (*G. cingulata*) is a sexual species, *C. acutatum* was recently reported to undergo sexual reproduction under natural conditions on highbush blueberry (Talgø *et al*., 2007), and *C. fragaria* has no known teleomorph. Not surprisingly, the authors showed that the population of *C. gloeosporioides* displayed a high diversity and low linkage disequilibrium, suggesting *G. cingulata*, the teleomorph, is responsible for the epidemics on strawberry plants. In contrast, the population of *C. acutatum* and *C. fragaria* showed lower diversity, suggesting their main reproductive strategy was clonality.

The case of *C. lindemuthianum* is different. This pathogen was shown to display important variability, especially when it comes to pathogenicity. For example, Silva *et al.* (2007) reported over 100 pathotypes. Various mechanisms were proposed to explain this variability. They include sexual reproduction (Rodriguez-Guerra *et al*., 2005), parasexuality (Roca *et al*. 2003) and mutation (Tu, 1992). Although the teleomorphic stage exists, it is rarely reported from the field. However, considering that some populations were at or close to gametic equilibrium, it

was suggested that periodic cycles of sexual reproduction were a key mechanism for production of variability in this species (Souza *et al*., 2010).

2.4. Prologue to Chapter 3

The following chapter is part of a manuscript accepted for publication in Mycologia (Appendix 1). It describes two different approaches undertaken to investigate the mating system of *C. truncatum*. The first approach relied on classical crosses between field isolates in order to determine the modalities for cross-fertility. The second approach used primers targeting the mating type idiomorphs in a selection of field isolates. The classical crossings, as well as screening of the isolates with the primers, are described in Chapter 3. However, the information pertaining to the development of those primers was not included as they were developed by Drs. Adrian Cabral and Perumal Vijayan.

CHAPTER 3

MATING SYSTEM OF *COLLETOTRICHUM TRUNCATUM* (*GLOMERELLA TRUNCATA*)

3.1. Introduction and objectives

Ascomycete fungi display various types of mating systems. They can be either homothallic or heterothallic (Kronstad and Staben, 1997), and in most heterothallic species, two mating types exist, determining a bipolarity. Incompatibility is homogenic, which means that two isolates with the same mating type cannot mate. Mating types are controlled by a single locus (MAT), with two alleles (MAT1-1 and MAT1-2; Metzenberg and Glass, 1990). Many heterothallic ascomycetes were shown to require partners carrying different alleles at the MAT locus for fertile crosses, as expected in the case of homogenic incompatibility (reviewed in Souza *et al*., 2003).

Yet, cases have been reported in the genus *Glomerella* that are inconsistent with homogenic incompatibility (Wheeler 1954, Vaillancourt *et al.,* 2000a, Rodriguez-Guerra *et al*., 2005). Tests for sexual compatibility have been performed in a few *Glomerella* species. Well before molecular tools were available, suspicions arose that this genus contained species with a mating system very different from what was known then in other ascomycetes. Earlier work by Edgerton (1914) and Wheeler (1954) demonstrated that isolates of *G. cingulata* could not be placed into two cross-fertile groups. Based on the example of *G. cingulata*, Wheeler (1954) proposed the 'unbalanced heterothallism' hypothesis. In this process, each partner of a fertile cross carries mutated fertility gene(s) that can either complement each other (cross-fertility) or not (cross-sterility). Cross-fertility studies on *C. graminicola* revealed that isolates could be homo- or heterothallic, and the number of mating types was not limited to two (Vaillancourt *et al.*, 2000a). It was speculated that unbalanced heterothallism could be characteristic for the mating system of *C. graminicola* as well (Vaillancourt *et al.*, 2000b). Current information available on the MAT idiomorphs in *Glomerella* spp. also supports the hypothesis that the genetic determinism for mating types in this genus differs from the typical ascomycete model.

The teleomorphic stage of *C. truncatum* has never been observed under field conditions.

This situation is common in the genus *Colletotrichum*, where teleomorphic stages of many species are known *in vitro* only, with a few notable exceptions like *Glomerella cingulata* (Sutton, 1992). In the case of *C. truncatum*, the sexual stage (*G. truncata* Armstrong-Cho and Banniza) was obtained under laboratory conditions (Armstrong-Cho and Banniza, 2006). Crossing experiments in this species, conducted on a small number of isolates, showed self-sterility for all isolates tested, but some combinations of isolates resulted in the production of perithecia, supporting the hypothesis of a heterothallic mating system (Armstrong-Cho and Banniza, 2006). More extensive crossing studies were needed to determine if *C. truncatum* follows the typical bipolar ascomycete mating system, or if, as in the case *C. graminicola,* sexual reproduction is likely determined by another system.

Colletotrichum truncatum sensu lato is not only pathogenic to lentil, but also to several other species, including soybean (*Glycine max*) and the weed scentless chamomile (*Matricaria perforata*). Almost all the isolates from chamomile tested were found to be specific to that species and another species in the genus *Matricaria*. Molecular studies showed that *C. truncatum* isolates from lentil formed a monophyletic, well defined cluster, distinct from isolates from other hosts (Ford *et al.*, 2004; Forseille, 2011). *Colletotrichum truncatum* isolates from *M. perforata*, pathogenic to this species, but not to any crop species, have been evaluated as a mycoherbicide against this invasive weed (Peng *et al.*, 2005). It is therefore of importance to ensure no sexual reproduction can happen between isolates from *M. perforata* and isolates pathogenic to lentil. Though mating between strains isolated from different hosts seems to be rare in the genus *Colletotrichum*, it has been demonstrated for *G. cingulata* (Cisar *et al.*, 1994, 1996). Cross-fertility or sterility between those different isolates can also give an indication of the genetic barriers between isolates from different species.

The objectives of this study were to examine the mating system of *C. truncatum* by performing sexual compatibility tests between isolates collected from lentil tissues and other hosts, and to determine if the mating type idiomorphs MAT1-1 and MAT1-2 are present in partners of a fertile cross.

3.2. Materials and methods

3.2.1. Biological materials and media

Monoconidial isolates of *C. truncatum* used in this study are listed in Table 3.1. Isolates were routinely cultured on oatmeal-agar medium (OMA: 30 g oatmeal flour [Quick Oats Robin Hood, Smucker Food of Canada, Markham, Ontario, Canada], 8.8 g granulated agar[Difco™, Becton, Dickinson & Company, Sparks, MD, USA], 1L distilled water), incubated at 22°C with a 12 h photoperiod. Liquid cultures were obtained by inoculating 40 mL centrifuge tubes containing 25 mL glucose yeast medium (1 g $NH_4H_2PO_4$ [EM Science], 0.2 g KCl [OmniPur®, EMD™], 0.2 g $MgSO_4$ $7H_2O$ [EM Science], 10 g glucose [BDH®], 5 g yeast extract, 0.01 g $ZnSO_4$ $7H_2O$ [EM Science], 0.005 g $CuSO_4$ $5H_2O$ [EM Science], 1 L distilled water) with small amounts of mycelium from 5-7 days-old cultures and incubating in a shaker (Model SI-600, Lab Companion, Jeio Tech, Seoul, Korea) at 23°C and 130 rpm for 5-7 days. Mycelia were harvested by centrifugation, stored at -80°C for 48h in 2mL microcentrifuge tubes and subsequently lyophilized for 2 days in a Labconco cryofreezer (Labconco Corp., Kansas City, MO, USA).

3.2.2. Tests for sexual compatibility

The susceptible lentil cultivar 'Eston' was grown under controlled conditions (23ºC, 16 h photoperiod) until senescence. The lignified stems were harvested, cut in 5 cm long segments and sterilized. Stems of healthy *M. perforata* were harvested from the wild and processed the same way. Isolates of *C. truncatum* obtained from lentil listed in Table 3.1. were tested for self-fertility, as well as cross-fertility in all possible combinations. CT-20 and CT-21, which are cross-fertile (Armstrong-Cho and Banniza, 2006) were used as positive controls for perithecia production. Isolates from *G. max* and from *M. perforata* were tested for cross-fertility with CT-21 and CT-30. Isolates from *M. perforata* were also crossed with each other. All crosses involving isolates from *M. perforata* were performed both on lentil stems and on scentless chamomile stems.

Conidial suspensions were prepared by flooding 2-week old cultures with sterile de-ionized water. The spore concentration was determined with a haemocytometer, and suspensions were diluted to 2×10^5 spores mL^{-1}. Five lentil stems were soaked for 2 hours in a mixture of 5 mL of spore suspension of each of the two isolates to be tested, or 10 mL of a single spore suspension in the case of selfings. The five stems were placed on Whatman No. 1 filter paper

overlying sterile water agar in Petri dishes and incubated under optimum conditions for perithecium formation as described by Armstrong-Cho and Banniza (2006). Four replicate Petri dishes were prepared for each combination. To identify fertile crosses, stems were screened for the presence of perithecia under × 40 magnification (SMZ-U, Nikon, Japan) 10 and 14 days after incubation. A cross was considered fertile if at least one perithecium was found in any of the replicates. Isolates were placed into incompatibility groups (IG) based on cross-fertility patterns.

3.2.3. DNA extraction

Freeze-dried mycelium was coarsely powdered with a pipette tip and DNA was extracted following a protocol modified from Raeder and Broda (1985). In a microcentrifuge tube, 1 mL of extraction buffer constituted of 200 mM Tris HCl pH 8.5, 250 mM NaCl, 25 mM EDTA and 0.5% SDS was added to approximately 1 mL of powdered mycelium. The tubes were manually shaken. The mixture obtained was incubated for 30 minutes at 37ºC. After adding 500 µL of 24:24:1 phenol/chloroform/isoamyl alcohol, the tubes were shaken again and centrifuged for 30 minutes at 13,000 g. The upper aqueous phase was transferred to a new microcentrifuge tube and 25 µL of RNAse A were added followed by incubation at 37ºC for 10 minutes. An equal volume of chloroform was added and the solution was centrifuged for 10 minutes at 13,000 g. The upper aqueous phase was transferred into a new microcentrifuge, and DNA was precipitated with × 0.5 that volume of ice-cold isopropanol. The solution was then kept at -20ºC for at least two hours to allow DNA to settle. After a short centrifugation, the liquid was discarded carefully to avoid disturbing the pellet. The pellet was washed with 70% ethanol, dried overnight and resuspended in 100 µL sterile 10 mM Tris HCl at pH 8, and 1 mM EDTA.

Table 3.1: Isolates of *Colletotrichum* spp. used in sexual compatibility tests and for amplification of the conserved regions of the mating type idiomorphs.

Isolate name	Species	Host	Source	Crossings	HMG box	Alpha box
CT-13	*Colletotrichum truncatum*	*Lens culinaris*	This study	X		
CT-20	*Colletotrichum truncatum*	*Lens culinaris*	Armstrong-Cho & Banniza, 2006	X	X	
CT-21	*Colletotrichum truncatum*	*Lens culinaris*	Armstrong-Cho & Banniza, 2006	X	X	
CT-28	*Colletotrichum truncatum*	*Lens culinaris*	Armstrong-Cho & Banniza, 2006	X		
CT-30	*Colletotrichum truncatum*	*Lens culinaris*	Armstrong-Cho & Banniza, 2006	X	X	
CT-31	*Colletotrichum truncatum*	*Lens culinaris*	Armstrong-Cho & Banniza, 2006	X	X	
CT-32	*Colletotrichum truncatum*	*Lens culinaris*	Armstrong-Cho & Banniza, 2006	X		
CT-34	*Colletotrichum truncatum*	*Lens culinaris*	Armstrong-Cho & Banniza, 2006	X		
CT-35	*Colletotrichum truncatum*	*Lens culinaris*	Armstrong-Cho & Banniza, 2006	X		
CT-37	*Colletotrichum truncatum*	*Lens culinaris*	This study	X		
CT-38	*Colletotrichum truncatum*	*Lens culinaris*	This study	X		
CT-39	*Colletotrichum truncatum*	*Lens culinaris*	This study	X	X	
CT-43	*Colletotrichum truncatum*	*Lens culinaris*	This study	X		
CT-44	*Colletotrichum truncatum*	*Lens culinaris*	This study	X		
CT-45	*Colletotrichum truncatum*	*Lens culinaris*	This study	X	X	
CT-46	*Colletotrichum truncatum*	*Lens culinaris*	This study	X		
CT-47	*Colletotrichum truncatum*	*Lens culinaris*	This study	X		
CT-48	*Colletotrichum truncatum*	*Lens culinaris*	This study	X		
CT-58	*Colletotrichum truncatum*	*Lens culinaris*	This study	X	X	

Table 3.1. continued.

Isolate name	Species	Host	Source	Crossings	HMG box	Alpha box
CT-59	*Colletotrichum truncatum*	*Lens culinaris*	This study	X	X	
CT-60	*Colletotrichum truncatum*	*Lens culinaris*	This study	X	X	
CT-49	*Colletotrichum truncatum*	*Gycine max*	K. Anderson (AAFC)	X		
CT-50	*Colletotrichum truncatum*	*Gycine max*	K. Anderson (AAFC)	X		
CT-51	*Colletotrichum truncatum*	*Gycine max*	K. Anderson (AAFC)	X		
CT-52	*Colletotrichum truncatum*	*Gycine max*	K. Anderson (AAFC)	X		
CT-53	*Colletotrichum truncatum*	*Gycine max*	K. Anderson (AAFC)	X		
CT-54	*Colletotrichum truncatum*	*Gycine max*	K. Anderson (AAFC)	X		
CT-55	*Colletotrichum truncatum*	*Gycine max*	K. Anderson (AAFC)	X		
CT-56	*Colletotrichum truncatum*	*Gycine max*	K. Anderson (AAFC)	X		
CT-61	*Colletotrichum truncatum*	*Matricaria perforata*	AAFC	X		
CT-62	*Colletotrichum truncatum*	*Matricaria perforata*	AAFC	X		
CT-63	*Colletotrichum truncatum*	*Matricaria perforata*	AAFC	X		
CT-64	*Colletotrichum truncatum*	*Matricaria perforata*	AAFC	X		
CT-65	*Colletotrichum truncatum*	*Matricaria perforata*	AAFC	X		
M1.001	*Colletotrichum graminicola*		J-A Crouch		X	
F-18	*Fusarium oxysporum*		This study			X
F-20	*Fusarium oxysporum*		This study			X

3.2.4. Amplification of conserved regions of the MAT genes

Three isolates of the incompatibility group 1 (IG-1) as determined above (CT-20, CT-30 and CT-59) and six of IG-2 (CT-21, CT-31, CT-39, CT-45, CT-58 and CT-60) were selected for amplification of the HMG box and the alpha domain. DNA of an isolate of *C. graminicola* (M1.001) carrying the MAT1-2 idiomorph (Vaillancourt *et al.*, 2000b), was obtained from Dr. J.A. Crouch (University of Minnesota, St. Paul, MN, USA) and included as a positive control. DNA amplifications of the HMG box by PCR were performed using two pairs of primers: a pair of degenerate primers (DeLCP2 : forward 5'-CCCCGGCCTCCCaaygcntwyat-3', reverse 5'-GCCGCTTCTCGGAGggyttnckngg-3') designed based on the alignment of conserved regions of the HMG box domain of the MAT1-2 locus of *C. lindemuthianum, G. cingulata* and *C. graminicola*, and a pair of *C. truncatum* -specific HMG primers (CT21HMG) (Adrian Cabral, pers. comm., Annexe 1).

The 20 µL reaction comprised 1X PCR buffer (50 mM KCl, 10 mM Tris-HCl, 0.1% Triton X-100), 200 µM of each dNTP, 2.5 mM of $MgCl_2$, 10 pM each forward and reverse primer, 1.25 U of Taq polymerase and 100 ng of genomic DNA. The amplification program included a denaturation step at 94°C for 3 minutes, followed by 35 cycles at 94°C for 45 seconds, 47°C for 30 seconds and 72°C for 1 minute, following a 'Touchdown' PCR (Don *et al.*, 1991) of 10 cycles, with an initial annealing temperature of 82°C, and subsequent decrements of 1°C per cycle. The program was terminated by a final elongation step at 72°C for 7 minutes.

Amplification of the alpha domain of MAT1-1 was attempted on the same nine *C. truncatum* isolates using the degenerate primers Falpha1 and Falpha2, and the protocols described by Arie *et al*. (2000). Two isolates of *Fusarium oxysporum* (F-18 and F-20) of mating type 1 and 2 respectively were used as controls. Each PCR reaction mixture (final volume 50 µl) contained 1X PCR buffer (50 mM KCl, 10 mM Tris-HCl, 0.1% Triton X-100), 2.5 mM $MgCl_2$, 200 µM of each dNTP, 2 µM of each forward and reverse primer, 1.25 U of Taq polymerase, and approximately 100 ng of fungal genomic DNA. The amplification program included a denaturation step at 95°C for 15 minutes; 35 cycles at 94°C for 1 minute, 55°C for 30 seconds, and 72°C for 1 minute and a final extension at 72°C for 10 minutes. Electrophoresis of the PCR products was conducted in 2% agarose gels stained with ethidium bromide and viewed on a UV transilluminator.

3.3. Results

3.3.1. Crossing experiments

For the tester cross CT-20 × CT-21, mature perithecia appeared on the lentil stems approximately ten days after inoculation (Figure 3.1). For some other fertile crosses, perithecia were slower to mature, so the stems were screened again for all crosses at day 14 postinoculation.

Figure 3.1: Fertile cross between CT-20 and CT-21 on a sterile lentil stem segment. Arrows indicate perithecia.

No isolate among the 21 tested displayed self-fertility (Table 3.2.). Cross-fertility was observed in 50 combinations out of a total of 231, which corresponded to 22%. Two incompatibility groups were observed. Seven isolates (CT-20, CT-30, CT-32, CT-38, CT-44, CT-48 and CT-59), cross-fertile with CT-21, were assigned to IG-1. Nine isolates (CT-13, CT-21, CT-37, CT-39, CT-43, CT-45, CT-46, CT-58 and CT-60) were cross-fertile with CT-20 and were assigned to IG-2. Three isolates (CT-28, CT-31 and CT-35) are putative IG-2 isolates, as they were cross-fertile with neither CT-20 nor CT-21, but displayed fertility when crossed with some isolates of IG-1. Two isolates (CT-34 and CT-47) displayed no fertility. The pattern of fertile crosses is consistent with that of a unilocular, biallelic mating system, characterized by two cross-compatibility groups. However, 34 crosses that should be fertile under a perfect bipolar model showed no fertility.

Fertility was highly variable among crosses. For example, the cross CT-38 × CT-45 was considered fertile although only one perithecium formed in four replicate dishes, whereas others,

like CT-20 × CT-21, were producing over one hundred perithecia per replicate. Fertility was also variable within replicates of the same cross.

Only two isolates from soybean (CT-51 and CT55), as well as two from scentless chamomile (CT-64 and CT-65), produced enough spores to permit crossings to be performed. In the case of the two soybean isolates sporulation was so low that only cross-fertility could be tested. Numerous attempts to grow the other isolates on various media were unsuccessful in terms of spore production. No fertile crosses between the lentil tester isolates, CT-21 and CT-30, and isolates from soybean were observed (Table 3.3). Chamomile isolates did not cross with lentil isolates either, nor with each other (Table 3.4 and 3.5). CT-20 and CT-21 produced perithecia both on lentil stems and on scentless chamomile stems, but on the latter, the number of perithecia was lower (2 perithecia within the 4 replicates on chamomile stems compared to 36 on lentil stems).

Table 3.2: Sexual compatibility of twenty-one *Colletotrichum truncatum* isolates from lentil. (+): presence of at least 1 perithecium within four repeats; (-): absence of perithecia.

	CT-13	CT-20	CT-21	CT-28	CT-30	CT-31	CT-32	CT-34	CT-35	CT-37	CT-38	CT-39	CT-43	CT-44	CT-45	CT-46	CT-47	CT-48	CT-58	CT-59	CT-60
CT-13	-																				
CT-20	+	-																			
CT-21	-	+	-																		
CT-28	-	-	-	-																	
CT-30	+	-	+	-	-																
CT-31	-	-	-	-	-	-															
CT-32	-	-	+	-	-	-	-														
CT-34	-	-	-	-	-	-	-	-													
CT-35	-	-	-	-	+	-	-	-	-												
CT-37	-	+	-	-	+	-	-	-	-	-											
CT-38	-	-	+	+	-	-	-	-	+	-	-										
CT-39	-	+	-	-	+	-	+	-	-	-	+	-									
CT-43	-	+	-	-	+	-	-	-	-	-	+	-	-								
CT-44	-	-	+	-	-	+	-	-	+	+	-	+	+	-							
CT-45	-	+	-	-	-	-	-	-	-	-	+	-	-	-	-						
CT-46	-	+	-	-	-	-	-	-	-	-	+	-	-	-	-	-					
CT-47	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-				
CT-48	+	-	+	-	-	-	-	-	+	-	-	+	+	-	+	+	-	-			
CT-58	-	+	-	-	+	-	-	-	-	-	+	-	-	-	-	-	-	+	-		
CT-59	+	-	+	-	-	-	-	-	-	-	-	+	+	-	-	+	-	-	+	-	
CT-60	-	+	-	-	+	-	-	-	-	-	-	-	-	+	-	-	-	+	-	+	-

Table 3.3. Sexual compatibility of *Colletotrichum truncatum* isolates from *Glycine max* with lentil isolates CT-20 and CT-21. Positive (+): presence of at least 1 perithecium within the repeats; Negative (-) : absence of perithecia. (/) not tested.

	CT-20	CT-21
CT-20	/	+
CT-51	_	_
CT-55	_	_

Table 3.4. Sexual compatibility of *Colletotrichum truncatum* isolates from *Matricaria perforata* with lentil isolates CT-20 and CT-21 on lentil stems. Positive (+): presence of at least 1 perithecium within the repeats; Negative (-) : absence of perithecia. (/) not tested.

	CT-20	CT-21	CT-64	CT-65
CT-20	/	+	_	_
CT-64	_	_	_	_
CT-65	_	_	_	_

Table 3.5. Sexual compatibility of *Colletotrichum truncatum* isolates from *Matricaria perforata* with lentil isolates CT-20 and CT-21 on scentless chamomile stems. Positive (+): presence of at least 1 perithecium within the repeats; Negative (-) : absence of perithecia. (/) not tested. [1]Only two perithecia in one replicate (less than on lentil);

	CT-20	CT-21	CT-64	CT-65
CT-20	/	+[1]	_	_
CT-64	_	_	_	_
CT-65	_	_	_	_

3.3.2. Amplification of conserved regions of the MAT genes

The primer pair DeLCP2 generated two DNA fragments of approximately 290 and 340 bp for all nine *C. truncatum* isolates, and a unique fragment for *C. graminicola.* Direct sequencing of the PCR products for isolates CT-21 (IG-2) and CT-30 (IG-1) (Annexe 1) gave a consistent single sequence suggesting that the two fragments obtained for each isolate correspond to the same gene. The *C. truncatum*-specificity of the primer pair CT21HMG was confirmed by the presence of a single band for each *C. truncatum* isolates, and a corresponding lack of amplification in the *C. graminicola* isolate (Figure 3.2 A and B). Regarding the alpha-box

of the MAT1-1 gene, primers Falpha1 and Falpha2 produced, as expected, a weak band just over 350bp in the *F. oxysporum* isolate of mating type 1 only (Arie *et al*., 2000). No band was obtained from any of the *C. truncatum* isolates or the *F. oxysporum* isolate of mating type 2 (Figure 3.2. C).

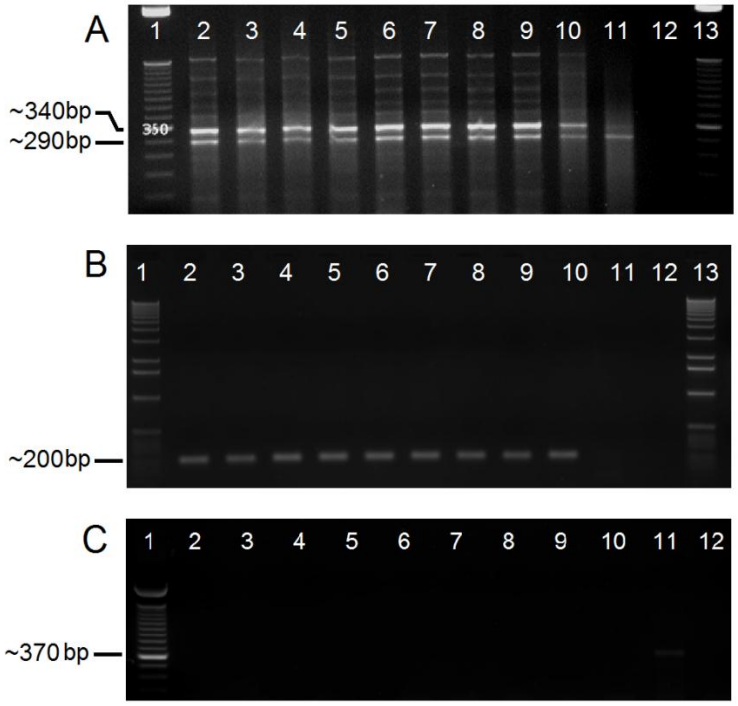

Figure 3.2. PCR amplification of conserved regions of the mating type idiomorphs. A and B: amplification of the HMG box of MAT1-2 with primer pair DeLCP2 (A) and CT21HMG (B). Lane 1 and 13: 1Kb DNA ladder. Lane 2 to 10: *Colletotrichum truncatum* isolates CT-20, CT-21, CT-30, CT-31, CT-39, CT-45, CT-58, CT-59 and CT-60. Lane 11: negative control (*C. graminicola*). Lane 12: negative control (water). C: amplification of the alpha domain of MAT1-1 with primers pair Falpha. Lane 1: 100pb DNA ladder. Lane 2 to 10: *C. truncatum* isolates CT-20, CT-21, CT-30, CT-31, CT-39, CT-45, CT-58, CT-59, and CT-60. Lane 11: positive control (*Fusarium oxysporum* of mating type 1). Lane 12: negative control (*F. oxysporum* of mating type 2).

3.4. Discussion

In this study, the inability of all 21 isolates of *C. truncatum* from lentil tested to self-fertilize suggests that the species is heterothallic. The pattern of fertile combinations is compatible with a bipolar self-incompatibility mating system. Some crosses were recorded as sterile when fertility was expected under the hypothesis of a bipolar mating system, but this could be explained by a fertility level below that detectable with the number of replications used in this study. Some combinations were considered fertile with only one perithecium counted; therefore combinations of isolates with an even more reduced fertility may have been considered sterile. Variability in fertility is a common feature in filamentous ascomycetes. Female sterility, in particular, was reported to be common in *Gibberella fujikuroi* field populations (Leslie, 1995), whereas male sterility was rare. The high differentiation of the female reproductive structures, as opposed to the male ones, and the complexity of their genetic control, means numerous loci can be mutated and be the cause of a sterile phenotype (Leslie and Klein, 1996). Several such genes were identified in the *G. fujikuroi* species complex (Hornok *et al*. 2007). In the genus *Glomerella,* significant variation in fertility has been reported for *G. cingulata* and *C. graminicola* (Edgerton 1914, Bryson *et al*., 1992, Vaillancourt *et al*., 2000a).

Genetic analyses of mating system have been performed for a few *Glomerella* spp., but the genetic control of sexual compatibility remains mainly unresolved. Both heterothallic and homothallic mating strategies were reported in the genus, sometimes within the same species (Vaillancourt and Hanau, 1991). Additionally, information obtained for three species, *G. cingulata* (Wheeler, 1954; Cisar and Tebeest, 1999), *C. graminicola* (Vaillancourt *et al.*, 2000b) and *C. lindemuthianum* (Rodriguez-Guerra *et al.*, 2005) suggests that those species all have unusual mating systems that do not follow the classical bipolar self-incompatibility mating system of filamentous ascomycetes. Multilocus systems were proposed for *C. graminicola* (Vaillancourt and Hanau, 1991, Vaillancourt, 2000b) and *G. cingulata* from *Ipomoea* spp. (Wheeler and McGahen, 1952), whereas a unilocus, multiallelic model was proposed by Cisar and Tebeest (1999) for *G. cingulata* from pecan.

The genus *Glomerella* is a very challenging genus in regard to its taxonomy. The genus as such is well-defined, considering that *Glomerella* is the only genus in the family Glomerellaceae (Zhang *et al.*, 2006). However, determining species within this genus has proven

to be very complicated (Skipp et *al.*, 1995). This is of concern in *C. truncatum sensu lato* as the species has been isolated from diverse hosts, but the relationships among isolates is poorly understood, hence the inclusion of cross-fertility experiments with isolates from different hosts in this study.

The biological species concept as proposed by Mayr in 1940 defines the species as 'groups of actually or potentially interbreeding natural populations which are reproductively isolated from other groups', but this diagnostic tool for species was already in use earlier (Shear and Dodge, 1927). The major limitation of this method in fungi is that it is only applicable to species for which sexual reproduction exists. Additionally, there are certain precautions to be taken when working with the biological concept, as mating tests in the laboratory have the tendency to artificially overestimate mating compatibility. Hybridization is sometimes possible between species that may be genetically isolated under natural conditions (reviewed in Harrington and Rizzo, 1999; Taylor *et al*., 2000). Furthermore, some distinct species may retain ancestral traits of reproductive compatibility (Rosen, 1979). However, the crossing of compatible isolates can be a powerful tool when coupled with other methods of species recognition.

No fertile crosses were recorded between *C. truncatum* isolated from lentil and isolates from soybean or scentless chamomile, but the very small number of crossings performed do not permit to draw conclusions. More crossings should be performed to confirm cross-sterility. It is noteworthy that cross-fertile isolates from lentil were able to mate both on lentil and on scentless chamomile stems, but that on the latter, the number of reproductive structures was lower, indicating that cues from the host tissue may be involved in triggering sexual reproduction. Not much direct evidence for specific host recognition by fungal pathogens have been described to date (Bignell *et al*., 2004), and any indirect evidence presented, including in *Colletotrichum* species, is mostly related to the phase of the initial infection (Kolattutudy *et al.,* 2000, Tucker and Talbot, 2001). Perithecia induction has been studied on various natural and synthetic media in many fungal species, but the involvement of specific plant-derived compounds in the switch from asexual to sexual behavior is poorly understood. In *Pestalotiopsis microspora*, an ascomycete associated with yew, production of perithecia occurred on whole *Taxus cuspidata* needles, but not after the needles were treated with methylene chloride. However, the methylene

chloride extract induced the formation of perithecia, suggesting the existence of one or several hydrophobic perithecial-stimulating factor(s) derived from the host (Metz *et al.*, 2000).

The presence of the HMG box of the MAT1-2 idiomorph in isolates of both compatibility groups of *C. truncatum* is consistent with the results obtained for *C. graminicola* and *C. lindemuthianum* where a MAT1-2 idiomorph was found in both partners of fertile crosses. However, it contradicts the assumption of a bipolar self-incompatibility mating system controlled through the MAT locus as described for other ascomycetes, and as suggested by the cross-fertility pattern of *C. truncatum* in the classical crossing experiment. Hence, for each of the four *Glomerella* species studied to date, a different model of sexual reproduction has been proposed, but none are matching the usual ascomycete mating system. Those types of atypical mating systems have not been reported from other genera, including closely related genera in the Hypocreales and the Microascales. However, the latter includes the genus *Ceratocystis*, were mating-type switching has been reported (Harrington and McNew, 1997).

True heterothallism is controlled by mating-type loci (Raju, 1992), but a heterothallic phenotype can be obtained through different genetic control mechanisms. Wheeler (1954) proposed that *G. cingulata* showed what he called 'unbalanced heterothallism'. In this model, each partner of a fertile cross carries mutated fertility genes that can complement each other resulting in cross-fertility. Based on this model, it has been suggested that heterothallism in a genus like *Glomerella* could be the result of a mutation in the developmental pathway for homothallism, and this mating system has been proposed for *C. graminicola* (Vaillancourt *et al.*, 2000a, 2000b).

There is no direct molecular evidence for the kind of genetic process that controls the unbalanced heterothallic phenotype proposed for *Glomerella* species. However, recombinations and fusions between both MAT idiomorphs have been documented, and are believed to be the key process in shifting between heterothallism and homothallism (Yun *et al.*, 1999). There has been much debate about which reproductive mode, homothallism or heterothallism, is ancestral (Metzenberg and Glass, 1990; Coppin *et al.*, 1997; Geiser *et al.*, 1998). Based on phylogenic evidence pertaining to the mating type locus organization, it has been suggested that the ascomycetous species *Neurospora crassa* and *Podospora anserina* may have been derived from a single homothallic ancestor, and that heterothallism was acquired concomitantly in a second step by both species, possibly because sexual reproduction was conferring some fitness

advantage (Coppin *et al.*, 1997). In contrast, studies by Yun *et al.* (1999) on *Cochliobulus* suggested that heterothallism was ancestral, and that homothallism developed through rare homologous recombination and unequal crossover events made possible by short identical sequences in the open reading frame of the MAT genes. This was based on the observation that in the genus *Cochliobolus*, the heterothallic species share a same organization at MAT loci, whereas each homothallic species has its own arrangement, strongly supporting the evolution of homothallism from heterothallism (Yun *et al.*, 1999). Although these findings are not directly applicable to the genus *Glomerella*, where both partners of a fertile cross are carrying the HMG box, they indicate a high plasticity at the mating type loci, and a potential for recombination in these areas.

In conclusion, isolates of *C. truncatum* tested in this study exhibited a cross-fertility pattern consistent with a mating system that is determined by one single locus with two alleles, but differed in that all isolates had the MAT1-2 idiomorph. Studies in other *Glomerella* species suggested that these mating systems could be explained through unbalanced heterothallism which could also apply to *C. truncatum*. To better understand the mating system of *C. truncatum,* studies on the inheritance of the mating types as determined by classical mating studies are required. Sequencing of the MAT1-2 idiomorph is currently in progress (Adrian Cabral, pers. comm.).

3.5. Prologue to chapter 4

This mating study among 21 isolates suggested that *C. truncatum* is a heterothallic fungus because fertile combinations occurred only when two different isolates were in contact. The data also support the hypothesis of a bipolar mating system. However, the existence of a copy of the MAT1-2 gene in partners of a fertile cross suggests that *C. truncatum* does not share the typical ascomycete genetic control for sexual reproduction, and several questions about the sexual reproduction in *C. truncatum* remain unanswered. First, considering the fact that this species belongs to a genus where induction of selfing has been reported, it is essential to verify that the progeny obtained from a fertile cross is effectively issued from both parents. Secondly, the experiments described in Chapter 3 were performed under laboratory conditions, between isolates differing in terms of sampling locations and dates, which does not permit to draw conclusions on whether sexual reproduction occurs under natural conditions. The following study was performed to provide some answers to these questions.

CHAPTER 4

GENETIC BASIS FOR CROSS-FERTILITY AND CROSS-FERTILITY OF FIELD ISOLATES OF *COLLETOTRICHUM TRUNCATUM*

4.1 Introduction and objectives

Induction of selfing of an isolate by the presence of another isolate (induced homothallism) is known to happen in *G. cingulata* (reviewed in Vaillancourt *et al.*, 2000a). This fact raises a real concern when working on species within the *Glomerella* genus. Traditionally, when two different isolates are crossed in classical mating studies, the development of sexual structures is considered proof of a heterothallic cross resulting in a progeny issued from two parents as long as such fruiting structures do not arise during selfing. In case of induced homothallism this is not the case, and it is imperative to perform genetic studies in order to demonstrate the presence of genetic material from both parents in the progeny. This can be achieved by following markers differing between the parents in the progeny. Historically, mutations responsible for morphological differences between parental isolates were used, and are still a powerful tool in certain cases nowadays (Vaillancourt *et al.*, 2000b). However, obtaining of mutants with useful morphological markers is a random and often long process. Molecular markers have numerous advantages, being easier to develop and giving direct access to the genotype of the individual studied.

The ability of *C. truncatum* from lentil to sexually reproduce under laboratory conditions that are not very different from those potentially encountered in the field raises the possibility for mating of *C. truncatum* to occur in nature. Sexual structures have not been reported from *C. truncatum* in natural conditions. Rather than attempting to detect ascospores or perithecia in lentil fields, this study was undertaken to investigate the potential for mating to occur in the field by determining whether the two mating incompatibility groups are coexisting on a small scale in nature, and if co-existing isolates are effectively cross-fertile.

To confirm heterothallism and better understand the mechanisms of cross fertility in *C. truncatum*, random spore analysis was performed. Crosses and backcrosses between parents and

progeny of one fertile cross were performed, as well as genetic analysis using AFLP fingerprinting.

4.2. Materials and methods

4.2.1. Collection and maintenance of isolates

Cross-fertile isolates CT-21 and CT-30 (Armstrong and Banniza, 2006) were used as mating type tester isolates, as well as parental isolates for obtaining progeny. Isolate CT-21 was collected during the crop year 2001 in the region of North Battleford, SK, Canada. Isolate CT-30 was sampled the same year at Drinkwater, SK, Canada, around 350 kilometers south-east of North Battleford. Isolates cannot be designated as mating types (MAT) 1 and 2 as described for other ascomycetes because cross-fertile *C. truncatum* isolates tested to date carry the same MAT idiomorph. Thus, CT-21 was assigned to be of Incompatibility Group 2 (IG-2), and CT-30, of IG-1.

For the field population study, a total of 27 monoconidial cultures were obtained from a field near Dysart in south-east Saskatchewan, Canada, in 2008 following a hierarchical sampling strategy. Infected lentil material was collected in three sampling spots across the field, collecting samples from five neighbouring plants, and isolating up to four isolates per plant (Fig. 4.1.). This sampling strategy was chosen to include isolates from restricted areas, within which watersplash dispersion can occur over short distances (neighbouring plants from the same sampling spot), as well as isolates sampled over greater distances from one another (sampling spots up to 44 metres apart).

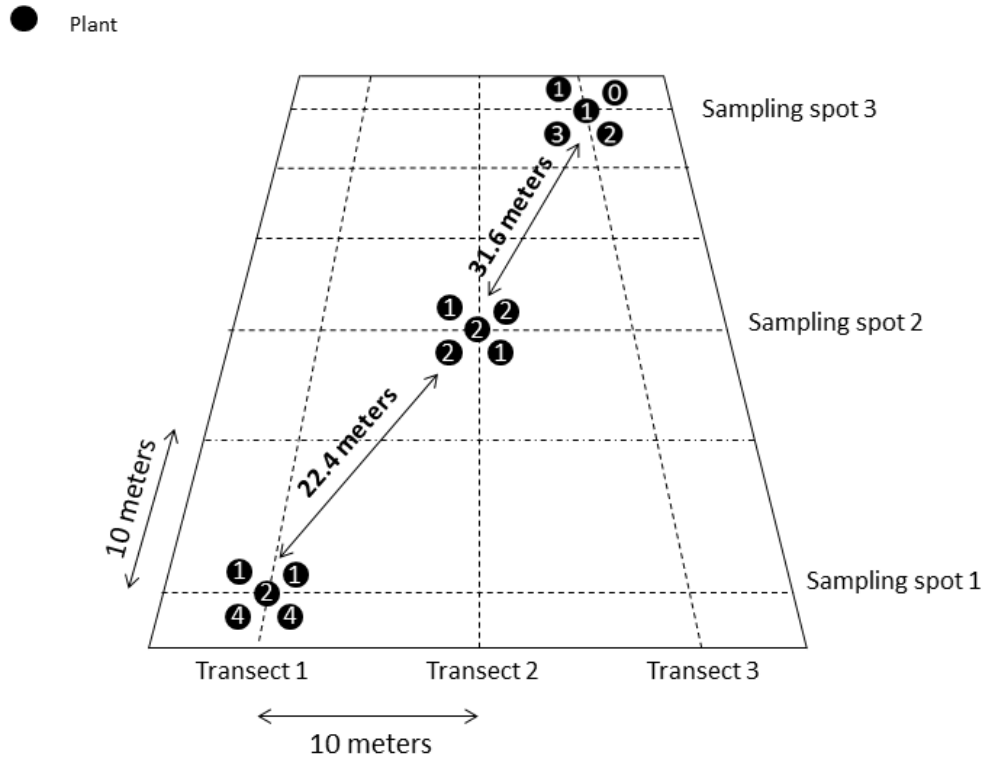

Figure 4.1. Hierarchical sampling of a lentil field infested with *Colletotrichum truncatum* near Dysart, SK, Canada showing the three sampling spots. The five plants sampled per spot are indicated by black circles. The numbers inside the circles indicate the number of isolates recovered per plant.

To obtain monoconidial isolates, stem lesions were excised from the plant and were surface-sterilized for 3 minutes in a 10% sodium hypochlorite solution, washed in sterile water and placed in Petri dishes containing PDA (potato dextrose agar) amended with streptomycin. After a few days of incubation at 22°C, colonies looking like *C. truncatum* were examined under the microscope for morphological features characteristic of *C. truncatum*. For some tissues heavily contaminated by other fungal species, conidial masses from acervuli thought to be of *C. truncatum* were dislodge with a scalpel and streaked onto PDA amended with antibiotics. After 24 h, three germinated conidia were transferred individually to new plates with the help of a dissecting microscope. The exact location of each isolate in the field was recorded.

For the progeny study, isolates obtained in earlier studies were used (Aurélie Cohen-Skali, pers. comm.). Thirteen isolates were obtained randomly from a single perithecium issued from the cross CT-21 × CT-30. Ascospore derived isolates were named GT for *Glomerella*

truncata to emphasize the fact they were obtained by sexual reproduction between the two field isolates. The thirteen GT isolates used in this study are GT-147, GT-148, GT-149, GT-150, GT-151, GT-152, GT-153, GT-155, GT-156, GT-157, GT-158, GT-162 and GT-163. All isolates were maintained as described in 3.2.1.

4.2.2. Mating of isolates

The mating of isolates was performed as described in 3.2.2.

For the field population study, the 27 *Colletotrichum* isolates were crossed with the tester strains CT-21 and CT-30. Additionally, one of the field isolates, CT-188, was crossed with field isolates CT-185, CT-186, CT-187, CT-189, CT-190, CT-191 and CT-192.

For the progeny study, all *Glomerella* isolates were crossed with both parental isolates, CT-21 and CT-30. Additionally, five *Glomerella* isolates (GT-147, GT-149, GT-150, GT-153 and GT-163) were arbitrarily chosen to be crossed in all possible combinations, including selfings.

4.2.3. Statistical analysis

To test the null hypothesis of an equal ratio of both mating types in both the field isolate population and the progeny population, as well as the segregation of parental markers in the progeny, the values of one-dimensional chi-square goodness of fit test were calculated. The statistic (χ^2) was defined by the following equation:

$$\chi^2 = \Sigma [(O - E)^2 / E]$$

where O_i is the observed frequency count for the categorical variable, and E_i is the expected frequency count for the categorical variable. The statistic was then tested against a χ^2 distribution. This permitted to examine deviation from the expected 1:1 ratio of both mating compatibility groups or parental markers.

4.2.4. DNA extraction

Procedures for mycelium production in liquid culture and for DNA extraction from the parental isolates CT-21 and CT-30, as well as the 13 *G. truncata* isolates were as described in 3.2.2.

4.2.5. AFLP analysis

AFLP analysis was performed using the DNA obtained from the 13 *G. truncata* isolates as well CT-21 and CT-30, according to Vos *et al.* (1995) with some modifications. Digestion and ligation of DNA were performed with the IRDye Fluorescent AFLP kit for Large Plant Genome Analysis (LI-COR Biosciences). A 1:10 dilution of the PCR products was performed for use in pre-amplification.

For pre-amplification, the oligonucleotides primers EcoRI + A and MseI + C were used. PCR was performed as follows: for a 50 µL reaction, 2.5 µL of diluted digested/ligated mixture were added to 5 µL 10X PCR reaction buffer (100 mM Tris-HCl (pH 8.3), 150 mM Mg-acetate, 500 mM K-acetate)), 1.5 µL of 50 mM $MgCl_2$, 1 unit of taq polymerase and 40 µL of pre-amplification primer mix containing nDTPs and both primers. The PCR reactions were adjusted to 50 µL with double distilled sterile water. Pre-amplification primer mix contained 10 µL of MseI primer at 300 ng/µL, 10 µL of EcoRI primer, 800 µL of dNTP (1.25 mM each) and 3180 µL double distilled sterile water. For amplification, a Perkin Elmer 9600 Thermal Cycler was used. The program was set as follows: 96°C for 30 seconds, 56°C for 60 seconds and 72°C for 60 seconds, for twenty cycles. A 1:50 dilution of the PCR products was performed for use in selective amplifications.

The preamplification step was followed by a selective amplification PCR. It was performed as follows: for a 20 µL reaction, 2.5 µL of diluted pre-amplified template were added to 2 µL 10X PCR reaction buffer (100 mM Tris-HCl (pH 8.3), 150 mM Mg-acetate, 500 mM K-acetate)), 0.6 µL of 50 mM $MgCl_2$, 1 unit of taq polymerase, 0.1 µL of 300 pM E-primer, 0.09 µL of 350 pM M-primer (Table 2) and 1.6 µL of 10 mM nDTPs. The PCR reactions were adjusted to 20 µL with double distilled sterile water. The program was set as follows: 94°C for 60 second, 65°C for 60 seconds and 72°C for 90 seconds, for ten cycles, decreasing the annealing temperature by 1°C per cycle, then, 94°C for 30 seconds, 56°C for 30 seconds and 72°C for 60 seconds for 23 cycles.

Denaturing 6% polyacrylamide gel electrophoresis was prepared with 29.4 g urea, 6 mL ×10 TBE, 9mL acrylamide/bisacrylamide mix and 24 mL distilled water. To catalyze the polymerization of acrylamide, 70 µL of TEMED and 70 µL of ammonium persulfate were added just before casting. The gels were run for 2 to 3 hours depending on the primers, to obtain the best band separation. DNA bands were visualized by silver staining.

Eleven primer pairs were tested. Five primer pairs generated easily scored, polymorphic bands, and were used to screen the two parental isolates CT-21 and CT-30 and their thirteen progeny. Those primer pairs are: E-AC / M-CA, E-AC /M-CT, E-AG / M-CG, E-AG / M-CT and E-AG / M-CA (Table 4.1.).

Table 4.1. Selective primers used to screen parental *Colletotrichum truncatum* isolates CT-21 and CT-30, and the 13 ascospore-derived progeny isolates.

Name of primer in text	Primer sequence
E-AC	5'-GACTGCGTACCAATTCAC-3'
E-AG	5'-GACTGCGTACCAATTCAG-3'
M-CA	5'-GATGAGTCCTGAGTAACA-3'
M-CG	5'-GATGAGTCCTGAGTAACG-3'
M-CT	5'-GATGAGTCCTGAGTAACT-3'

4.3. Results

4.3.1. Progeny study

Each of the thirteen *G. truncata* isolates was tested for their ability to mate with parental isolates CT-21 and CT-30. All of them were cross-fertile with one and only one of the parents. Six isolates were cross-fertile with CT-21 and were thus IG-1 isolates, whereas seven isolates crossed with CT-30, thus belonged to IG-2 (Table 4.2). Five progeny isolates were also crossed in all possible combinations. No isolate displayed self-fertility. The pattern of cross fertility between those five isolates was compatible with a bipolar mating system with two groups of cross-fertile isolates (Table 4.3.). The ratio of 6:7 IG-1/IG-2 did not differ significantly from the expected ratio of 1:1 (χ^2 observed = 0.1; $\chi^2_{0.05}$ 1:1 = 3.84,), suggesting the mating types are present at equal frequencies.

Table 4.2. Sexual compatibility between *Colletotrichum truncatum* tester strains CT-21 and CT-30, and thirteen *Glomerella truncata* isolates. Positive (+): presence of at least 1 perithecium within the four replicate; Negative (-): absence of perithecia.

	CT-21	CT-30
GT-147	+	-
GT-148	+	-
GT-149	+	-
GT-150	+	-
GT-151	-	+
GT-152	+	-
GT-153	+	-
GT-155	-	+
GT-156	-	+
GT-157	-	+
GT-158	-	+
GT-162	-	+
GT-163	-	+

Table 4.3. Sexual compatibility between five *Glomerella truncata* isolates. Positive (+): presence of at least 1 perithecium within the four replicates; Negative (-): absence of perithecia.

	CT-21	CT-30	GT-147	GT-149	GT-150	GT-153	GT-163
CT-21							
CT-30	+						
GT-147	+	-	-				
GT-149	+	-	-	-			
GT-150	+	-	-	-	-		
GT-153	+	-	-	-	-	-	
GT-163	-	+	+	+	+	+	-

4.3.2. AFLP analysis

Each of the five primer pairs used to screen the isolates lead to a single strong marker polymorphic between the parents. For each marker, both parental types were found in the progeny.

Eight of the progeny isolates had the same haplotype as one of the parents: seven *Glomerella* isolates shared their haplotype with CT-21 and one with CT-30. The five other isolates showed non-parental haplotypes of 3 different kinds. Among those, isolates GT-148 and GT-153 were displaying bands typical of both parents (Table 4.4.).

None of the five markers were linked to mating incompatibility groups.

Table 4.4. Haplotypes and mating type of parental *Colletotrichum truncatum* isolates CT-21 and CT-30 and their progeny. Blue and pink shading designates parental haplotypes. * indicate deviation of parental markers from the 1:1 ratio (χ^2 test, P<0.05).

Isolates	Mating type	Primer pairs				
		E-AC/M-CA	E-AC/M-CT	E-AG/M-CG	E-AG/M-CT	E-AG/M-CA
CT-21	B	0	1	0	0	0
CT-30	A	1	0	1	1	1
GT-147	A	0	1	0	0	0
GT-148	A	1	0	1	1	1
GT-149	A	1	1	1	1	0
GT-150	A	0	1	0	0	0
GT-151	B	0	1	0	0	0
GT-152	A	0	1	0	0	0
GT-153	A	1	1	1	1	0
GT-155	B	0	0	0	0	1
GT-156	B	0	0	0	0	0
GT-157	B	0	0	0	0	0
GT-158	B	0	1	0	0	0
GT-162	B	0	1	0	0	0
GT-163	B	0	1	0	0	0
Segregation of markers in progeny (1:0)		3:10	9:4	3:10	3:10	2:11*

4.3.3. Study of field isolates

Twenty-seven field isolates were crossed with tester isolates CT-21 and CT-30. One isolate (CT-274) did not grow. Of the remaining 26 isolates, 7 (27%) isolates crossed with CT-21 and belong to IG-1, 9 (35%) isolates crossed with CT-30 (IG-2), and 10 (38%) did not cross with either tester isolates. Both mating types were found at each sampling spot, and on three occasions even on the same plant (Fig. 4.2). Isolate CT-188, which was determined to be of IG-1, was crossed with the seven isolates of IG-2 sampled at the same spot. In three cases, perithecia were formed (Table 4.5). Fertility varied but was of the same magnitude as that of the control cross CT-21 × CT-30.

The ratio of 7:9 for IG-1 : IG-2 did not differ significantly from the expected ratio of 1:1 (χ^2 1:1 = 0.06, P>0.01), suggesting the mating types are present at equal frequencies.

Table 4.5. Sexual compatibility between *Colletotrichum truncatum* isolates. Positive (+): presence of at least 1 perithecium within the four replicates; Negative (-): absence of perithecia.

	CT-188
CT-185	-
CT-186	+
CT-187	+
CT-189	+
CT-190	-
CT-191	-
CT-192	-

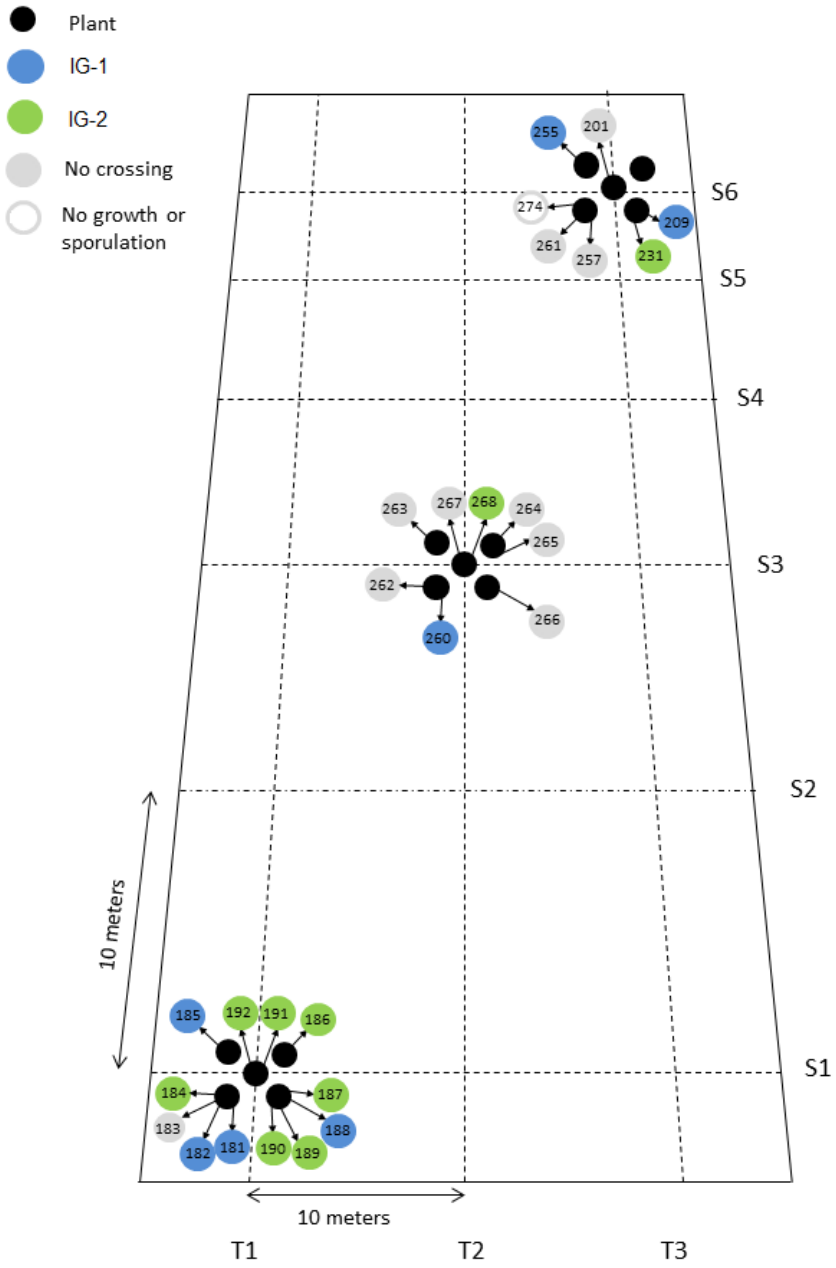

Figure 4.2. Sampling location and mating types of *Colletotrichum truncatum* isolates collected in a lentil field near Dysart, SK, in 2008. IG-1: incompatibility group 1; IG-2: incompatibility group 2.

4.4. Discussion

In *C. truncatum*, agent of lentil anthracnose, the teleomorphic state can be induced under laboratory conditions (Armstrong-Cho and Banniza, 2006), and the previous study (Chapter 3) suggested that this species is heterothallic. However, as the induction of selfing has been reported in the genus *Glomerella*, classical crossing studies are not sufficient proof for heterothallism in this genus. This study on *C. truncatum* isolates issued from sexual reproduction used molecular makers to show the occurrence of recombinant progeny, and thus confirmed that this species is heterothallic.

Sexual reproduction seems to occur infrequently in *Colletotrichum* species. To date, most species lack a teleomorph. In sexual species, both heterothallic and homothallic mating strategies have been reported. Extensive mating studies performed in *G. cingulata* resulted in the discovery of both homo- and heterothallic strains (Driver and Wheeler, 1955). The same holds true for *C. graminicola* (Vaillancourt and Hanau, 1991). The teleomorph of *C. destructivum*, *G. glycines*, was obtained under laboratory conditions by Manandhar and colleagues (1986). The results of their study seem to indicate that this species is homothallic. Other species where homothallic isolates were recovered are *C. lindemuthianum* (Shear and Wood, 1913) and *C. falcatum* (von Arx and Muller, 1954 in Cai *et al*., 2009). However, *G. cingulata*, *C. lindemuthianum* and *C. graminicola* seem to prefer outcrossing (Bryson *et al*., 1992).

In *G. cingulata*, induction of selfing of sterile isolates by fertile isolates was reported early (Chilton *et al*., 1945). Under specific laboratory conditions, self-sterile or near self-sterile isolates of *G. cingulata* could be induced to self-fertilize after application of a filtrate of the liquid media in which self-fertile isolates were grown, suggesting the implication of a sexual hormone (Driver and Wheeler, 1955). Induction of homothallism has also been studied in the oomycete genus *Phytophthora*. This genus is peculiar in that sexual reproduction is easy to obtain between morphologically and physiologically well distinguished taxa. Ko (1988) showed under laboratory conditions in several species the absence of genetic exchange in seemingly heterothallic intraspecific crosses, suggesting these *Phytophthora* isolates are in fact homothallic, but are unable to self without an exogenous hormonal induction from an isolate of the opposite mating type. Interestingly, selfing occurs also when isolates of *P. cinnamomi* of mating type A2 are put in presence of species of the ascomycetous genus *Trichoderma* (Brasier, 1978).

Various methods were used to demonstrate outcrossing in crosses between *Colletotrichum* isolates. Recombinants were identified in the progeny obtained from the cross between two *C. lindemuthianum* isolates using RAPD markers (Camargo Jr *et al*., 2007). In *C. musae*, heterothallism was shown by crossing a chlorate-resistant mutant with a chlorate-sensitive wild isolate. Results were confirmed with RAPD markers (Rodriguez and Owen, 1992). RFLP markers were used to confirm heterothallism in *G. cingulata* (Cisar and TeBeest, 1999). In the current study on *C. truncatum* isolated from lentil, genetic markers for both parental isolates were found in the progeny obtained from a cross involving CT-21 and CT-30, indicating outcrossing. Some of the progeny was sharing its haplotype with one or the other parent, while others were recombinant and carried markers originating from both parents. Perithecial development has been studied in detail in *G. cingulata* (McGahen and Wheeler, 1951). The authors showed that all asci of a given perithecium of this species are arising from a single dikaryotic cell issued from plasmogamy, hence all asci in a perithecium are of the same origin. Experimental data suggested an identical development in *C. graminicola* (Vaillancourt *et al.*, 2000a). Assuming that the same applies to *C. truncatum*, the genetic diversity observed within the progeny of the cross CT-21 x CT-30 is probably the result of the meiosis immediately following karyogamy. The presence of ascospore-derived progeny with markers of both parents confirms that the progeny are the product of a recombination of genes from both parents as all isolates are issued from the same perithecium, hence the same genetic stock. The high proportion of non-parental haplotypes in the progeny suggests that four of the five markers are either very loosely linked, or unlinked, possibly being located on different chromosomes. Additionally, at least one parental marker does not seem to segregate normally. However, the sample size, while sufficient for confirming heterothallism, is not adequate for segregation studies. A larger sample would be required to confirm these results. Both mating types are present in the progeny and the pattern of fertile crosses within the progeny and in backcrosses with the parents is compatible with a bipolar mating system as proposed in Chapter 3.

In the genus *Colletotrichum*, the sexual state has been reported in the following species: *C. acutatum* (*G. acutata*), *C. destructivum* (*G. glycines*), *C. falcatum* (*G. tucumanensis*), *C. fioriniae* (*G. acutata* var. fioriniae), *C. fructicola* (*Glomerella* sp.), *C. gloeosporioides* (*G. cingulata*), *C. gossypii* (*G. gossypii*), *C. graminicola* (*G. graminicola*), *C. lindemuthiana* (*G. lindemuthiana*), *C. musae* (*G. musarum*), *C. orbiculare* (*G. lagenaria*), *C. phormii* (*G. phormii*),

C. sublineola (*Glomerella* sp.)(compiled in Hyde *et al.*, 2009), but in most cases the sexual stage has been obtained under laboratory conditions. Few have been reported under natural conditions. Perithecia of *G. cingulata* from banana were observed in the field in 1964 and 1965 by Kaiser and Lukezic (1966). They were localized on leaves, petioles, bracts and fruits. *Glomerella cingulata* is also responsible for avocado anthracnose in various countries (Prusky, 1996), and the same crop can also be infected by *G. acutata* in New Zealand, Australia and Mexico (Avila-Quezada *et al*., 2007). *Glomerella acutata* has also been determined to cause disease in chili peppers fields in China (Xia *et al.*, 2011), and highbush blueberry in Norway (Talgø *et al*., 2007) and a *Glomerella* species phylogenetically resembling *C. acutatum* was isolated from Norway Maple trees in the USA (LoBuglio and Pfister, 2008). *Glomerella miyabeana* was recorded in Australia on willows (Cunnington *et al.*, 2007), and an undetermined species of *Glomerella* was recovered from postharvest anthracnose lesions on passionfruit (Tarnowski and Ploetz, 2010). The multiplication of recent reports of *Glomerella* in the field suggests that the occurrence of the teleomorphic stage of *Colletotrichum* may be more common than originally thought, at least in some species, and opens the possibility that the teleomorph could play a part in the epidemics of anthracnoses.

Sexual reproduction of *C. truncatum* on lentil has not been reported under natural conditions, but this does not indicate it is absent. While numerous fields in the lentil-growing regions of Canada have been regularly scouted for anthracnose since the emergence of the disease in the late 1980s, there has been no focus on the search for sexual structures. Moreover, technical difficulties hamper identification of sexual structures in the field. Temporally irregular cycles of sexual reproduction, scarcity of reproductive structures, and contamination of plant material by other organisms including saprophytes are some of the many challenges that can be encountered. In this study, isolates from a single field were shown to belong to both mating compatibility groups, and both were found in close physical proximity on single plants. The fact that these isolates are coexisting at such a small scale, coupled with the fact that several of them were cross-fertile under laboratory conditions, indicate a potential for sexual recombination to occur under natural conditions.

In conclusion, this study confirmed that *C. truncatum* is outcrossing by demonstrating that both parental haplotypes were present in the progeny of one perithecium. It also showed that

isolates of both mating types were available in the same field, sometimes on the same plant, suggesting that geographic isolation is not a cause for lack of sexual reproduction.

4.5. Prologue to chapter 5

The previous study confirmed the heterothallism of *C. truncatum* under laboratory condition, highlighting the possibility of genetic recombination in this pathogen if sexual reproduction indeed occurs in the field. It also showed that isolates of different incompatibility groups, that were shown to be cross-fertile under laboratory conditions, coexist in a lentil field, and even on the same plant. This suggests a strong possibility, but no definite proof, that sexual recombination may very well occur in *C. truncatum* in the field. The following study has been undertaken to obtain indirect information about potential sexual reproduction in the field.

CHAPTER 5

ANALYSIS OF THE GENETIC STRUCTURE OF A POPULATION OF *COLLETOTRICHUM TRUNCATUM*, THE AGENT OF LENTIL ANTHRACNOSE

5.1. Introduction and objectives

Despite the fact that sexual reproduction in *C. truncatum* has only been observed under laboratory conditions, and has never been reported in the field, information obtained in Chapter 4 indicated that there was the potential for mating to occur under natural conditions. This is a concern because of the possible consequences of sexual reproduction in the field with regard to the production of air-borne ascospores and the appearance of new races of *C. truncatum*.

Although disease management options like crop rotation and / or fungicide applications are available, control of anthracnose relies significantly on the use of cultivars carrying resistance genes, as this is considered to be the most effective, economically viable and environmentally-friendly strategy. However, this strategy has some limitations when applied to sexually reproducing pathogen populations with high variability, and thus, high plasticity to overcome plant resistance. For this reason, is it essential for a successful breeding program to take into account the biology of the pathogen and its genetic diversity (McDonald and Linde, 2002a). One specific concern in *C. truncatum* is the existence of two pathogenic races, Ct0 and Ct1, with different levels of aggressiveness. Lentil germplasm partially resistant to race Ct1 are available (Buchwaldt *et al*., 1999, 2004), and cultivars have been developed with partial resistance to Ct0. Thus, the emergence of any new race with different modalities of aggressiveness and different responses to available resistant cultivars could have important implications for lentil cultivation in the Prairies.

Additionally, the presence or absence of wind-blown ascospores is an important parameter in epidemics at spatial and temporal levels. Ascospores can potentially be blown over long distances, whereas conidia are usually water-splashed over short distances only. In several species, eg. *Mycosphaerella graminicola* (Eriksen and Munk, 2003), asexual spores have a prominent role in the early development of epidemics, whereas the role of ascospores is initially minimal but gradually increases in importance as the growing season progresses. Nothing is

known yet about the dispersal of *G. truncata* ascospores, but *G. cingulata*, a species with a teleomorphic stage present under natural conditions, produces ascospores that are wind dispersed (Sutton and Shane, 1983). In the absence of direct evidence of sexual reproduction in the form of reproductive structures under natural conditions, studying the population structure of an organism can reveal information about its reproductive strategy, whether sexual, asexual, or both.

The objectives of this experiment were to study the population structure of Saskatchewan field isolates of *C. truncatum* and to determine whether outbreeding occurs in the field by estimating the extent of the linkage disequilibrium using AFLP data.

5.2. Materials and methods

5.2.1. Collection and maintenance of isolates:

Lentil tissue infected with *C. truncatum* was collected from naturally infected lentil stems from fields in different locations in south Saskatchewan from 2001 to 2008. The collection comprised isolates from a hierarchical sampling of a lentil field near Dysart (south-east of SK) in 2008, and isolates sampled in diverse locations across Saskatchewan (Table 5.1 and Fig. 5.1 and Annex 2).

For isolates obtained during hierarchical samplings, the exact location of each isolate in the field was recorded. Infected plant material was collected from 3 transects approximately 10 meters apart, with 6 sampling points approximately 10 meters apart on each transect.

Plant tissues were surface-sterilized for 3 minutes in a 10% sodium hypochlorite solution, washed in sterile water and placed in Petri dishes containing potato dextrose agar (PDA) amended with antibiotics. After a few days of incubation at 22°C, colonies resembling *C. truncatum* were examined under the microscope for morphological features characteristic of *C. truncatum*. For some tissues heavily contaminated by other fungal species, conidial masses from acervuli thought to be of *C. truncatum* were dislodged with a scalpel and streaked onto PDA amended with antibiotics. After 24 h, three germinated conidia were transferred individually to new plates with the help of a dissecting microscope. A total of 179 isolates were used in this study.

Table 5.1: Origin and year of collection of isolates of *Colletotrichum truncatum* collected from lentil fields in Saskatchewan and used in the population study.

Location	Number of isolates	Year
Belle Plaine	2	2008
Clavet	3	2008
Delisle	1	2008
Dinsmore	2	2008
Drinkwater	1	2001
Dysart	131	2008
Forgan (2 fields)	4	2008
Harris	1	2008
Handel	2	2008
Liberty	2	2008
North Battleford	1	2001
Osage	1	2001
Oungre	1	2001
Regina	3	2004
Regina	2	2008
Rouleau	2	2008
Silton	4	2008
Simpson (2 fields)	2	2008
Tuxford	2	2008
Vanscoy	1	2008
Wilkie	2	2008
Zealandia (2 fields)	2	2008
Other locations (7 fields)	7	2004, 2008
Total	179	

5.2.2 Mycelium production and DNA extraction

Mycelium was grown in liquid cultures and DNA extraction was conducted on the 179 isolates listed in Table 5.1 as described in 3.2.2.

5.2.3. AFLP analysis

To detect strong polymorphic markers, eight isolates *of C. truncatum* (CT-20, CT-21, CT-30, CT-31, CT-39, CT-58, CT-59, CT-60) were chosen as they represented a highly variable set in terms of geographical origin and crop year to maximize the potential for genetic variability and to represent every available combination of mating incompatibility and race identity available. Screening was performed as described in 4.2.5. The primers used for selective amplification are listed in Table 5.2.

Table 5.2. Selective primers used to screen the collection of 179 *Colletotrichum truncatum* isolates.

Name of primer in text	Primer sequence
E-AC	5'-GACTGCGTACCAATTCAC-3'
E-AG	5'-GACTGCGTACCAATTCAG-3'
M-CA	5'-GATGAGTCCTGAGTAACA-3'
M-CG	5'-GATGAGTCCTGAGTAACG-3'
M-CT	5'-GATGAGTCCTGAGTAACT-3'

To test reproducibility of the AFLP profiles, analyses were repeated on a subset of 16 isolates, corresponding to the first line of each 96-well PCR plates. This test showed a high repeatability of the AFLP pattern (100%).

5.2.4. Data analysis:

AFLP profiles were scored visually for presence (1) or absence (0). Only high intensity DNA fragments were used, and each one was considered as a marker. Data were arranged into a binary matrix. Isolates were considered as sharing a haplotype only if all (100%) of the bands were identical and were considered to belong to the same clone.

The genotypic diversity, defined as the probability for two individuals taken randomly to have different genotypes, was also calculated. The value of this index is 0 if the population is totally clonal and 1 if every individual is different.

A clone corrected dataset was used for all additional analyses.

Prior to gametic disequilibrium analyses, population differentiation tests were performed to confirm that the collection of isolates could be considered as a single population. Two datasets comprising two subsamples based on 1) crop years and 2) sampling locations were tested. In the first dataset, isolates from crop years 2001 and 2004 were considered one subpopulation, those from 2008 the other subpopulation. The second dataset encompassed a subsample of isolates from Central Saskatchewan and a subsample from the South-East (Figure 5.1). An exact test of sample differentiation based on haplotype frequencies (Raymond and Rousset, 1995) was performed with the program Arlequin 3.11 using 10 000 Markov chain steps and 5000 dememorization steps. The exact test of sample differentiation tests the hypothesis of random distribution of isolates between pairs of populations. Populations are considered significantly different if the significance level is higher than the P-value of 0.05.

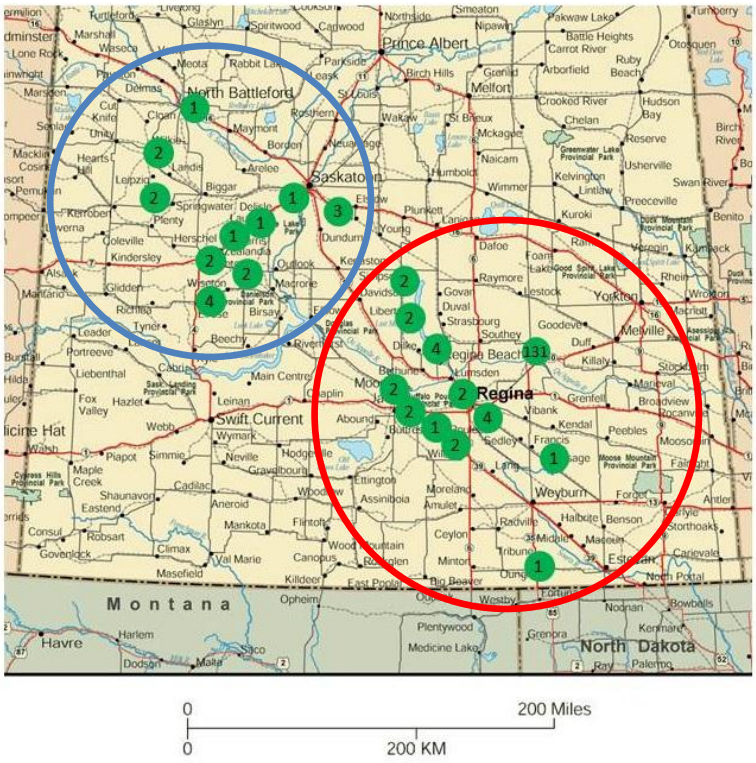

Figure 5.1: Geographical origin of *Colletotrichum truncatum* isolates. Numbers in green dots indicate the number of isolates sampled at each location. The circles correspond to the subsamples created to test for population differentiation based on sampling location.

To analyze gametic disequilibrium, the program MULTILOCUS 1.3b (Agapow and Burt, 2001) was used to calculate the index of association I_A (Brown *et al*., 1980). The multilocus connection disequilibrium $\overline{r}_d$ was also calculated. It is a modification of the index of association I_A that is independent of the number of sampled loci, and thus unbiased (Burt *et al.*, 1999). For both indexes, 1000 artificially recombined datasets were created to determine the statistical value of the test (Agapow and Burt, 2001).

A dendrogram was generated with NTSYS- PC 2.10e software (Exeter Software) using the UPGMA (unweighted pair-group method with arithmetical averages) procedure based on Dice's genetic similarity coefficient (Dice, 1945).

5.3. Results

The five AFLP primer combinations resulted in 91 scorable markers, including 15 (16.48%) that were polymorphic across the dataset. This corresponds to an average of 18.2 markers per primer. A total of 29 distinct haplotypes were found among the 179 *C. truncatum* isolates analyzed. Genotypic diversity was equal to 0.47.

All 131 isolates sampled in a single field in Dysart in 2008 shared the same haplotype, except one isolate, CT-233 which differed from the others by a single marker. However, other isolates did not always group according to geographic location based on the UPGMA cluster analysis (Fig. 5.2). The maximum genetic distance was 0.06.

There was no population differentiation based on geographical location or crop year (p-value = 1 for both set of samples, non-significant at a 0.05 significance level). Accordingly, all isolates were considered as belonging to the same population and used to estimate the gametic disequilibrium indexes. The index of association I_A was equal to 1.274, and the multilocus connection disequilibrium $\overline{r}_d$ was equal to 0.094. In the case of total absence of association of alleles, I_A and $\overline{r}_d$ are expected to equal 0. Here, both were significantly different from 0 ($p<0.001$), indicating the presence of linkage disequilibrium.

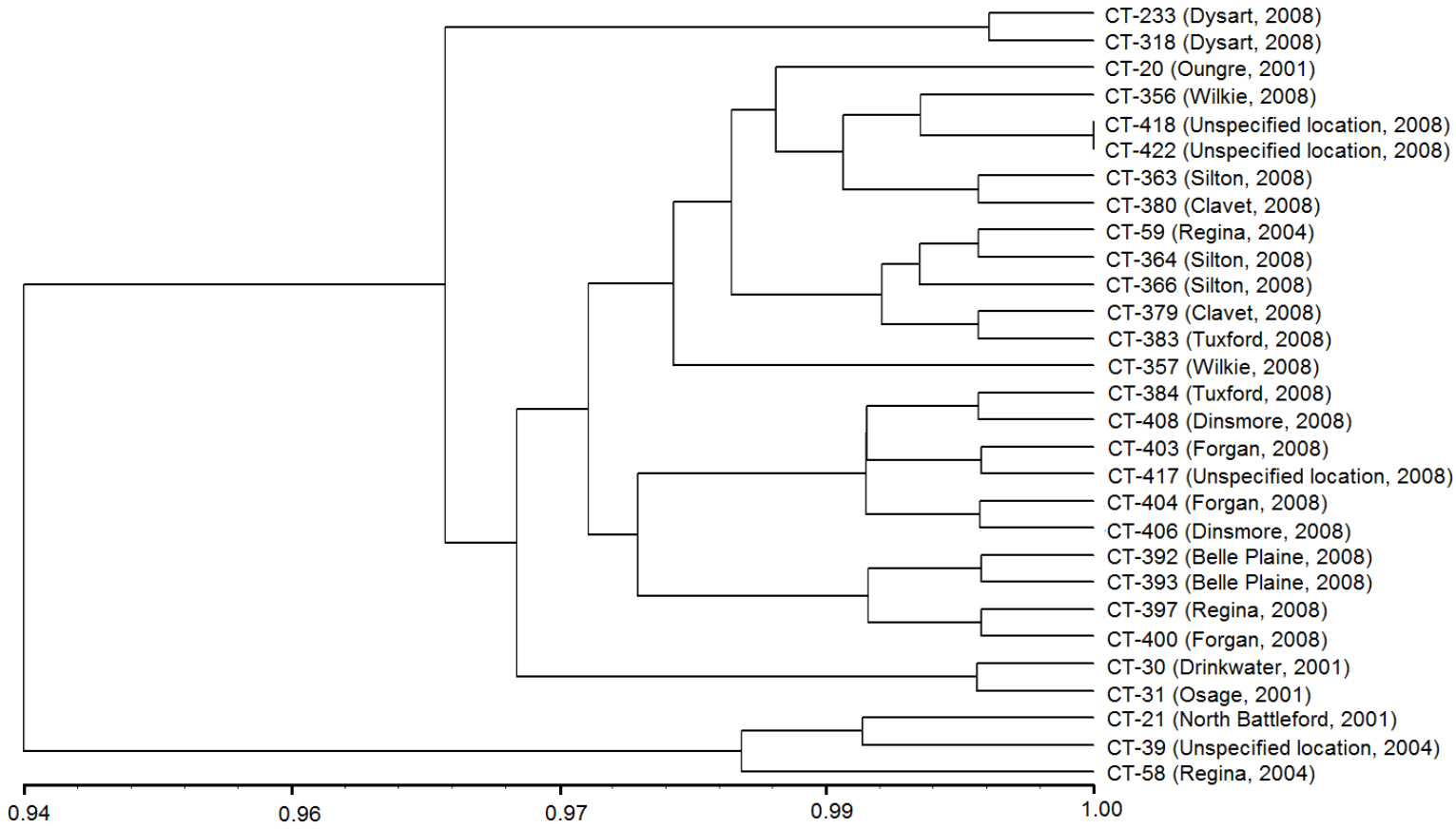

Figure 5.2: UPGMA cluster tree based on Dice genetic distance among 29 isolates of *Colletotrichum truncatum* collected from lentil fields in Saskatchewan, representing each of the 29 distinct haplotypes obtained after clone correction.

5.4. Discussion

This is the first molecular population study performed on *C. truncatum* responsible for lentil anthracnose. Prior, the only indications of variability in this species were the presence of two races (Buchwaldt *et al.*, 2004) and the existence of two mating incompatibility groups (Armstrong-Cho and Banniza, 2006; Chapters 3 and 4 of this study), as well as morphological differences in cultures noticed under laboratory conditions. This study was undertaken to determine if sexual reproduction has a role in the life cycle of this species, and also to assess the level of variability available in field isolates of *C. truncatum*. The studied population consisted of isolates from diverse geographical locations in Saskatchewan, sampled from 2001 to 2008, to maximize the potential for variability.

The reported diversity in *Colletotrichum* species varies greatly depending on species and populations. In a population of *C. lindemuthianum* from common bean, Dice's coefficient of genetic similarity varied between 0.42 and 0.97. Some association was found between genetic similarity, and geographical origin and pathogenicity, as isolates belonging to the same pathotype had a tendency to group together, (Barcelos *et al*., 2011). In *C. gloeosporioides* from mango, a population was shown to have genetic similarity varying between 0.71 and 1, with very virulent isolates clearly separated from the moderately and weakly virulent isolates (Rojas-Martinez *et al*., 2008). Similar values of 0.70 to 1 were obtained for a collection of *C. gloeosporioides* isolated from strawberry (Xiao *et al*., 2004). Even more variability (Dice coefficient 0.33-1) was discovered in the same pathogen on coffee berries in Vietnam. In this case, the isolates clustered by geographical location (Nguyen et al., 2009). Chala *et al*. (2011) studied a collection of *C. sublineolum* from sorghum sampled from different regions of Ethiopia. Genetic similarity spanned the range 0.45 to 0.96 and the isolates also grouped based on geographic origin.

In the current study, in contrast, the lowest level of similarity between *C. truncatum* isolates from lentil was very high (0.94), and the majority of isolates were 97% similar or more.

It can be difficult to assess whether a population is clonal or whether recombination occurs. If a single recombinant period occurs between many asexual generations, an important proportion of individuals in the population is derived from asexual reproduction and the

population's genetic constitution may appear unchanged (Burnett, 2003). The fact that the 131 isolates of *C. truncatum* sampled from a single lentil field shared the same haplotype suggests that the sampling of that field was performed after a period of asexual reproduction, but does not preclude the occurrence of sexual reproduction at other periods and/or other locations.

Population structure and the presence of sexual reproduction in the field vary among *Colletotrichum* species. *Glomerella cingulata* is a sexual species on some hosts, which has led to high levels of genetic diversity in some of its populations (Weeds *et al.*, 2003), but this pattern is uncommon in the *Colletotrichum* genus. *Colletotrichum graminicola* from sorghum (= *C. sublineola*) is a species for which a teleomorph was obtained in vitro (Vaillancourt and Hanau, 1992), but is not considered to play a role in the field. Rosewich *et al*. (1998), studying a collection of 411 isolates sampled in a nursery in three consecutive years, obtained only nine distinct haplotypes, demonstrating a low genotypic diversity. However, gene diversity was moderately high. The gene diversity of some studied populations of *C. graminicola* from sorghum was even higher than that reported for sexually reproducing fungi. Two hypotheses were suggested: either this could indicate that there is no direct relationship between genetic diversity and reproduction strategy in this species (Rosewich *et al.*, 1998), or that sexual reproduction occurs, hence suggesting the need to search for the sexual stage in nature starting where high genetic variability is reported (Chala *et al.*, 2011).

Parasexual recombination has been suggested to explain the genetic variability in *Colletotrichum* species when no sexual reproduction appears to take place. The parasexual cycle is a process that can create recombination independently of meiosis in fungi. It starts with a plasmogamy event between two haploid hyphae, resulting in a heterokaryotic mycelium. Subsequent karyogamy results in the formation of a diploid nucleus in an otherwise haploid mycelium. During the following meiosis, recombination is created by crossing over, and the haploid state is restored (Cole, 1996).

In the case of *C. lindemuthianum*, sexual reproduction is possible under laboratory conditions, but there is no consensus on the situation in the field. Numerous pathotypes occur (Hernández-Godinez *et al.*, 1998), but it was suggested that mutation and parasexuality, not sexual reproduction, were responsible for this high genetic variability (Rodriguez-Guerra, 2003). However, a study by Sousa *et al.* (2010) suggested that even if linkage disequilibrium was found in several *C. lindemuthianum* populations - it ranged from low to moderate - this was not

inconsistent with the presence of some sexual reproduction. In another population of *C. lindemuthianum* isolated from common bean in various regions of Brazil (Barcelos *et al*., 2011), a $\overline{r}_d$ value of 0.1 was reported, and the authors did not reject the possibility that sexual reproduction events occurred in this population. The value obtained here in *C. truncatum* ($\overline{r}_d$ = 0.094, closelycomparable. Although demonstrating some gametic disequilibrium, this value is low compared to what could be expected in totally clonal populations, and by analogy with *C. lindemuthianum*, some level of recombination can be suspected. However, the value of $\overline{r}_d$ obtained for the population of *C. truncatum* is 2 to 3 times higher than what was estimated by Rau *et al.* (2003) in *Pyrenophora teres* f.sp. *teres*, a fungus known to have regular sexual reproduction, suggesting that asexual reproduction is predominant and that random mating, if present, must be a rare occurrence. In a study on a panel of *Colletotrichum* isolates grouped as *Colletotrichum acutatum sensu lato*, Guerber *et al.* (2003) were able to identify distinct groups on the basis of gene introns, mtDNA and intronRFLP data. They found that two groups, while encompassing isolates with the ability to mate under laboratory conditions, had probably not mated recently and that genetic isolation might have occurred before reproductive isolation.

An observation described from *G. cingulata* f.sp. *phaseoli* (=*G. lindemuthiana*) could also partially explain the low genetic variability and existence of low levels of linkage disequilibrium in *C. truncatum* from lentil. In that species, Camargo *et al*. (2007) showed that plants inoculated with ascospores exhibited milder symptoms than those inoculated with conidia. Additionally, in *C. gloeosporioides* isolated from *Stylosanthes guianensis*, the anamorphic and teleomorphic stages infect the host in a similar manner, but the anamorph accomplishes more successful penetrations (Ogle *et al.*, 1986). This suggests that the individuals originating from sexual reproduction may have a reduced fitness and, as a consequence, the associated recombinant characters may not be maintained in the gene pool of the population.

It is not possible at this stage to determine if the genetic variability and the relatively low levels of linkage disequilibrium observed in *C. truncatum* are due to outcrossing or to other mechanisms reported for *C. lindemuthianum*. However, the presence of two races and two sexual incompatibility groups in an overall genetically rather homogeneous population raises the question of the origin of *C. truncatum ex* lentil in Canada.

In conclusion, the collection of *C. truncatum* isolates displayed a low diversity compared to what was observed in populations of other *Colletotrichum* species. Although *C. truncatum* is a

sexual species under laboratory conditions and both mating types coexist in the field, the measurements of linkage disequilibrium suggest that the studied population is mostly clonal and that sexual reproduction, if existing, remains a rare event

CHAPTER 6

GENERAL DISCUSSION

6.1. Properties and consequences of sexual reproduction in *Colletotrichum truncatum*

6.1.1. Specificity of the sexual reproduction of *Glomerella* species

The agent of lentil anthracnose, *C. truncatum*, belongs to a genus with notoriously atypical sexual reproduction. Ironically, one of the first filamentous ascomycetes investigated in regard to its mating system was *G. cingulata* (Edgerton, 1914), hence, the exception was studied before the rule. Edgerton described two types of self-fertile isolates that had morphological differences and that were cross- fertile. Further analysis by Wheeler during the 1950s led him to propose the theory of 'unbalanced heterothallism', stipulating that this species is basically homothallic, and that the heterothallic strains were derived from those homothallic strains. Under this hypothesis, mutations in genes involved in sexual reproduction would be the cause for the existence of heterothallic isolates rather than classical, distinct mating type genes. Those mutations could happen anywhere, and isolates with complementary mutations would restore fertility (either homothallic or heterothallic). The number of mating types would thus be unlimited.

Since then, several other species in the genus were shown to display atypical mating systems, and the development of molecular biological methods permitted more thorough investigation of the validity of the typical mating type determination for this specific genus. As most *Colletotrichum* species do not have a sexual state, classical genetic analysis has been performed for very few species and the genetic control of sexual compatibility in the genus remains mainly unresolved.

With this study on *G. truncata*, the number of *Glomerella* species for which mating systems has been studied has increased to four (*G. cingulata*: Wheeler, eg. 1956; Cisar & Tebeest, 1999; *C. graminicola*: Vaillancourt *et al.*, 2000b; and *C. lindemuthianum*: Rodriguez-Guerra *et al.*, 2005). Reported modalities for sexual reproduction are different for each species.

The results of the crossing experiments among isolates of *C. truncatum* are consistent with a heterothallic, bipolar mating system as classically described in most ascomycetes, but differ from what has been described until now in other *Colletotrichum* species. The information obtained for the three other species suggests that those species have unusual mating systems that do not seem to follow that of most ascomycetes because fertile interactions are not always consistent with the typical single locus bipolar system. For example, in the study by Cisar and Tebeest (1999), four isolates of *G. cingulata* from pecan crossed in all possible combinations displayed a pattern compatible with a bipolar mating system and could be classified into two mating compatibility groups, but a fifth one was able to mate with every other isolate, independently of mating group. The authors proposed a model characterized by a single mating type locus with multiple alleles, but a model with two mating type loci (A and B), with 3 and 2 alleles respectively, was also proposed for explaining the crossing pattern of other isolates of the same species (Wheeler, 1954). In *G. cingulata*, the 'B' locus shares a major characteristic with a classical MAT locus because the two partners of a fertile cross need to carry opposite alleles. The 'A' locus is different in that a particular allele needs to be present in only one partner to obtain fertility. The same type of system was described in *G. graminicola* (Vaillancourt and Hanau, 2000b). In this species, the authors described two loci, called *Crf1* and *Crf2* (for 'cross-fertility'). Based on the results of sibling crosses and backcrosses, they hypothesize that their cross-fertile tester isolates, M1.001 and M5.001 have the genotypes *Crf1-1/Crf2-1* and *Crf1-2/Crf2*-2, respectively. Backcrosses and sibling crosses showed that under this hypothesis, fertility is achieved when one or both parents carry the *Crf1-2* allele and when parents have different alleles at the *Crf2* locus. The authors suggest that the *Crf1* locus could correspond to a gene involved in the developmental pathway for fertility, and propose two hypotheses concerning *Crf2*. The first possibility is that this gene could be an idiomorphic mating type locus. However, if it is, it would function in a different manner to that in other ascomycetes, as the MAT1-2 idiomorph is present in both M1.001 and M5.001 and is unlinked to *Crf2*. The second possibility is that the *Crf2* locus actually consists of two or more linked genes necessary for fertility and that self-sterile, cross-fertile parents carry null alleles at different loci. The existence of self-fertile recombinant progeny seems consistent with this hypothesis. Regarding the case of the MAT locus, the authors suggested that *G. graminicola* could carry both MAT idiomorphs, as in a homothallic species, and that the inability to amplify the alpha-box of a MAT1-1 idiomorph

could be due to an alpha-box being not as well conserved as expected. Alternatively, the MAT1-1 idiomorph could simply be absent. Glass *et al.* indeed reported in 1990 the case of homothallic ascomycetes missing a MAT idiomorph. Homothallic isolates usually carry both idiomorphs in the same nucleus (Yun *et al.*, 1999), but *Neurospora africana*, *N. dodgei*. *N. galapagosensis* and *N. lineolata* appear to be missing MAT1-2. However, MAT1-2 is the idiomorph carrying a unique gene controlling mating specificity in many species, whereas MAT1-1 is a cluster with a mating specificity gene, but also one or more genes involved in post-plasmogamy fertility. As a consequence, the absence of MAT1-1 could have drastic effects on fertility.

Although conclusive evidence is still lacking to confirm if unbalanced heterothallism occurs in *C. truncatum,* and the genetic control of such a mating system is still unclear, it is noteworthy that both the classical mating studies performed in the 1950's on *G. cingulata* by Wheeler and his team and the hypothesis of evolution of unbalanced heterothallism from homothallism are consistent with the data obtained for the four *Glomerella* species studied to date and could represent a plausible explanation for the variety of mating modalities present in this genus.

6.1.2. Environmental factors influencing perithecium production in *Glomerella* species

Perithecium production in *Colletotrichum* appears to be sensitive to environmental conditions. The temperatures reported for successful induction of perithecia in *Colletotrichum* species range from 15 to 28°C. In *C. lindemuthianum,* light was shown to inhibit perithecia production, but not in *C. graminicola*. In *G. cingulata*, grown under laboratory conditions, light was shown to be essential, and few or no ascospores were produced in darkness. Ascospore production was earlier and higher at 2000 lux for 24 hours/day than at 1000 lux for 12 hours/day, 1000 lux for 24 hours/day or 3000 lux for 24 hours/day (Ann, 1995). Under laboratory conditions, high humidity was shown to be beneficial, albeit not essential, for perithecia formation in *G. cingulata* from banana (Kaiser and Lukezic, 1966). Under natural conditions, the authors report that perithecia and acervuli were preferentially formed during the rainy season in Honduras, but rarely during the dry season. The preferred temperature ranged between 23 and 28°C; no perithecia formed below 12°C or over 30°C, and ascospore discharge occurred between 18 and 28°C. Ascospore discharge was shown to be insensitive to light conditions, but very sensitive to humidity, occurring only at 100% humidity or when the perithecia were wet. However, viable spores could be emitted during 6 weeks of alternate periods of humidity and

dryness, suggesting inoculum could be maintained through unfavorable periods. Rodriguez and Owen (1992) obtained successful crosses of *G. musae* under laboratory conditions by incubating conidia at 23°C under continuous fluorescent light for 21 to 30 days. In *C. truncatum* from lentil grown under controlled conditions, a temperature of 22°C and a relative humidity of 70% are adequate, and perithecia are produced in the darkness (Armstrong–Cho and Banniza, 2006; this study). It has been suggested that sexual reproduction could occur in the field, considering the relative simplicity of the requirement for perithecia formation in *C. truncatum* from lentil (Armstrong–Cho and Banniza, 2006). The temperature range within which perithecia of *C. truncatum* from lentil are produced has not been determined in this study, but it can be hypothesized that the lower end of the range should not be higher than that of *G. cingulata*, a tropical pathogen of banana, which produces perithecia at temperatures as low as 12 to 13°C (Kaiser and Lukezic, 1966).

Weather conditions can vary substantially from year to year in southern Saskatchewan, the primary lentil growing area of Saskatchewan. Average daily temperatures in south Saskatchewan are generally above 12°C from mid-May to mid-September, but the difference between day and night temperatures can be substantial. For example, according to Environment Canada (http://www.weatheroffice.gc.ca) for the 1971-2000 period in Regina, daily maximum temperatures for the five months from May to September (18.8, 23.2, 25.7, 25.3 and 18.7°C) are consistently about 14°C higher than daily minimum temperatures (4.6, 9.6, 11.8, 10.7 and 4.6°C respectively). Information obtained for other species, with regards to temperatures adequate for perithecia production, are mostly based on laboratory studies where the isolates were maintained at constant temperatures; therefore it is not clear to what extent they apply to field conditions, where temperature differentials between day and night are the rule. The average monthly number of days with significant rainfall for the same period in Regina is 10.1, 12.8, 10.9, 9.6 and 8.

Based on studies in other species regarding temperature and moisture conditions conducive to perithecia development, June, July and August appear to be the most favourable months for perithecium production and ascospore discharge in Saskatchewan. As lentil is seeded in Saskatchewan in late April or early May, and mature lesions usually develop during the reproductive stages of the crop, it is unlikely that perithecia would form before late June. In July and August, temperatures are potentially high enough for perithecia formation, and several periods of wet conditions exists that could permit successful ascospore discharge.

6.1.3. Influence of sexual reproduction on epidemics and disease control

The presence of a teleomorphic state can affect the life cycle and epidemics of a pathogen. For example, *Didymella rabiei*, the teleomorph of *Ascochyta rabiei* has had a profound influence on the epidemics and control of this pathogen in Spain and in the USA (Trapero-Casas *et al*., 1996). Sexual reproduction can generate genetic diversity in the pathogen, thereby increasing the risk of breakdown of crop resistance. Ascospores are also a powerful agent of disease dispersal and can aid the survival of a fungus from one crop year to another (Kaiser, 1997).

While water-splashed conidia of ascomycetes are generally spread over short distances, ascospores are often wind-dispersed, sometimes over very long distances. For example, over 50% of *Ascochyta lentis* conidia spread by water splashing within a 15 cm radius of spore production. Very few spores are dispersed beyond 70 cm, although strong winds could transport them in aerosol droplets as far as 100 m (Pederson *et al.*, 1993). In comparison, ascospores from the closely related *Didymella rabiei* are suspected to be responsible for new infection foci located between 10 and 15 km from the ascospore source (Kaiser, 1992).

Information is scarce on the dispersal of ascospores in the genus *Colletotrichum*. *Colletotrichum gloeosporioides* is responsible for yam anthracnose in Nigeria. Based on the high levels of genetic diversity, the absence of subdivision in the population, and the random spatial pattern of infections consistent with long-distance ascospore dispersal, as well as the observation of perithecia on senescent yam leaves, Abang (2003) hypothesized that ascospores are a significant source of inoculum in yam anthracnose, but no direct observations of ascospore dispersal were made.

Control of lentil anthracnose is currently achieved through diverse methods including cultural methods, fungicides, and the use of resistant cultivars. Resistance to lentil anthracnose is partial and under the control of several genes (Tullu *et al*., 2003). Sources of resistance to race Ct1 are available in several cultivated lentil cultivars, and some partially resistant cultivars adapted to the Prairies, such as CDC Robin and CDC Redberry, have been developed. However, sources of resistance to race Ct0 are very rare and not very effective in *L. culinaris*, but exist in the secondary gene pool constituted by the wild species *L. ervoides*, *L. lamottei* and *L. nigricans*.

Pathogens that reproduce sexually often have a high genetic diversity and a risk model proposed by McDonald and Linde (2002a) hypothesizes that pathogens with the greatest

evolutionary potential are also the ones with the more potential to cause break-down of resistance and counteract fungicides or antibiotics. Several evolutionary forces are involved, including mutation, genetic drift, gene flow, and mating systems. Regular recombination, either by sexual reproduction or other phenomena such as parasexuality, can create new combinations of virulence alleles and other characters that increase fitness. A pathogenicity study on *G. truncata* isolates issued from a cross between a race Ct0 and a race Ct1 conidial *C. truncatum* seems to indicate that the pathogenicity of *G. truncata* isolates all display phenotypes relative to pathogenicity and that no visible new races emerged. However, only isolates of the first generation of sexual strains were studied (A. Cohen-Skali, Dept. of Plant Sciences, University of Saskatchewan, personal communication).

6.2. Relationships between isolates of *Colletotrichum truncatum* sensu lato and the nature of the *Colletotrichum* species causing lentil anthracnose in Canada

The main theoretical concept used to describe a species is the evolutionary species concept (ESC) first detailed by Simpson (1951). Wiley (1978), within the ESC, defined a species as 'a single lineage of ancestor-descendant populations which maintains its identity from other such lineage and which has its own evolutionary tendencies and historical fate'. While relatively straightforward, this definition is not usable directly to test species in the laboratory. Therefore, other species definitions that can be considered as derived from the ESC are used. Mayden (1997) called them 'operational species concepts' to stress the fact that these are actually usable in practice.

The more widely used concepts for species recognition are the morphological species concept, the phylogenetic species concept, and the biological species concept.

The morphological species concept is based on the observation of phenotypic characters including morphological characters, preferred growth temperature, and production of pigments and secondary metabolites (reviewed in Harrington and Rizzo, 1999). This has historically prevailed for the diagnoses of fungal species, and continues to prove useful. However, in numerous cases, it fails to elucidate relationships at the species level (Taylor *et al*. 2000), which, as seen earlier, may be the case for *C. truncatum*.

The definition of the species in the phylogenetic species concept as proposed by Nixon and Wheeler (1990) is 'the smallest aggregation of populations (sexual) or lineages (asexual) diagnosable by a unique combination of character states in comparable individuals'. However, it should be noted that not all lineages should necessarily be considered a distinct species; hence it is important to also take in to account biological and ecological information when describing species (Harrington and Rizzo, 1999). Phylogenetic analysis can be performed using characteristics of any nature, but modern studies rely mostly on molecular markers. A careful choice of markers is required to allow discrimination between closely related species (Harrington and Rizzo, 1999).

The third operational species concept is the biological species concept, based on interfertility, as described in 3.4.

The first two operational species concepts were applied to *Colletotrichum truncatum* previously, and the third, the biological species concept, was utilized here with lentil, soybean and chamomile isolates.

It was shown that isolates from lentil induced disease on lentil and field pea when inoculated artificially under control conditions, but not on scentless chamomile, whereas isolates from scentless chamomile caused symptoms only on scentless chamomile (Forseille, 2009). Morphological features such as conidia and the size and shape of appressoria are typically used for species determination in *Colletotrichum*. Conidia of *C. truncatum sensu lato* from different hosts were compared (Ford et al., 2004; Forseille, 2011). Conidia of isolates from lentil and scentless chamomile had a similar slightly falcate shape, but the spores from scentless chamomile were slightly more truncated. The conidia of isolates from soybean were more falcate, longer and narrower; those from alfalfa were more ovoid. The size of appressoria was similar for all isolates, except for those from soybean that were much larger.

Phylogenetic studies (Ford *et al.*, 2004; Forseille, 2011) showed that isolates from different hosts cluster in well separated groups. Recently, it has been suggested that *C. truncatum sensu lato* is a polyphyletic taxon and that the isolates from pea, lentil and faba bean do not belong to this species, but rather to the *C. destructivum* aggregate (Latunde-Dada and Lucas, 2007). A multilocus molecular analysis suggested that isolates obtained from the following plant species belong to the species *C. truncatum*: *Phaseolus lunatus, P. vulgaris, Glycine max, Medicago sativa, Arachis hypogaea, Vigna unguiculata, Vigna sinensis, Stylosanthes hamata,*

Clitoria ternatea, *Crotalaria spectabilis*, *C. juncea*, *Capsicum frutescens*, *C. annuum*, *Brassica* sp., *Peperomia magnoliifolia*, *Corchorus capsularis*, *Bougainvillea* sp., *Limonium* sp., *Opuntia* sp., *Basella rubra*, *Cyperus rotundus* (Damm *et al.*, 2009). Indeed, the description of *C. truncatum* by Andrus and Moore was that of an isolate on *P. lunatus* (Damm et al., 2009). *Colletotrichum destructivum*, on the other hand, has been reported on lentil, as well as other legume species such as *Lotus pedunculatus*, *Medicago sativa*, *Melilotus alba*, *Trifolium fragiferum*, *T. pratense*, *T. repens* and several *Vicia* species (Lenné, 1992). Based 18-25S rDNA data, Ford et al (2004) showed that lentil isolates of *C. truncatum* were distinct from *C. destructivum* originating from *Serradella ornithopus*, but it is unknown how the latter is related to other isolates in the *C. destructivum* aggregate. It is also not known if the *C. destructivum* isolates collected on several occasions on lentil belong to the same species as the agent of lentil anthracnose in Canada. Interestingly, one of the Herbarium specimens identified as *C. destructivum* at the International Mycological Institute had been sampled on lentil in 1983 in Manitoba, Canada (http://www.indexfungorum.org/). Although limited in the number of isolates tested, crossing experiments here with isolates from different hosts indicated that isolates from lentil are genetically distinct. In a genus like *Colletotrichum*, where studies based on a single taxonomical method often fail to discriminate at the species level, obtaining the teleomorph of *C. truncatum* could prove to be a powerful tool to identify the relationships among isolates currently within this species.

6.3. History of lentil anthracnose in Canada

6.3.1. Origin of lentil anthracnose

Two hypotheses have been suggested to explain the origin of lentil anthracnose in Canada (Morrall, 1997). Either the pathogen is an indigenous species present originally on a native legume host(s), or it could be an introduced pathogen, possibly imported on seed of lentil or other legume crops. Supporting the first hypothesis, isolates morphologically identical to those found on lentil were found on *Vicia* spp. (Morrall *et al*., 1989), and anthracnose-like symptoms were reported on the native narrow leaf vetch (*V. americana*). Furthermore, under controlled conditions, *C. truncatum* isolates from lentil are pathogenic to *V. americana* (Gossen *et al*., 2009).

Alternatively, if *C. truncatum* is an exotic pathogen, it could either have been introduced with lentil itself or with another pulse crop. Transmission of *C. truncatum* from seed is very low on lentils, with only 2% becoming infected of seed produced by heavily infected plants, and a quasi-null seed-to-seedling transmission (Gibson, 1994). This is the antithesis of the ascochyta blight situation, where *Ascochyta lentis*, a seed-borne pathogen with a high level of host specificity and seed-to-seedling transmission, is thought to have been introduced in Canada with contaminated seed (Morrall, 1997). However, even low levels of *C. truncatum* on introduced lentil seeds could have been sufficient to start the Canadian epidemics.

If lentil anthracnose was introduced into Canada with another crop, good candidates for such crops would be faba bean and field pea, because isolates of *C. truncatum* from lentil are also pathogenic to these two crops. Several *Colletotrichum* spp. were also recovered from various other exotic crops. Isolates of *Colletotrichum* isolated from lentil, field pea and faba bean obtained from commercial fields in Manitoba in 1990 were all identified as *C. truncatum* by the Biosystematics Research Institute, Ottawa (Gibson *et al.*, 1991). Faba bean is a relatively new crop to Canada. The first North American commercial crop of faba bean is thought to have been sown in Nova Scotia in 1967. Cultivation started in Western Canada in 1972, three years after that of lentil. An isolate of *Colletotrichum* sp. was recovered on faba bean in the district of Outlook, Saskatchewan as early as 1973 (McKenzie and Morrall, 1975). In contrast to lentil and faba bean, field peas have long been grown in North America. The first peas are thought to have been introduced in the late 15th century and their cultivation was documented in the Montreal region in 1535 (Fraser and Wallen, 1956 in Ali-Khan and Slinkard, 1995). Occasionally, *Colletotrichum* isolates have been recovered from field pea in Canada. The exact species of some isolates was never determined, but others were identified as *C. truncatum* or *C. pisi*, a species that belongs to the *C. gloeosporioides* complex.

On alfalfa, an exotic forage legume introduced into Canada in 1871 (Goplen *et al.*, 1995), *Colletotrichum* was occasionally observed, with some isolates having been identified as *C. trifolii*. However, *C. destructivum* (or a species of the *C. destructivum* complex) is a secondary pathogen of alfalfa reported in North America, Europe, North and South Africa (compiled in Latunde-Dada *et al*., 1997), and can be considered a primary pathogen under warm conditions and high humidity (Latunde-Dada *et al*., 1997). *Colletortrichum destructivum* overwinters readily on stems in Maryland, USA (Graham *et al.*, 1976). A *Colletotrichum* sp. was also found

on Birdsfoot trefoil, another introduced forage crop species, in Manitoba in 2009 (Desjardins, 2010).

6.3.2. Post-introduction mechanisms

When a species colonizes a new environment, the founder population is characterized by a subset of alleles, creating a genetic bottleneck leading to generally restricted variability. The level of variability in such founder populations can be lowered further by subsequent genetic drift, depending on the effective size of the population and the number of generations. In the case of a very small founding population, genetic drift can lead to a severe decrease in genetic diversity. The number of alleles can increase again in time with migration or mutation (Rogers and Rogers, 1999). For example, the genetic diversity of the chestnut blight fungus, *Cryphonectria parasitica*, appears to be lower in its introduced range (Europe and America) than in Japan and China, its native range (Liu and Milgroom, 2007). In *C. gloeosporioides* from *Stylosanthes* spp., a high level of genetic variability was found in the native range of the hosts, as well as where the host was naturalized. In Australia, where *Stylosanthes* is cultivated, but is not a native genus, the pathogen is associated with lower diversity (Weeds *et al.*, 2003).

Species invasions can be accomplished in a single founder event or in several successive ones (multiple introductions). Multiple introductions can limit the reduction of genetic diversity in the introduced population, especially if the different founder events originate from genetically different populations in the native range of the species (Novak, 2005).

Regardless of whether the pathogen of lentil anthracnose originates from a native species or is an introduced fungus, it may have undergone a change of host. Host range expansion has been proposed as an evolutionary process in several biotrophic pathogens (eg. Voglmayr, 2003). The case of powdery mildew caused by *Golovinomyces* is interesting because this plant pathogenic genus bears some similarities with *Colletotrichum*. The taxonomy of both genera is complex and the host ranges are uncertain (Matsuda and Takamatsu, 2003). A study suggested that the most ancient hosts are probably in the plant family Asteraceae, and that the isolates found on other hosts belong to the most recently evolved groups in *Golovinomyces* (Matsuda and Takamatsu, 2003). For example, in Hungary, *Torenia fournieri* (Scrophulariaceae), originating from South-East Asia and cultivated only experimentally in small numbers in Hungary, is often infected by powdery mildew caused by *Golovinomyces* spp. It was suggested that this is the

result of a very recent host range expansion (Vagi *et al*., 2007). Matsuda and Takamatsu (2003) suggest that the evolution of *Golovinomyces* followed two different but simultaneous paths: 1) the pathogen evolves following the evolution of Asteraceae; 2) the pathogen also evolves by expansion of its host range.

In the *C. destructivum* complex, host ranges of isolates vary. Globally, it was shown that the host range of an isolate from tobacco had a similar range of hosts to that from a legume species, and that legume isolates were also pathogenic to tobacco (Cronin, 1958 in Shen *et al*., 2001), suggesting they belonged to the same species. Nonetheless, some host specificity exists. Latunde-Dada *et al*. (1997) showed that isolates from cowpea were not able to infect alfalfa, whereas in reverse, isolates from alfalfa were pathogenic to cowpea. However, while alfalfa isolates produced lesions on alfalfa leaves in 3 days, it took 7 days for lesions to appear on cowpea leaves. Tiffany and Gilman (1954) performed inoculations with isolates of *C. destructivum* from sweet clover, red clover and pea on the following species: soybean, alfalfa, sweet clover, red clover, and lespedeza. Under moist greenhouse conditions, the isolates from the three species were pathogenic to all species tested.

In a study in simulated natural conditions on live Northern Jointvetch (*Aeschynomene virginica*), an isolate of *G. cingulata* from Northern Jointvetch was able to mate with several isolates from pecan (*Carya illinoensis*). The progeny was shown to be non-pathogenic on vetch, but varying levels of pathogenicity were displayed on apple fruits (Cisar *et al.*, 1996). Successful crosses between isolates of Northern Jointvetch and winged water primrose (*Ludwigia decurrens*) were also recorded. The authors stated that all three species can be found in the same environment, that is, could be cohabiting at close proximity (Cisar *et al.*, 1994). These examples demonstrate the potential for fungal species to change or expand their host range through sexual recombination. This could also explain the existence of three combinations of race and mating types (Ct0/IG-1, Ct0/IG-2 and Ct1/IG-2). Under this hypothesis, the fourth potential combination (Ct1/IG-1) could be missing simply by the absence of a recombination event leading to this specific gene association, or possibly by poor fitness of these recombinants.

Although it is not possible to reject the possibility of sexual reproduction in *C. truncatum* from lentil, the results of the population study presented in this thesis suggest sexual reproduction does not occur frequently in the field. The predominance of asexual reproduction in a species can be due to a deficiency in sexual reproduction (sexual failure) or to the effectiveness

of asexual reproduction (clonal success). Sexual failure occurs in the absence of a compatible mating type or if the environment (e.g. climatic conditions) is unfavourable for sexual reproduction to occur. Clonal success occurs in case of pre-adaptation, high plasticity, or a high mutation rate of one or a few clonal lineages (Silvertown, 2008). In a meta-analysis based on 284 studies covering 218 plant species, Silvertown (2008) showed that exotic introduced species display higher frequencies of clonality, and that the perfect parameters for clonal success would be found in a plant species that is a "rare, aquatic, alien apomict living in an undisturbed, geographically marginal habitat". Due to the extreme restrictiveness of these conditions, the author suggested that this should be seen as a set of parameters for sexual failure rather than as parameters for clonal success. In the case of the chestnut blight, the pathogenic agent *C. parasitica* was first observed in Europe in 1938 (Biragi, 1946 in Dutech *et al.*, 2010). This heterothallic ascomycete is also capable of selfing (Marra *et al.*, 2004), and several studies in southern France suggested that outcrossing is limited despite the presence of both mating types (Dutech *et al*., 2008, 2010). Several explanations were proposed to explain this lack of (or reduction in) outcrossing: a preference for selfing, a reduced fertility due to a known viral disease, or inconducive environmental conditions. In *C. truncatum*, neither geographical nor reproductive isolation seems to be responsible for lack of sexual recombination, and climatic conditions appear to be rather favourable for perithecia production and ascospore dispersal. However, reduced aggressiveness has been reported in isolates of *C. lindemuthianum* and *C. gloeosporioides* produced through sexual reproduction, compared to the aggressiveness of clonal isolates (Camargo *et al*., 2007, Ogle *et al.*, 1986). Therefore, it is possible that the low genetic variability and the existence of moderate linkage disequilibrium in *C. truncatum* populations from lentil are not due to the absence of sexual reproduction, but rather due to the poor fitness of recombinant individuals.

If *C. truncatum* was introduced with another exotic crop (for example, faba bean), it is possible that both the founder effect and host range expansion occurred sequentially, assuming that the original host range was restricted to faba bean. However, the fact that *Colletotrichum* spp. were known from lentil in the Old World prior to the Canadian anthracnose epidemic also leaves open the possibility that *C. truncatum*, even if introduced on faba bean, could always have been a pathogen of lentil. The natural and cultivated ranges of lentil and faba bean in the Old World overlap, and both plant species are relatively closely related. Their range also overlap that

of cowpea (*Vigna unguiculata*), a crop susceptible to several *Colletotrichum* spp., including *C. destructivum*.

A founder effect, associated or not with a host range change, could explain the low genetic diversity observed in populations of *C. truncatum* from lentil, irrespective of whether *C. truncatum* was introduced from abroad on lentil or another crop species, or originated as a native pathogen of an indigenous species such as a *Vicia* sp. The existence of two races and two mating types despite a very restricted genetic variability in the Canadian population suggests that these alleles were present in the founding population(s). The overall low variability makes it unlikely that those characters appeared subsequently. These alleles could have been introduced either through a single introduction event or their presence could be the consequence of multiple introductions. It is interesting to note that no isolate of race Ct1 was found to belong to IG-1. This fact, combined with the globally very low genetic variability of *C. truncatum* populations from lentil, could indicate that all three available race-incompatibility group combinations (Ct0/IG-1; Ct0/IG-2 and Ct1/IG-2) were initially introduced.

6.4. Future research

The study presented in Chapter 3 indicated that in *C. truncatum* from lentil, in contrast to what occurs in typical ascomycetes, both partners of a fertile cross carried the idiomorph MAT1-2 at the MAT locus. Attempts to localize MAT1-1 were unsuccessful, however, it is not known if this is due to the absence of MAT1-1, or to the fact this gene is not well conserved in *C. truncatum* in comparison with other species. At this stage, it would be beneficial to sequence the entire genome of one isolate of each mating incompatibility group. Assuming overall relatively low sequence variation, the full sequence could then be inspected for the presence of MAT1-1, and for identification of potential mutations and recombinations expected under the 'unbalanced heterothallism' hypothesis.

Results from crossing experiments on field isolates determined that isolates cohabiting in close physical proximity under natural conditions were cross-fertile under laboratory conditions. However, sexual reproduction was not demonstrated under natural conditions. A population study suggested that reproduction, if it occurs, is a rare event. In addition, several technical difficulties in species identification and in isolating sexual structures on natural, contaminated lentil tissue reduce the likelihood of finding direct evidence of sexual reproduction in the field. To gain a better understanding of the potential of sexual reproduction to occur under natural conditions, it would be interesting to repeat the crossings performed in the present study under conditions simulating diurnal temperature fluctuations as experienced during the growing season in Saskatchewan. Incubation temperatures in this study were constantly comparable to the average daytime temperature, but cooler night temperatures in the field may have an effect on the sexual reproduction process. It may also be useful to perform the tests on live plants at different growth stages. Indeed, the possibility that perithecia can form on live plants should not be neglected as in *G. cingulata*, perithecia were found on live tissues (Kaiser and Lukezic, 1966).

Recent studies suggested that the species pathogenic on lentil in Canada is not *C. truncatum* (see Chapter 2), and that some evidence links it to the *C. destructivum* species complex. It is important to correctly identify the species of the lentil anthracnose pathogen for various reasons. Firstly, the genus *Colletotrichum* is quite heterogeneous regarding certain characteristics, e.g. *Colletotrichum* encompasses species with very different infection strategies. Consequently, information gathered for one species may be of no relevance for another. *Colletotrichum higginsianum*, a species that has recently been linked to the *C. destructivum*

group, has drawn renewed research interest in recent years because it is pathogenic to the model plant species *Arabidopsis thaliana,* and its entire genome has been sequenced (*Colletotrichum higginsianum* Genome Project, Max Planck Institute (http://www.mpiz-koeln.mpg.de). If the lentil anthracnose pathogen is indeed closely related to the same complex rather than to *C. truncatum*, genome information from *C. higginsianum* could potentially be very useful in future research on *C. truncatum* from lentil. Furthermore, correct species identification would allow for clear delineation of isolates from lentil from those obtained from other plant species. The latter isolates are currently identified as *C. truncatum*, e.g. isolates from scentless chamomile researched as a bio-herbicide for that plant. Additionally, a clearer understanding of low-rank relationships in *Colletotrichum* species from legumes could shed more light on the history of the introduction of lentil anthracnose to Canada.

6.5. Conclusions

Lentil anthracnose constitutes a severe threat to Canadian lentil production. To address the problems caused by this relatively recent disease, most research efforts were initially focused on the screening of fungicides and the development of resistant cultivars. However, information is missing regarding parts of the life cycle of this species under natural conditions. The research described in this thesis was aimed at answering some of the central questions pertaining to the sexual reproduction and diversity of *C. truncatum* populations in Saskatchewan.

Mating studies indicated that both *C. truncatum* field isolates and their laboratory-borne progeny are heterothallic and display a bipolar mating system. However, this bipolarity does not seem to be determined by the homogenic incompatibility characteristic of the typical genetic system of ascomycetous fungi, as a marker for the same mating-type idiomorph was found in both parents of fertile crosses. Genetic markers studied in a parental cross and its progeny confirmed that the developing ascospores were not the product of homothallic reproductive events induced by the presence of another isolate, but true recombinant offspring from a cross between two different parental nuclei. The confirmation of heterothallism is an important aspect of the pathogen's biology, indicating the potential for sexual recombination that could give rise to new races in the population of *C. truncatum*.

Another aspect of the biology of *C. truncatum* addressed in this study was the spatial distribution of sexually compatible isolates in lentil fields. It was shown that both incompatibility groups were present in the field at the scale of single plants. Although sexual structures were not identified on field material, several of these closely cohabiting isolates were shown to be sexually compatible under laboratory conditions. This suggests that neither geographical nor reproductive isolation restrict sexual reproduction of *C. truncatum* in the field. However, a population study using AFLP markers on isolates obtained from diverse locations and crop years revealed low levels of variability in *C. truncatum*. It also showed moderate levels of linkage disequilibrium, indicating that the population is not panmictic, but neither is this incompatible with some levels of sexual reproduction.

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APPENDICES

APPENDIX 1:

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Glomerella truncata: Another _Glomerella_ species with an atypical mating system.

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Abstract: In the genus *Glomerella*, all species studied to date do not fit the usual mating system of heterothallic ascomycetes. This study investigated the mating system of *G. truncata* (anamorph *Colletotrichum truncatum*), a pathogen responsible for lentil anthracnose. Twenty-two field isolates from the Canadian prairies were crossed in all possible combinations, including selfings. All isolates were also screened for the presence of the MAT1-1 and MAT1-2 idiomorphs by targeting small conserved areas of the MAT genes (the alpha domain and the high mobility group HMG box) with degenerate primers, and a pair of *G. truncata* -specific HMG primers (CT21HMG) was designed. The results of the classical mating study suggested that *G. truncata* is heterothallic. Isolates fell into two incompatibility groups, which is consistent with a bipolar mating system, but different from what has been described in other *Glomerella* species. Molecular screening showed that the HMG box used as a marker for the MAT1-2 idiomorph was present in both partners of fertile crosses in *G. truncata*, unlike in the typical ascomycete system,

but as previously described for two other *Glomerella* species. *G. truncata* therefore appears to share unusual mating system characteristics with the other Glomerella species studied to date.
Key words: ascomycete, MAT1-2, mating-types, true heterothallism, unbalanced heterothallism.

INTRODUCTION

Ascomycete fungi display various types of mating systems. Generally, they are either homothallic or heterothallic (Kronstad and Staben 1997), and in most heterothallic species, the mating system is controlled by a single locus (MAT) occupied by one of two different genes or sets of genes (MAT1-1 and MAT1-2), determining a two mating type polarity (Metzenberg and Glass 1990). These alleles have been called "idiomorphs" to emphasize the fact that they have almost no sequence similarity, and clearly different functions (Glass et al. 1988). Both MAT1-1 and MAT1-2 idiomorphs were shown to include DNA-binding protein domains that are highly conserved amongst species: an alpha domain in the case of the MAT1-1 idiomorph and a high mobility group (HMG) box in the MAT1-2 idiomorph (Zhong and Steffenson 2001). These domains have been exploited for the development of PCR-based methods to amplify and characterize the mating type idiomorphs (Arie et al. 1997, 2000). Since then, many heterothallic ascomycetes have been shown to require partners carrying different alleles at the MAT locus for fertile crosses, as expected in the case of homogenic incompatibility (reviewed in Souza et al. 2003).

Yet, cases have been reported in the genus *Glomerella* that are inconsistent with homogenic incompatibility (Wheeler 1954, Vaillancourt et al. 2000a, Rodriguez-Guerra et al., 2005). Tests for sexual compatibility have been performed in a few *Glomerella* species. Well before molecular tools were available, suspicions arose that this genus contained species with a mating system very different from what was known then in other ascomycetes. Earlier work by Edgerton (1914) and Wheeler (1954) demonstrated that isolates of *G. cingulata* (anamorph *C. gloeosporioides*) could not be placed into two cross-fertile groups. Based on the example of *G. cingulata*, Wheeler (1954) proposed the 'unbalanced heterothallism' hypothesis. In this process, each partner of a fertile cross carries mutated fertility gene(s) that can either complement each other (cross-fertility) or not (cross-sterility). Cross-fertility studies on *G. graminicola* (*C. graminicola*) revealed that isolates could be homo- or heterothallic, and the number of mating types was not limited to two (Vaillancourt 2000b). It was speculated that unbalanced

heterothallism could be characteristic for the mating system of *G. graminicola* as well (Vaillancourt et al. 2000a).

Current information available on the MAT idiomorphs in *Glomerella* spp. also supports the hypothesis that the genetic basis of mating type in this genus differs from the typical ascomycete model. The HMG box of the MAT1-2 idiomorph has been successfully amplified in several *Glomerella* species, mostly for phylogenetic analyses (eg. Crouch et al. 2006; Du et al. 2005), but the presence of the idiomorphs in relation to cross-fertility has been studied only in *G. cingulata* and *G. graminicola*, where both partners of a fertile cross carried the MAT1-2 idiomorph (Vaillancourt 2000a, Rodriguez-Guerra et al. 2005). To date, the MAT1-1 idiomorph has never been reported from the genus *Glomerella* despite several attempts to amplify the alpha-domain (Vaillancourt 2000a, Rodriguez-Guerra 2005) and the development of at least two genome sequences (*Colletotrichum* sequencing project, Broad Institute of Harvard and MIT ; http://www.broadinstitute.org).

Colletotrichum truncatum (Schwein.) Andrus & Moore is the pathogenic agent of anthracnose on lentil (*Lens culinaris* Medik.), a disease responsible for severe yield losses in Canada (Morrall and Pedersen, 1991, Anderson et al. 2000). The teleomorph has never been observed under field conditions. This situation is common in the genus *Colletotrichum*, where teleomorphs of many species are known *in vitro* only, with a few notable exceptions like *Glomerella cingulata* (Sutton 1992). In the case of *C. truncatum* from lentil, the sexual stage (*Glomerella truncata* Armstrong-Cho & Banniza) was obtained under laboratory conditions (Armstrong-Cho and Banniza 2006). Crossing experiments in this species, conducted on a small number of isolates, showed self-sterility for all isolates tested, but some combinations of isolates resulted in the production of perithecia, supporting the hypothesis of a heterothallic mating system (Armstrong-Cho and Banniza, 2006). More extensive crossing studies, coupled with the search for the mating-type gene idiomorphs, were needed to determine if *G. truncata* follows the typical bipolar ascomycete mating system, or if, as in the case *G. graminicola* and G. *lindemuthiana*, sexual reproduction is likely determined by another system. The objectives of this study were to examine the mating system of *G. truncata* by performing sexual compatibility tests, and to determine if the mating type idiomorphs MAT1-1 and MAT1-2 are present in partners of a fertile cross.

MATERIALS AND METHODS

Biological materials and media. —Monoconidial isolates of *G. truncata* used in this study are listed in Table I. Isolates were routinely cultured on oatmeal-agar medium (OMA: 30g oatmeal flour [Quick Oats Robin Hood, Smucker Food of Canada, Markham, Ontario, Canada], 8.8g granulated agar [Difco™, Becton, Dickinson & Company, Sparks, MD, USA], 1L distilled water), incubated at 22 C with a 12 h photoperiod. Liquid cultures were obtained by inoculating 40 mL centrifuge tubes containing 25 mL glucose yeast medium (1 g $NH_4H_2PO_4$ [EM Science], 0.2 g KCl [OmniPur®, EMD™], 0.2 g $MgSO_4$ $7H_2O$ [EM Science], 10 g glucose [BDH®], 5 g yeast extract, 0.01 g $ZnSO_4$ $7H_2O$ [EM Science], 0.005 g $CuSO_4$ $5H_2O$ [EM Science], 1 L distilled water) with small amounts of mycelium from 5-7 days-old cultures and incubating in a shaker (Model SI-600, Lab Companion, Jeio Tech, Seoul, Korea) at 23 C and 130 rpm for 5-7 days. Mycelia were harvested by centrifugation, stored at -80 C for 48 h in 2 mL microcentrifuge tubes and subsequently lyophilized for 2 days in a Labconco cryofreezer (Labconco Corp., Kansas City, MO, USA).

TABLE I: Isolates of *Glomerella* spp. used in sexual compatibility tests and for amplification of conserved regions of the MAT genes.

Isolate name	Species	Source	Crossings[a]	HMG	alpha box[c]
CT-13	*Glomerella truncata*	This study	X		
CT-20	*Glomerella truncata*	Armstrong-Cho & Banniza,	X	X	X
CT-21	*Glomerella truncata*	Armstrong-Cho & Banniza,	X	X	X
CT-28	*Glomerella truncata*	Armstrong-Cho & Banniza,	X		
CT-30	*Glomerella truncata*	Armstrong-Cho & Banniza,	X	X	X
CT-31	*Glomerella truncata*	Armstrong-Cho & Banniza,	X	X	X
CT-32	*Glomerella truncata*	Armstrong-Cho & Banniza,	X		
CT-34	*Glomerella truncata*	Armstrong-Cho & Banniza,	X		
CT-35	*Glomerella truncata*	Armstrong-Cho & Banniza,	X		
CT-37	*Glomerella truncata*	This study	X		
CT-38	*Glomerella truncata*	This study	X		
CT-39	*Glomerella truncata*	This study	X	X	X
CT-43	*Glomerella truncata*	This study	X		
CT-44	*Glomerella truncata*	This study	X		
CT-45	*Glomerella truncata*	This study	X	X	X
CT-46	*Glomerella truncata*	This study	X		
CT-47	*Glomerella truncata*	This study	X		
CT-48	*Glomerella truncata*	This study	X		
CT-58	*Glomerella truncata*	This study	X	X	X
CT-59	*Glomerella truncata*	This study	X	X	X
CT-60	*Glomerella truncata*	This study	X	X	X

M1.00	*Glomerella graminicola*	J-A Crouch	X	
F-18	*Fusarium oxysporum*	This study		X
F-20	*Fusarium oxysporum*	This study		X

[a] Isolates used for sexual compatibility tests
[b] Isolates used for amplification of the HMG box
[c] Isolates used for amplification of the alpha box

Tests for sexual compatibility. — The susceptible lentil cultivar 'Eston' was grown under controlled conditions (23 C, 16 h photoperiod) until senescence. The lignified stems were harvested, cut in 5 cm long segments and sterilized. Isolates of *G. truncata* listed in Table I were tested both for self-fertility and cross-fertility in all possible combinations. CT-20 and CT-21, which are cross-fertile (Armstrong-Cho and Banniza, 2006) were used as positive controls for perithecium production. Conidial suspensions were prepared by flooding 2-week old cultures with sterile deionized water. The spore concentration was determined with a haemocytometer, and suspensions were diluted to 2×10^5 spores mL^{-1}. Five lentil stems were soaked for 2 h in a mixture of 5 mL of spore suspensions of each of the two isolates to be tested, or 10 mL of a single spore suspension in the case of selfings. The five stems were placed on Whatman No. 1 filter paper overlying sterile water agar in Petri dishes and incubated under optimum conditions for perithecium formation as described by Armstrong-Cho and Banniza (2006). Four replicate Petri dishes were prepared for each combination. To identify fertile crosses, stems were screened for the presence of perithecia under a 40 × magnification (SMZ-U, Nikon, Japan) 10 and 14 d after incubation. A cross was considered fertile if at least one perithecium was found in any of the replicates.

DNA extraction.— Mycelium was coarsely powdered with a pipette tip and DNA was extracted following the method described by Raeder and Broda (1985), modified by adding a 30 min incubation step at 37°C after adding the extraction buffer.

Amplification of the conserved regions of the mating type idiomorph.— The protein sequences representing the HMG box domain of the MAT1-2 locus of three *Glomerella* spp. and *Verticillium dahliae* were used for sequence alignment. Sequences of *G. lindemuthiana* (Accession No. ABY84976), *G. cingulata* (Accession No. AAQ62647) and *V. dahliae* (Accession No. BAH66364.1) were obtained from the NCBI database, whereas the fourth sequence, Transcript_3769 from *C. graminicola*, was obtained from the Broad Institute's online database

(http://www.broadinstitute.org/annotation/genome/colletotrichum_group/MultiHome.html). Sequences were aligned using ClustalW (Larkin et al. 2007), and degenerate primers were designed based on conserved regions using the iCODEHOP software (Boyce et al. 2009). Based on the sequence obtained with the primer pair DeLCP2 for the isolate CT-21, a pair of *G. truncata* -specific HMG primers (CT21HMG) was designed (Table II).

TABLE II: List of primers used for the HMG box amplification.

Name[a]	Sequence forward	Sequence reverse
DeLC1	5'-CAAGGTGCCCCGGccnccnaaygc-3'	5'-TTCCGAGGGGTGTACckrtartcngg-3'
DeLCP1	5'-CAAGGTGCCCCGGccnccnaaygc-3'	5'-GCTTCCGAGGGGTGtanckrtartc-3'
DeLCP2	5'-CCCCGGCCTCCCaaygcntwyat-3'	5'-GCCGCTTCTCGGAGggyttncknggg-3'
DeLCVP1	5'-CAAGATCAAGGTCCCCmgnccnccnaa-3'	5'-TTCCGAGGGGTGTACckrtartcngg-3'
DeLCVP2	5'-CCTCCCAACGCCTACathytntaymg-3'	5'-CCGAGGGGTGTACCkrtartcnggrt-3'
CT21HMG	5'-TGAACCCGCACATCCAAAACC-3'	5'-TTCTCGGAGGGCTTGCGGGG

[a]Names of degenerate primers include the name of the species used for alignment: L = *G. lindemuthiana*; G = *G. cingulata*, P = *G. graminicola* predicted gen3769; V = *Verticillium dahliae*.

Three isolates of the incompatibility group 1 (IG-1), as determined above (CT-20, CT-30 and CT-59) and six of IG-2 (CT-21, CT-31, CT-39, CT-45, CT-58 and CT-60) were selected for amplification of the HMG box and the alpha domain. DNA of an isolate of *G. graminicola* (M1.001) carrying the MAT1-2 idiomorph (Vaillancourt et al. 2000a), was obtained from Dr. J.A. Crouch (University of Minnesota, St. Paul, MN, USA) and included as a positive control. DNA amplifications of the HMG box by PCR were performed using each of the four pairs of degenerate primers and the pair of selective primers CT21HMG (Table II). The 20 µL reaction comprised 1X PCR buffer (50mM KCl, 10mM Tris-HCl, 0.1% Triton X-100), 200 µM of each dNTP, 2.5 mM of $MgCl_2$, 10 pM each forward and reverse primer, 1.25 U of Taq polymerase and 100 ng of genomic DNA. The amplification program included a denaturation step at 94 C for 3 min, followed by 35 cycles at 94 C for 45 s, 47 C for 30 s and 72 C for 1 min, following a 'Touchdown' PCR (Don et al. 1991) of 10 cycles, with an initial annealing temperature of 82 C, and subsequent decrements of 1 C per cycle. The program was terminated by a final elongation step at 72 C for 7 min.

Amplification of the alpha domain of MAT1-1 was attempted on the same nine *G. truncata* isolates using the degenerate primers Falpha1 and Falpha2 and the protocols described by Arie *et al*. (2000). Two isolates of *Fusarium oxysporum* (F-18 and F-20) of mating type 1 and 2 respectively, were used as controls.

Electrophoresis of the PCR products was conducted in 2% agarose gels stained with ethidium bromide and viewed on a UV transilluminator.

Sequencing of the HMG box.—The PCR products of the HMG box obtained for isolates CT-21 and CT-30 were purified using PolyEthylene Glycol (PEG) 8000 precipitation, and were directly sequenced with forward and reverse primers DeLCP2 at the Plant Biotechnology Institute of the National Research Council of Canada (Saskatoon, Canada). The sequence of the HMG box were BLASTed against NCBI's nucleotide (nr/nt) and protein (nr) databases of *G. graminicola*, using TBLASTX and BLASTX features, respectively (Altschul et al. 1997; http://blast.ncbi.nlm.nih.gov/Blast.cgi).

RESULTS

Tests for sexual compatibility. — For the tester cross CT-20 × CT-21, mature perithecia appeared on lentil stems approximately ten days after inoculation. For some other fertile crosses, perithecia were slower to mature, so stems of all crosses were screened again at day 14. No isolate among the 21 tested displayed self-fertility (TABLE III). Cross-fertility was observed in 50 combinations out of a total of 231, which corresponded to 22%. Two incompatibility groups were observed. Seven isolates (CT-20, CT-30, CT-32, CT-38, CT-44, CT-48 and CT-59), cross-fertile with CT-21, were assigned to IG-1. Nine isolates (CT-13, CT-21, CT-37, CT-39, CT-43, CT-45, CT-46, CT-58 and CT-60) were cross-fertile with CT-20 and were assigned to IG-2. Three isolates (CT-28, CT-31 and CT-35) are putative IG-2 isolates, as they were cross-fertile with neither CT-20 nor CT-21, but displayed fertility when crossed with some isolates of IG-1. Two isolates (CT-34 and CT-47) displayed no fertility. The pattern of fertile crosses is consistent with that of a unilocular, biallelic mating system, characterized by two cross-compatibility groups. However, 34 crosses that should be fertile under a perfect bipolar model showed no fertility.

Fertility was highly variable among crosses. For example, the cross CT-38 × CT-45 produced only one perithecium formed in four replicate dishes, whereas others, like CT-20 × CT-

21, produced over one hundred perithecia per replicate. Fertility was also variable within replicates of the same cross.

TABLE III: Sexual compatibility of twenty-one *Glomerella truncata* isolates from lentil.

	CT-13	CT-21	CT-37	CT-39	CT-43	CT-45	CT-46	CT-58	CT-60	CT-20	CT-30	CT-32	CT-38	CT-44	CT-48	CT-59	CT-28	CT-31	CT-34	CT-35	CT-47
CT-13	-[a]																				
CT-21	-	-																			
CT-37	-	-	-																		
CT-39	-	-	-	-																	
CT-43	-	-	-	-	-																
CT-45	-	-	-	-	-	-															
CT-46	-	-	-	-	-	-	-														
CT-58	-	-	-	-	-	-	-	-													
CT-60	-	-	-	-	-	-	-	-	-												
CT-20	+[b]	+	+	+	+	+	+	+	+	-											
CT-30	+	+	+	+	+	-	-	+	+	-	-										
CT-32	-	+	-	+	-	-	-	-	-	-	-	-									
CT-38	-	+	-	+	+	+	+	+	-	-	-	-	-								
CT-44	-	+	+	+	+	-	-	-	+	-	-	-	-	-							
CT-48	+	+	-	+	+	+	+	+	+	-	-	-	-	-	-						
CT-59	+	+	-	+	+	-	+	+	-	-	-	-	-	-	-	-					
CT-28	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-				
CT-31	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-			
CT-34	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-		
CT-35	-	-	-	-	-	-	-	-	-	-	+	-	+	+	+	-	-	-	-	-	
CT-47	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

[a]-: absence of perithecia; [b]+: presence of at least 1 perithecium within four repeats

Mating-type idiomorphs. — All four pairs of degenerate primers produced DNA amplicons of a size expected for the HMG box region in all isolates. The cleanest amplicons were obtained with primer pair DeLCP2, which generated two DNA fragments of 275- 325 bp for all nine *C. truncatum* isolates, and a unique fragment for *G. graminicola*. Direct sequencing of the PCR products for isolates CT-21 (IG-2) and CT-30 (IG-1) gave a consistent single sequence suggesting that the two fragments obtained for each isolate correspond with the same gene. A gap in the fifth nucleotide position at the 5' end on the CT-30 sequence was attributed to a sequencing error, as sequencing with the reverse primer produced the exact same sequence for both isolates. The BLASTX query returned a best hit of 76% identity with the mating type protein sequence of *G. graminicola* (Accession No. AAY16426.2). Further, the protein query sequence was BLASTed against NCBI's Conserved Domain (cds) database and returned a match with the HMG Box superfamily of eukaryotic chromosomal proteins. Additionally, the BLASTX search confirmed sequence similarities with mating-type proteins of several other *Glomerella* species, including *G. lindemuthiana* (up to 61% depending on the isolate of *G. lindemuthiana*), *C. coccodes* (up to 72%), *G. cingulata* (up to 73%), with a portion of the *C. graminicola* M1.001genome corresponding to the HMG box of MAT1-2 (FIG. 2). As degenerate primers also generated unspecific bands, species-specific primers were developed based on sequence information. The *G. truncata*-specificity of the primer pair CT21HMG was confirmed by the presence of a single band for each *G. truncata* isolates, and a corresponding lack of amplification in the *C. graminicola* isolate.

Regarding the alpha-box of the MAT1-1 gene, primers Falpha1 and Falpha2 produced a band just over 350bp in the *F. oxysporum* isolate of mating type 1 only, as expected (Arie et al. 2000). No band was obtained from any of the *Glomerella* isolates or the *F. oxysporum* isolate of mating type 2 (FIG. 3).

FIG 1: Alignment of the nucleotide sequences of the conserved HMG box from two isolates of *Glomerella truncata*, CT-21 and CT-30.

```
CT-30 GATG-ACCCGCACATCCAAAACCATGACATATGTAAGCGGAGAAGCCTGCTTTGACACGA
      |||| ||||||||||||||||||||||||||||||||||||||||||||||||||||||
CT-21 GATGAACCCGCACATCCAAAACCATGACATATGTAAGCGGAGAAGCCTGCTTTGACACGA

CT-30 GCGCCATGACTAACCTTTGCATAGCCAAAAGACTAGGCGCCGCCTGGAATTCCGAGTCAC
      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
CT-21 GCGCCATGACTAACCTTTGCATAGCCAAAAGACTAGGCGCCGCCTGGAATTCCGAGTCAC

CT-30 ACGAGGTTCGAGAGAGATACAGAGCGCTCGCAAAAGCCTACAAAGAGCGCCACAACAAGA
      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
CT-21 ACGAGGTTCGAGAGAGATACAGAGCGCTCGCAAAAGCCTACAAAGAGCGCCACAACAAGA

CT-30 TGCACCCGGACTATCGGTATTCTCCCCGCAAGCCCTCCGAGAAGCG
      |||||||||||||||||||||||||||||||||||||||||||||
CT-21 TGCACCCGGACTATCGGTATTCTCCCCGCAAGCCCTCCGAGAAGCG
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FIG 2: Results of the tblast translated nucleotide query. A: Translated protein sequence for *Glomerella truncata* isolate CT-21. B: Alignment with *G. graminicola* isolate M1.001

```
A:      86  AKRLGAAWNSESHEVRERYRALAKAYKERHNKMHPDYRYSP  208

B:  CT-21          A+ LG  AWN+ESHEVRE+YRALAKAYKERHNK+HP  YRY+P
    G.g. M1.001    AQLLGKAWNAESHEVREKYRALAKAYKERHNKLHPHYRYNP  243
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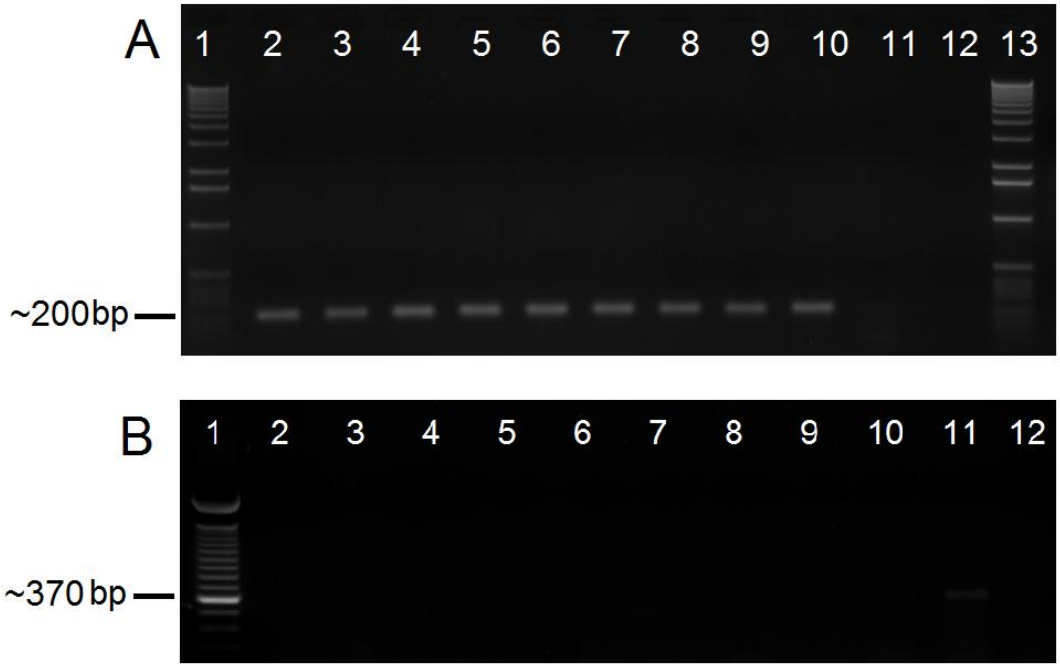
FIG 3: PCR amplification of conserved regions of the mating type idiomorphs. A: amplification of the HMG box of MAT1-2 with primer pair CT21HMG. Lane 1 and 13: 1Kb DNA ladder. Lane 2 to 10: *Glomerella truncata* isolates CT-20, CT-21, CT-30, CT-31, CT-39, CT-45, CT-58, CT-59 and CT-60. Lane 11: negative control (*G. graminicola*). Lane 12: negative control (water). B: amplification of the alpha domain of MAT1-1 with primers pair Falpha. Lane 1: 100pb DNA ladder. Lane 2 to 10: *G. truncata* isolates CT-20, CT-21, CT-30, CT-31, CT-39, CT-45, CT-58, CT-59, and CT-60. Lane 11: positive control (*Fusarium oxysporum* of mating type 1). Lane 12: negative control (*F. oxysporum* of mating type 2).



DISCUSSION

Genetic analyses have been performed for a few *Glomerella* spp., but the genetic control of sexual compatibility remains mainly unresolved. The information obtained for three species, *G. cingulata* (Wheeler 1954, Cisar and Tebeest 1999), *G. graminicola* (Vaillancourt et al. 2000b) and *G. lindemuthiana* (Rodriguez-Guerra et al. 2005), suggests that those species all have unusual mating systems that do not seem to follow the usual bipolar self-incompatibility mating system of filamentous ascomycetes. In *G. lindemuthiana*, all but one combination of 19 isolates were sterile (Rodriguez-Guerra et al. 2005). In *G. cingulata*, a study by Cisar and Tebeest (1999) suggested that most combinations of isolates were fertile and incompatible with a bi-allelic single locus basis. A unilocus, multiallelic model was proposed for *G. cingulata* by Cisar and Tebeest. (1999), whereas a multilocus system was proposed for *G. graminicola* (Vaillancourt and Hanau 1991, Vaillancourt, 2000a).

In this study, in contrast, results of classical mating studies of *G. truncata* suggested that the species is heterothallic, with a pattern of fertile combinations compatible with a bipolar self-incompatibility mating system. Crosses recorded as sterile when fertility was expected under the hypothesis of a bipolar mating system could be explained by a lack of fertility in some isolates or combinations of isolates, or by a fertility level below that detectable with the number of

replications used in this study. Some combinations were considered fertile with only one perithecium counted; therefore combinations of isolates with an even more reduced fertility may have been considered sterile. Variability in fertility is a common feature in filamentous ascomycetes. Female sterility, in particular, has been reported to be common in *Gibberella fujikuroi* field populations (Leslie, 1995), whereas male sterility was rare. The high differentiation of the female reproductive structures, as opposed to the male ones, and the complexity of their genetic control, means numerous loci can be mutated and be the cause of a sterile phenotype (Leslie and Klein, 1996). Several such genes were identified in *G. fujikuroi* species complex (Hornok et al. 2007). In the genus *Glomerella,* significant variation in fertility has been reported for *G. cingulata* and *G. graminicola* (Edgerton 1914, Bryson et al. 1992, Vaillancourt et al. 2000b). In *G. graminicola*, it was also shown that the production of perithecia was highly sensitive to environmental conditions (Vaillancourt and Hanau 1991).

The presence of the HMG box of the MAT1-2 idiomorph in isolates of both incompatibility groups of *G. truncata* is consistent with the results obtained for *G. graminicola* and *G. lindemuthiana* where a MAT1-2 idiomorph was found in both partners of fertile crosses. However, it contradicts the assumption of a bipolar self-incompatibility mating system with a genetic control through the MAT locus as described for other ascomycetes, and as suggested by the cross-fertility pattern of *G. truncata* in the classical crossing experiment. Hence, for each of the four *Glomerella* species studied to date, a different model of sexual reproduction has been proposed, but none matching the usual ascomycete mating system. Similar atypical mating systems have not been reported from other genera, including closely related genera in the Hypocreales and the Microascales. However, the latter include the genus *Ceratocystis*, were mating-type switching has been reported (Harrington and McNew 1997).

True heterothallism is controlled by mating-type loci (Raju 1992), but a heterothallic phenotype can be obtained through different genetic control mechanisms. Wheeler (1954) proposed that *G. cingulata* showed what he called 'unbalanced heterothallism'. In this model, each partner of a fertile cross carries mutated fertility genes that can complement each other resulting in cross-fertility. Based on this model, it has been suggested that heterothallism in a genus like *Glomerella* could be the result of a mutation in the developmental pathway for homothallism, and this mating system has been proposed for *G. graminicola* (Vaillancourt et al. 2000a, 2000b).

There is no direct molecular evidence for the kind of genetic process that controls the unbalanced heterothallic phenotype proposed for *Glomerella* species. However, recombinations and fusions between both MAT idiomorphs have been documented, and are believed to be the key process in shifting between heterothallism and homothallism (Yun et al. 1999). There has been much debate about which reproductive mode, homothallism or heterothallism, is ancestral (Metzenberg and Glass 1990, Coppin et al. 1997, Geiser et al. 1998). Based on phylogenetic evidence pertaining to the mating type locus organization, it has been suggested that the ascomycetous species *Neurospora crassa* and *Podospora anserina* may have been derived from a single homothallic ancestor, and that heterothallism was acquired concomitantly in a second step by both species, possibly because sexual reproduction was conferring some fitness advantage (Coppin et al. 1997). In contrast, studies by Yun et al. (1999) on *Cochliobulus* suggested that heterothallism was ancestral, and that homothallism developed through rare homologous recombination and unequal crossover events made possible by short identical sequences in the ORF of the MAT genes. This was based on the observation that in the genus *Cochliobolus*, the heterothallic species share the same organization at MAT loci, whereas each homothallic species has its own arrangement, strongly supporting the evolution of homothallism from heterothallism (Yun et al. 1999). Although these findings are not directly applicable to the genus *Glomerella*, where both partners of a fertile cross are carrying the HMG box, they indicate a high plasticity at the mating type loci, and a potential for recombination in these areas.

In conclusion, isolates of *G. truncata* tested in this study exhibited a cross-fertility pattern consistent with a mating system that is determined by one single locus with two alleles, but differed in that all isolates carried an HMG box characteristic of the MAT1-2 idiomorph. Studies in other *Glomerella* species suggested that these mating systems could be explained through unbalanced heterothallism which could also apply to *G. truncata*. To better understand the mating system of *G. truncata*, studies on the inheritance of the mating types as determined by classical mating studies are required. Sequencing of the entire mating type genes of both compatibility groups would also allow for identification of mutations and recombinants that could support the model of unbalanced heterothallism.

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FOOTNOTES

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APPENDIX 2: List of *Colletotrichum truncatum* and *Glomerella truncata* isolates

Isolate name	Host	Sampling location	Crop Year
CT-13	*Lens culinaris*	unknown	2001
CT-20	*Lens culinaris*	Oungre, SK	2001
CT-21	*Lens culinaris*	North Battleford, SK	2001
CT-28	*Lens culinaris*	unknown	2001
CT-30	*Lens culinaris*	Drinkwater, SK	2001
CT-31	*Lens culinaris*	Osage, SK	2001
CT-32	*Lens culinaris*	Bateman, SK	2001
CT-34	*Lens culinaris*	Manitoba	1995
CT-35	*Lens culinaris*	Manitoba	1995
CT-37	*Lens culinaris*	Grandora, SK	2004
CT-38	*Lens culinaris*	Saskatoon	2004
CT-39	*Lens culinaris*	unknown	2004
CT-43	*Lens culinaris*	Regina	2004
CT-44	*Lens culinaris*	unknown	2004
CT-45	*Lens culinaris*	Regina	2004
CT-46	*Lens culinaris*	Regina	2004
CT-47	*Lens culinaris*	Regina	2004
CT-48	*Lens culinaris*	Regina	2004
CT-49	*Gycine max*	Quebec	2002
CT-50	*Gycine max*	Quebec	2002
CT-51	*Gycine max*	Quebec	2002
CT-52	*Gycine max*	Quebec	2002
CT-53	*Gycine max*	Quebec	2002
CT-54	*Gycine max*	Quebec	2002
CT-55	*Gycine max*	Quebec	2002
CT-56	*Gycine max*	Quebec	2002
CT-58	*Lens culinaris*	Regina	2004
CT-59	*Lens culinaris*	Regina	2004
CT-60	*Lens culinaris*	Regina	2004
CT-61	*Matricaria perforata*	Germany	unknown
CT-62	*Matricaria perforata*	Germany	unknown
CT-63	*Matricaria perforata*	Saskatchewan	unknown
CT-64	*Matricaria perforata*	Saskatchewan	unknown
CT-65	*Matricaria perforata*	Saskatchewan	unknown
CT-182	*Lens culinaris*	Dysart, SK	2008

Isolate name	Host	Sampling location	Crop year
CT-183	*Lens culinaris*	Dysart, SK	2008
CT-184	*Lens culinaris*	Dysart, SK	2008
CT-185	*Lens culinaris*	Dysart, SK	2008
CT-186	*Lens culinaris*	Dysart, SK	2008
CT-187	*Lens culinaris*	Dysart, SK	2008
CT-188	*Lens culinaris*	Dysart, SK	2008
CT-189	*Lens culinaris*	Dysart, SK	2008
CT-190	*Lens culinaris*	Dysart, SK	2008
CT-191	*Lens culinaris*	Dysart, SK	2008
CT-192	*Lens culinaris*	Dysart, SK	2008
CT-193	*Lens culinaris*	Dysart, SK	2008
CT-222	*Lens culinaris*	Dysart, SK	2008
CT-223	*Lens culinaris*	Dysart, SK	2008
CT-224	*Lens culinaris*	Dysart, SK	2008
CT-225	*Lens culinaris*	Dysart, SK	2008
CT-226	*Lens culinaris*	Dysart, SK	2008
CT-227	*Lens culinaris*	Dysart, SK	2008
CT-228	*Lens culinaris*	Dysart, SK	2008
CT-229	*Lens culinaris*	Dysart, SK	2008
CT-230	*Lens culinaris*	Dysart, SK	2008
CT-231	*Lens culinaris*	Dysart, SK	2008
CT-232	*Lens culinaris*	Dysart, SK	2008
CT-233	*Lens culinaris*	Dysart, SK	2008
CT-235	*Lens culinaris*	Dysart, SK	2008
CT-236	*Lens culinaris*	Dysart, SK	2008
CT-237	*Lens culinaris*	Dysart, SK	2008
CT-238	*Lens culinaris*	Dysart, SK	2008
CT-239	*Lens culinaris*	Dysart, SK	2008
CT-240	*Lens culinaris*	Dysart, SK	2008
CT-241	*Lens culinaris*	Dysart, SK	2008
CT-242	*Lens culinaris*	Dysart, SK	2008
CT-243	*Lens culinaris*	Dysart, SK	2008
CT-244	*Lens culinaris*	Dysart, SK	2008
CT-245	*Lens culinaris*	Dysart, SK	2008
CT-246	*Lens culinaris*	Dysart, SK	2008
CT-247	*Lens culinaris*	Dysart, SK	2008
CT-248	*Lens culinaris*	Dysart, SK	2008
CT-249	*Lens culinaris*	Dysart, SK	2008

Isolate name	Host	Sampling location	Crop year
CT-250	*Lens culinaris*	Dysart, SK	2008
CT-251	*Lens culinaris*	Dysart, SK	2008
CT-252	*Lens culinaris*	Dysart, SK	2008
CT-253	*Lens culinaris*	Dysart, SK	2008
CT-254	*Lens culinaris*	Dysart, SK	2008
CT-255	*Lens culinaris*	Dysart, SK	2008
CT-256	*Lens culinaris*	Dysart, SK	2008
CT-257	*Lens culinaris*	Dysart, SK	2008
CT-258	*Lens culinaris*	Dysart, SK	2008
CT-259	*Lens culinaris*	Dysart, SK	2008
CT-260	*Lens culinaris*	Dysart, SK	2008
CT-261	*Lens culinaris*	Dysart, SK	2008
CT-262	*Lens culinaris*	Dysart, SK	2008
CT-263	*Lens culinaris*	Dysart, SK	2008
CT-264	*Lens culinaris*	Dysart, SK	2008
CT-265	*Lens culinaris*	Dysart, SK	2008
CT-266	*Lens culinaris*	Dysart, SK	2008
CT-267	*Lens culinaris*	Dysart, SK	2008
CT-268	*Lens culinaris*	Dysart, SK	2008
CT-269	*Lens culinaris*	Dysart, SK	2008
CT-270	*Lens culinaris*	Dysart, SK	2008
CT-271	*Lens culinaris*	Dysart, SK	2008
CT-272	*Lens culinaris*	Dysart, SK	2008
CT-273	*Lens culinaris*	Dysart, SK	2008
CT-274	*Lens culinaris*	Dysart, SK	2008
CT-275	*Lens culinaris*	Dysart, SK	2008
CT-276	*Lens culinaris*	Dysart, SK	2008
CT-277	*Lens culinaris*	Dysart, SK	2008
CT-278	*Lens culinaris*	Dysart, SK	2008
CT-279	*Lens culinaris*	Dysart, SK	2008
CT-280	*Lens culinaris*	Dysart, SK	2008
CT-281	*Lens culinaris*	Dysart, SK	2008
CT-282	*Lens culinaris*	Dysart, SK	2008
CT-283	*Lens culinaris*	Dysart, SK	2008
CT-284	*Lens culinaris*	Dysart, SK	2008
CT-285	*Lens culinaris*	Dysart, SK	2008
CT-286	*Lens culinaris*	Dysart, SK	2008
CT-287	*Lens culinaris*	Dysart, SK	2008

Isolate name	Host	Sampling location	Crop year
CT-288	*Lens culinaris*	Dysart, SK	2008
CT-289	*Lens culinaris*	Dysart, SK	2008
CT-290	*Lens culinaris*	Dysart, SK	2008
CT-291	*Lens culinaris*	Dysart, SK	2008
CT-292	*Lens culinaris*	Dysart, SK	2008
CT-293	*Lens culinaris*	Dysart, SK	2008
CT-294	*Lens culinaris*	Dysart, SK	2008
CT-295	*Lens culinaris*	Dysart, SK	2008
CT-296	*Lens culinaris*	Dysart, SK	2008
CT-297	*Lens culinaris*	Dysart, SK	2008
CT-298	*Lens culinaris*	Dysart, SK	2008
CT-299	*Lens culinaris*	Dysart, SK	2008
CT-300	*Lens culinaris*	Dysart, SK	2008
CT-301	*Lens culinaris*	Dysart, SK	2008
CT-302	*Lens culinaris*	Dysart, SK	2008
CT-303	*Lens culinaris*	Dysart, SK	2008
CT-304	*Lens culinaris*	Dysart, SK	2008
CT-305	*Lens culinaris*	Dysart, SK	2008
CT-306	*Lens culinaris*	Dysart, SK	2008
CT-307	*Lens culinaris*	Dysart, SK	2008
CT-308	*Lens culinaris*	Dysart, SK	2008
CT-309	*Lens culinaris*	Dysart, SK	2008
CT-310	*Lens culinaris*	Dysart, SK	2008
CT-311	*Lens culinaris*	Dysart, SK	2008
CT-312	*Lens culinaris*	Dysart, SK	2008
CT-313	*Lens culinaris*	Dysart, SK	2008
CT-314	*Lens culinaris*	Dysart, SK	2008
CT-315	*Lens culinaris*	Dysart, SK	2008
CT-316	*Lens culinaris*	Dysart, SK	2008
CT-317	*Lens culinaris*	Dysart, SK	2008
CT-318	*Lens culinaris*	Dysart, SK	2008
CT-331	*Lens culinaris*	Dysart, SK	2008
CT-332	*Lens culinaris*	Dysart, SK	2008
CT-333	*Lens culinaris*	Dysart, SK	2008
CT-334	*Lens culinaris*	Dysart, SK	2008
CT-335	*Lens culinaris*	Dysart, SK	2008
CT-337	*Lens culinaris*	Dysart, SK	2008
CT-338	*Lens culinaris*	Dysart, SK	2008

Isolate name	Host	Sampling location	Crop year
CT-339	*Lens culinaris*	Dysart, SK	2008
CT-340	*Lens culinaris*	Dysart, SK	2008
CT-341	*Lens culinaris*	Dysart, SK	2008
CT-342	*Lens culinaris*	Dysart, SK	2008
CT-343	*Lens culinaris*	Dysart, SK	2008
CT-344	*Lens culinaris*	Dysart, SK	2008
CT-345	*Lens culinaris*	Dysart, SK	2008
CT-347	*Lens culinaris*	Dysart, SK	2008
CT-348	*Lens culinaris*	Dysart, SK	2008
CT-349	*Lens culinaris*	Dysart, SK	2008
CT-350	*Lens culinaris*	Dysart, SK	2008
CT-351	*Lens culinaris*	Dysart, SK	2008
CT-352	*Lens culinaris*	Dysart, SK	2008
CT-353	*Lens culinaris*	Dysart, SK	2008
CT-354	*Lens culinaris*	Dysart, SK	2008
CT-355	*Lens culinaris*	Dysart, SK	2008
CT-356	*Lens culinaris*	Wilkie, SK	2008
CT-357	*Lens culinaris*	Wilkie, SK	2008
CT-359	*Lens culinaris*	Handel, SK	2008
CT-361	*Lens culinaris*	Handel, SK	2008
CT-363	*Lens culinaris*	Silton, SK	2008
CT-364	*Lens culinaris*	Silton, SK	2008
CT-365	*Lens culinaris*	Silton, SK	2008
CT-366	*Lens culinaris*	Silton, SK	2008
CT-369	*Lens culinaris*	Zealandia, SK	2008
CT-370	*Lens culinaris*	Zealandia, SK	2008
CT-374	*Lens culinaris*	Harris, SK	2008
CT-375	*Lens culinaris*	Liberty, SK	2008
CT-376	*Lens culinaris*	Liberty, SK	2008
CT-377	*Lens culinaris*	Simpson, SK	2008
CT-378	*Lens culinaris*	Simpson, SK	2008
CT-379	*Lens culinaris*	Clavet, SK	2008
CT-380	*Lens culinaris*	Clavet, SK	2008
CT-381	*Lens culinaris*	Clavet, SK	2008
CT-383	*Lens culinaris*	Tuxford, SK	2008
CT-384	*Lens culinaris*	Tuxford, SK	2008
CT-386	*Lens culinaris*	Rouleau, SK	2008
CT-387	*Lens culinaris*	Rouleau, SK	2008

Isolate name	Host	Sampling location	Crop year
CT-392	*Lens culinaris*	Belle Plaine, SK	2008
CT-393	*Lens culinaris*	Belle Plaine, SK	2008
CT-396	*Lens culinaris*	Regina, SK	2008
CT-397	*Lens culinaris*	Regina, SK	2008
CT-400	*Lens culinaris*	Forgan, SK	2008
CT-401	*Lens culinaris*	Forgan, SK	2008
CT-403	*Lens culinaris*	Forgan, SK	2008
CT-404	*Lens culinaris*	Forgan, SK	2008
CT-406	*Lens culinaris*	Dinsmore, SK	2008
CT-408	*Lens culinaris*	Dinsmore, SK	2008
CT-410	*Lens culinaris*	Vanscoy, SK	2008
CT-411	*Lens culinaris*	Delisle, SK	2008
CT-417	*Lens culinaris*	unknown	2008
CT-418	*Lens culinaris*	unknown	2008
CT-419	*Lens culinaris*	unknown	2008
CT-420	*Lens culinaris*	unknown	2008
CT-421	*Lens culinaris*	unknown	2008
CT-422	*Lens culinaris*	unknown	2008
GT-147	ascospore-derived progeny		2007
GT-148	ascospore-derived progeny		2007
GT-149	ascospore-derived progeny		2007
GT-150	ascospore-derived progeny		2007
GT-151	ascospore-derived progeny		2007
GT-152	ascospore-derived progeny		2007
GT-153	ascospore-derived progeny		2007
GT-155	ascospore-derived progeny		2007
GT-156	ascospore-derived progeny		2007
GT-157	ascospore-derived progeny		2007
GT-158	ascospore-derived progeny		2007
GT-162	ascospore-derived progeny		2007
GT-163	ascospore-derived progeny		2007