THE INFECTION PROCESS OF *COLLETOTRICHUM TRUNCATUM* ON LENTIL

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ABSTRACT

The fungus *Colletotrichum truncatum* (Schw.) Andrus and Moore causes lentil anthracnose, which is a major challenge to lentil production in western Canada. The pathogen infects leaves and stems, resulting in defoliation, stem girdling, plant wilting, and possibly plant death. Two races, Ct0 and Ct1, have been identified in the pathogen population in Canada. However, the differences in the infection process between the two races have not been described in detail. Currently, several lentil cultivars, such as CDC Redberry, CDC Robin, CDC Rosetown, CDC Rouleau, and CDC Viceroy, have resistance against race Ct1, whereas there are no cultivars showing resistance to race Ct0. The objective of this study was to investigate differences in the infection process between race Ct0 and race Ct1 using the fully susceptible cultivar Eston and the race Ct1-resistant cultivar CDC Robin. Experiments on glass well slides showed that race Ct0 had no inherently different conidium germination rate compared to race Ct1, and that differences in conidium germination between the two races on lentil plants were the result of specific interactions between the two races and lentil resistance. Investigations of the infection process of the two races on detached and attached leaves of both lentil cultivars were conducted starting 12 h postinoculation (hpi) until 72 hpi, including conidium germination, appressorium formation, and leaf penetration. Results indicated that differences in virulence of the two races may be related to the ability of conidia to germinate and form appressoria, as well as the ability of primary infection hyphae to grow in response to cues from the lentil cultivars. Furthermore, resistance of lentil to isolates of race Ct1 appeared to involve an inhibition in and/or delay of the spread of primary infection hyphae inside the plant tissue. Results of infection studies of one isolate from each race on attached leaves did not completely agree with results of the same isolates on detached leaves. Based on this study, race Ct0 and race Ct1 do not appear to be classical physiological races, but may represent aggressive races or some intermediate forms.

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1. Introduction

Lentil (*Lens culinaris* Medikus) is originally from the Near East and has a long history of cultivation as a food crop (Webb and Hawtin, 1981). In Canada the history of lentil cultivation began in 1969 (Morrall, 1997), and the harvested area of lentil reached 532,200 ha in 2007 (FAO, 2009). In 2005, the harvested area even reached 785,000 ha which were the highest during the period of 2002-2007 in Canada (FAO, 2009). As one of the main export crops, Canadian lentil exports reached 683,022 tonnes in 2006 which were 106,144 tonnes more than those in 2005 (FAO, 2009). In 2007, Canadian lentil was mainly exported to India, Bangladesh, Algeria, Colombia, United Arab Emirates, Pakistan, Turkey, and Egypt (Agriculture and Agri-Food Canada, 2008b).

As a food, lentil seeds are characterized by their high protein content, which is the reason why lentil seeds are consumed as a substitute for meat (Agriculture and Agri-Food Canada, 2008b). Other nutrients identified include starch, fat, fibre, and minerals (Solanki et al., 1999; Wang and Daun, 2006). Identified minerals include calcium, copper, potassium, manganese, phosphorus and Zinc (Wang and Daun, 2006). In addition, lentil seeds also provide the B vitamin folate (Agriculture and Agri-Food Canada, 2008b). Thus, lentil seeds are of highly nutritional value.

Growing a lentil crop can be challenged by many abiotic and biotic stresses, including drought, water-logging, salinity, heat, low temperature, nutrient deficiency, diseases, insects, weeds, and nematodes, all of which restrict the normal development of lentil (Erskine et al., 1994; Muehlbauer et al., 2006; Erskine et al., 1994). Among diseases, lentil anthracnose, caused by the fungus *Colletotrichum truncatum* (Schwein.) Andrus and Moore, was initially identified in Manitoba in 1986 and subsequently reported in Saskatchewan in 1990 (Morrall, 1988; Morrall and Pedersen, 1991). Nowadays, this disease has spread across western Canada (Tullu et al., 2003). *Colletotrichum truncatum* can infect the above-ground parts of lentil plants starting at the seedling stage, causing defoliation, stem girdling, plant wilting and even death after

vascular tissue of stems is impaired. Thus, anthracnose can be a significant threat to lentil yields (Morrall, 1988; Buchwaldt et al., 1996; Kaiser et al., 1998; Bailey et al., 2003).

Colletotrichum species have demonstrated two main infection strategies to invade plants: intracellular hemibiotrophic infection and subcuticular intramural infection (reviewed by Bailey et al., 1992). The process of *C. truncatum* infection has been studied on pea and soybean (O'Connell et al., 1993; Manandhar et al., 1985). Like other *Colletotrichum* species, e.g. *C. destructivum* on lucerne, *C. truncatum* on pea displays the following infection process before the appearance of symptoms: conidium germination, appressorium formation, penetration into plant tissue, development of infection vesicles, development of primary and secondary hyphae (SH). However, the time course for these infection structures varies among *Colletotrichum* species (O'Connell et al., 1993; Latunde-Dada et al., 1997). The major difference between the infection processes of *C. truncatum* on pea and soybean is that for the latter hyphae spread below the cuticle during the initial infection phase, and then the fungus spreads into the cell wall (Manandhar et al., 1985).

Chongo et al. (2002) studied the infection processes of two *C. truncatum* isolates with different virulences on two lentil genotypes with different resistance reactions to the fungus, and showed that the resistant genotype limited the growth of the hyphae, evident in an eight- to eleven-day delay on symptom appearance, as well as showed fewer and smaller lesions on leaflets compared to the susceptible genotype (Chongo et al., 2002). Based on field experiments and tests under controlled conditions, Buchwaldt et al. (2004) identified 16 accessions with resistance to anthracnose among 1,771 lentil accessions. Among those 16 accessions, seven were chosen as host differentials and inoculated with 50 single-spored *C. truncatum* isolates. Based on the study, two pathogenic races were identified, i.e. race Ct0 and race Ct1. So far the differences in the infection process between race Ct0 and race Ct1 have not been reported. Currently, the lentil cultivars CDC Redberry, CDC Robin, CDC Rosetown, CDC Rouleau, and CDC Viceroy have showed resistance to race Ct1, but no cultivars are resistant to race Ct0 (Saskatchewan Ministry of Agriculture, 2008b).

The objective of this study was to investigate differences in the infection process of race Ct0 and race Ct1 using the fully susceptible cultivar Eston and the race Ct1-resistant cultivar CDC Robin.

2. Literature Review

2.1. Lentil

2.1.1. Lentil

Lentil (*Lens culinaris* Medikus) is one of the food crops with the longest history of cultivation and one of the earliest cultivated plant species in the Near East (Webb and Hawtin, 1981). The word “lentil” is originated from the Latin word “*lens*” which describes the seed shape of this plant (Cubero, 1981). Lentil is an annual plant and characterized by many branches, light green stems and leaves, and short plant height. It is 15 to 75 cm tall, which is determined by the cropping condition and genotype. Other basic morphological features include that one to eight pairs of obovate leaflets are arranged oppositely or alternatively on a leaf; the number of flowers varies from one to four per peduncle; one or two seeds are generated in an oblong pod; the seed is 2 to 9 mm in diameter and has green, orange, or yellow cotyledons; the seed coat shows different colours, e.g. slight red, green, and brown (Saxena and Hawtin, 1981). Indeterminate growth is another feature of lentil; flowers are still blossoming at higher branches, while pods are maturing at lower branches on the same plant (Slinkard et al., 1990; Erskine et al., 1990a). For optimal yield, lentil plants need proper growing conditions, with pest levels managed under economic threshold. Self-pollination is another feature (Muehlbauer et al., 2006), so lentil plants do not need insects as a vector to spread pollen.

Natural environmental conditions affect the growth of lentil. Different lentil genotypes showed different reactions to temperature and photoperiod with a preferrence of warmer temperature and longer day for flowering (Summerfield et al., 1985). Rainfall and winter temperatures were two determining factors of lentil yield in Mediterranean regions (Erskine and EL Ashkar, 1993). Based on differences in environmental conditions lentil crops are planted in different seasons in different regions worldwide. For

example, in India lentil crops are planted in winter, while in the USA and Canada they are planted in spring. In Turkey, elevation determines the time of planting, winter and spring plantings take place in the areas with lower and higher elevation, respectively (Saxena, 1981).

Besides natural environmental conditions, appropriate agronomic measures such as planting date, seeding rate, plant spacing, resistance to lodging, and weed control can also affect growing conditions and thereby the yield of lentil. Studies by Ali-Khan and Kiehn (1989) and Turk et al. (2003) showed that earlier seeding resulted in higher yield in Canada (early May) and Jordan (early January). In Australia, lentil yield was much higher when planted in May compared to July, August, and September) (Dean et al., 2003). A study on seeding rates indicated that 150 plants/m^2 resulted in the maximum economic return in Australia (Siddique et al., 1998). Lentil was found to have higher yields when row spacing was narrowed to 0.15 m (Canada) and 0.2 m (West Asia) (Ali-Khan and Kiehn, 1989; Silimet al., 1990). Thicker stems increased the resistance to lodging (Erskine and Goodrich, 1988). It was shown that applications of the herbicides linuron, prometryn, or a combination of the two raised lentil yields by 59%, 68%, and 49%, respectively, when compared to untreated weedy controls (Elkoca et al., 2005). When lentil seeding rate was raised, not only was the field used more effectively, but also growth of weeds was inhibited (Baird, 2007).

Lentil plants are facing many abiotic and biotic stresses during their growth. The former includes drought, water-logging, salinity, heat, low temperature, and nutrient deficiency (Erskine et al., 1994; Muehlbauer et al., 2006), and the latter includes diseases, insects, weeds, and nematodes (Erskine et al., 1994). Ascochyta blight, anthracnose, Stemphylium blight, Fusarium wilt, Sclerotinia white mold, and rust are important lentil diseases (Muehlbauer et al., 2006), of which the former three severely threaten lentil production in Canada.

2.1.2. Use of lentil

The seeds of lentil, which is one of the food legume crops, provide rich nutritional resources for humans (Sarker and Erskine, 2006). Protein, starch, fat, fibre, minerals, and

vitamins have been identified in lentil seeds (Solanki et al., 1999; Wang and Daun, 2006; Sarker and Erskine, 2006). Regarding the exact composition of these nutritients, research done by various researchers showed the different results (Solanki et al., 1999; Wang and Daun, 2006; Bhatty, 1984; Khan et al., 1987). There are 18 kinds of amino acids in lentils, among which glutamic acid and aspartic acid are the two most common ones (Wang and Daun, 2006). Compared to protein (27.2%) and starch (43.7%), the content of fat is rather low with only 1.1% (Wang and Daun, 2006). The content of total dietary fibre (including insoluble and soluble) in lentils is 19.2%, and insoluble dietary fibre covers about 90% of total dietary fibre (Perez-Hidalgo et al., 1997). The following minerals have been reported: calcium (Ca), copper (Cu), iron (Fe), magnesium (Mg), manganese (Mn), nickel (Ni), potassium (K), phosphorus (P), sodium (Na), and zinc (Zn) (Solanki et al., 1999; Wang and Daun, 2006; Bhatty, 1984; Khan et al., 1987). Among the minerals P, Ca, Mg, Na, and K, the content of Na is the lowest (Bhatty, 1984). The contents of thiamin, riboflavin, and niacin in germinated lentils were significantly higher than those in raw lentils (Urbano et al., 1995).

Whole or split lentils and lentil flour can be used as human food (Aw-Hassan et al., 2003). Two main characteristics of lentils, i.e. short cooking time and easy digestion (Aw-Hassan et al., 2003), plus the above mentioned beneficial nutritional composition, make lentils a valuable food item for human consumption. Studies by Bhatty (1984 and 1990) showed that cooking quality of lentil was closely related to Ca, Mg, and P, especially the ratio of Ca^{2+} + Mg^{2+} to P^{4+}, as well as the content of phytic acid in the seed. The content of phytic acid in poorly-cooked lentils was significantly lower than that in well-cooked lentils (Bhatty, 1990). Soaking seeds in water shortened the cooking time by 6.3-8.0 min compared to dry seeds (Khan et al., 1987).

Besides use as a food source for human being, lentil also provides feed for animals, e.g. poultry (Aw-Hassan et al., 2003), ram (Kalkan.and Karabulut, 2003), and sheep (Erskine et al., 1990b). Straw, pod walls and seed coats of lentil can be valuable animal feed sources. The content of protein in the seed coat can reach up to 13% (Aw-Hassan et al., 2003). Steamed at high temperature and treated with H_2SO_4, cell walls can be easily usable. Steaming at high temperatures alone accelerated degradation of cellulose and hemicellulose and gas releasing in lentil straw (Kalkan.and Karabulut,

2003). Studies by Erskine et al. (1990b) showed that on average 44.5% of lentil straw was digestible dry matter. Among leaves, branches, pods, and roots in straw, the percentage of leaves was highest with 38%, and the percentage of branches placed the second with 34%. It was shown that the digestible dry matter value of leaves was much higher than that of branches (Erskine et al., 1990b). Sometimes the lentil plants growing in the field are used as a fodder for livestock (Aw-Hassan et al., 2003).

2.1.3. Lentil production

2.1.3.1. Lentil production and trade in the world

World lentil production was 3.87 million tonnes (Mt) in 2007 (FAO, 2009). That year, Canada was the second largest lentil producing country in the world with a production of 0.67 Mt after India. Based on production, the following eight large lentil producing countries were Turkey, China, Syria, Nepal, the United States, Australia, Bangladesh, and Iran (FAO, 2009). In 2005, Canada was a leading lentil producing country in the world with a production of 1.16 Mt (FAO, 2009). Production of red and green lentil, the two main market classes, varies in different countries. Green lentils have traditionally been grown mainly in Canada and the US, whereas red lentils are produced in the other countries (Skrypetz, 2006).

In 2006, Canada (0.68 Mt), Australia (0.17 Mt), the US (0.13 Mt), India (0.12 Mt), and Syria (0.08 Mt) were the top five exporting countries (FAO, 2009). Lentil exports of Canada covered 51.6% of world exports in 2006 (FAO, 2009). In 2005, Canadian lentils were mainly exported to Algeria, Turkey, Colombia, Egypt, Spain, Venezuela, Morocco, Mexico, Italy, and India (FAO, 2009). In 2006, Bangladesh, United Arab Emirates, Pakistan, Egypt, Sri Lanka, Turkey, Colombia, India, Spain, and Algeria were the top ten lentil importing countries (FAO, 2009).

2.1.3.2. Lentil production in Canada

The history of lentil cultivation in Canada began as early as 1969 (Morrall, 1997). Nowadays, lentil production is across Saskatchewan, Albert and Manitoba. Saskatchewan ranked highest during the past 10 years, with over 95% of the national lentil production (Skrypetz, 2006). In 2007, the harvested area of lentil in Canada was 532,200 ha, with an average yield of 1,258.3 kg/ha, and a total production of 669.7 kt. The harvested area, yield, and production in 2005 were the highest of the 2002 – 2007 period in Canada (FAO, 2009).

Lentil crops show best performance in the brown and dark brown soil zones of western Canada. They can also perform well in the black soil zone when moisture is not too high (Skrypetz, 2006). Green lentils can be sub-classified into four groups based on the thousand seed weight (TSW): French green, small green, medium green, and large green. Common green lentils in Canada are CDC LeMay (French green), CDC Milestone (small green), CDC Vantage (medium green), and CDC Sovereign (large green). Similarly, red lentils are also divided into two groups based on TSW. They are small red and extra small red lentils. CDC Redberry and CDC Robin are common small red and extra small red lentils in Canada, respectively (Skrypetz, 2006; Saskatchewan Ministry of Agriculture, 2008b).

In 2006-2007, total domestic use of lentils was 127 kt, representing 20.2% of lentil production. Lentil is mainly used for food, feed, and seed domestically (Agriculture and Agri-Food Canada, 2008a).

2. 2. The Genus *Colletotrichum*

2.2.1. General features

The fungal genus *Colletotrichum* Corda is anamorphic, belonging to the family Melanconiaceae, the order Melanconiales, and the class Coelomycetes (Sharma, 2002), and includes about 900 species (Sutton, 1992). The genus *Glomerella* is teleomorphic, belonging to the family *Polystigmataceae* and the order Sphaeriales (Sharma, 2002), and

includes about 80 species, among which anamorphs of more than 20 species were identified as *Colletotrichum* species. For example, the imperfect stage of *G. graminicola, G. glycines*, *G. musae, G. cingulata, G. acutata,* and *G. truncata* are *C. graminicola, C. destructivum, C. musae, C. gloeosporioides, C. acutatum,* and *C. truncatum,* respectively (Politis, 1975; Manadhar et al., 1986; Rodriguez et al., 1992; Tan and Tow, 1992; Guerber and Correll, 2001; Armstrong-Cho and Banniza, 2006; Sutton, 1992)**.** Among the above species, the teleomorphs of *C. graminicola, C. destructivum* and *C. gloeosporioides* were only found in plant tissues after inoculation under controlled conditions (Politis, 1975; Manadhar et al., 1986; Tan and Tow, 1992). Roy's studies (1982) showed that *G. cingulata* and *G. glycines* could both infect soybean seedlings in the field, but *G. cingulata* was a bigger threat to soybean seedling than *G. glycines* (Roy, 1982). Vaillancourt and Hanau (1992) identified the teleomorph of a *Colletotrichum* species on sorghum leaves under controlled conditions, which closely resembled *G. graminicola* on maize in morphology (Vaillancourt and Hanau, 1992).

A conidium of *Colletotrichum* is a single cell, which has no colour and shows different shapes, e.g. ovoid, cylindric, or dumbbell-shaped, depending upon the species. In the area where a great number of conidia grow, pink or salmon colour is visible. The acervulus, in which condia are formed, develops under the epidermis of the infected plant tissue, and due to its growth, it will pierce the epidermis, thus releasing a lot of conidia that serve to spread the pathogen. In the acervulus, hyphae are dark and sterile (Agrios, 2005). The species of *Glomerella* form perithecia, asci, and ascospores. Each of these structures may differ in size and shape depending upon the species (Politis, 1975; Manadhar et al., 1986; Rodriguez et al., 1992; Tan and Tow, 1992; Guerber and Correll, 2001; Armstrong-Cho and Banniza, 2006; Agrios, 2005). Rodriguez et al. (1992) reported that the number of asci in a perithecium varied from 50 to 80 for *G. musae*. Within an ascus, eight ascospores are produced and they have been observed with the species *G. graminicola, G. musae, G. acutata,* and *G. truncata* (Politis, 1975; Rodriguez et al., 1992; Guerber and Correll, 2001; Armstrong-Cho and Banniza, 2006).

2.2.2. Host range

Species of *Colletotrichum* and its teleomorph *Glomerella* cause anthracnose symptom on many annual plants (e.g. lentil, bean, onion, tomato, strawberry, pasture grasses, and cereals) and also are the causal agents of canker, dieback, and rot on some perennial plants (e.g. camellia, apple, peach, and grape) (Agrios, 2005; Freeman, 2000). They are the pathogens of ornamental plants as well, e.g. *Asystasia gangetica,* which is also a vegetable in Southeast Asia (Agrios, 2005; Tan and Tow, 1992). Besides having a large number of hosts, the importance of these fungi is also evident in the following three aspects: I. The distribution is worldwide irrespective of differences in climate, from temperate to tropical regions. II. Pathogens in this genus can infect both young plant tissues and ripe fruits, thus diseases can occur either in the field or in the storage room. The diseases of young leaves and stems reduce the photosynthesis and transportation of nutrients, thereby indirectly influencing the formation and quality of fruits. Once fruits (either young or ripe) are infected, direct economic losses will be encountered. III. Pathogens in this genus can infect the entire plant, including aboveground and underground parts (Freeman, 2000). In different parts, plants may have different disease symptoms, e.g. anthracnose (on leaf, stem, and pod), pod spot, and root rot can be caused on legume plants following the infection of *Colletotrichum* species (Lenné, 1992). *Colletotrichum gloeosporioides* infects mango and results in blossom blight (Jeffries et al., 1990). When leaves of strawberry were attacked by *C. acutatum*, anthracnose would develop, whereas a necrosis of the roots would be observed when the roots were infected (Freeman and Katan, 1997).

More than one plant species may serve as hosts of a single species of *Colletotrichum,* e.g. *C. coccodes* can infect tomato and potato, *C. acutatum* is a pathogen of almond, peach, anemone, and lupin. Hosts of *C. gloeosporioides* include avocado, mango, banana, and orange. In contrast to the above, more than one species of *Colletotrichum* may be pathogenic on one particular plant species, e.g. *C. acutatum*, and *C. gloeosporioides* can infect mango, peach, and apple, and *C. fragariae, C. acutatum,* and *C. gloeosporioides* can all attack strawberry (Dillard, 1992; Adaskaveg

and Hartin, 1997; Freeman et al., 2000; Talhinhas et al., 2002; Mills et al., 1992; Prior et al., 1992; Bernstein et al., 1995; Freeman and Rodriguez, 1995**)**.

Among the *Colletotrichum* species which can attack weeds, *C. gloeosporioides* was studied most widely. For example, *C. gloeosporioides* f. sp. *malvae* was found to infect round-leaved mallow (Makowski and Mortensen, 1998), leaves of *Miconia calvescens* could be attacked by *C. gloeosporioides* f. sp. *miconiae* **(**Killgore et al., 1999; Seixas and Barreto, 2007**)**, and *C. gloeosporioides* f. sp. *clidemiae* was pathogenic to *Clidemia hirta* (Trujillo et al., 1986; Norman and Trujillo, 1995). When *Aeschynomene virginica* was infected by *C. gloeosporioides* f. sp. *aeschynomene*, anthracnose symptoms would be induced (Luo and TeBeest, 1998). Other common *Colletotrichum* species infecting weeds include *C. coccodes* and *C. orbiculare*, and they infect *Abutilon theophrasti* and *Xanthium spinosum*, respectively (Ditommaso and Watson, 1997; Klein and Auld, 1996). These pathogens represent potential mycoherbicides, and *C. gloeosporioides* f. sp. *aeschynomene* has been developed into one for the control of northern jointvetch (Luo and TeBeest, 1998). Being able to control weeds has constituted one of characteristics of the *Colletotrichum* species.

2.2.3. The infection process of Colletotrichum species

2.2.3.1. General infection process

The infection process of *Colletotrichum* species starts with the attachment of conidia to the surface of host plant tissues. Under suitable environmental conditions, germ tubes will be generated from conidia. This is called germination. With the continuous growth of the germ tubes, appressoria will be differentiated at their ends and gradually darken in colour (O'Connell et al., 2000). For penetration, *Colletotrichum* species need to overcome the restriction posed by the plant epidermal cell wall and other cell walls (Bailey et al., 1992). Underneath the appressoria a penetration peg is formed, by which the epidermis can be directly penetrated. For example, *C. gloeosporioides* attacks avocado fruits and *C. acutatum* invades the leaves of citrus by direct penetration (O'Connell et al., 2000; Coates et al., 1993; Zulfiqar et al., 1996). Besides this

penetration mode, wounds and stomata are selected by some *Colletotrichum* species to enter host tissues. For example, *C. gloeosporioides* attacks ripe mango fruits through wounds, as does *C. musae* infecting banana where it induces wound anthracnose (Dinh et al., 2003; Chillet et al., 2007). An unidentified *Colletotrichum* species, which has 95–96% of similarities to *C. gloeosporioides* in the nucleotide sequences of certain rDNA regions, can enter cowpea leaves by stomata (Latunde-Dada et al., 1999).

Before penetration, during the period from attachment of conidia to penetration of the pathogens into host tissues, the development of *Colletotrichum* species shows a lot of similarities. However, the way how conidia are attached to the plant tissue surface, the materials involved in the attachment, as well as the effects of the darkening of appressoria and cutinases on penetration may be used to distinguish among a limited number of species that have been studied in more detail. The main differences among *Colletotrichum* species in the infection process often occur after penetration. Many *Colletotrichum* species do not cause symptoms right after they penetrate into host plant tissues. After a varying period of time, lasting from one day or less to more than three days, symptoms begin to appear. Thus the infection process of these *Colletotrichum* species can be divided into two stages: the symptomless biotrophic stage and the necrotrophic stage where plant tissues are damaged. The first stage is characterized by large primary hyphae (PH) appearing inside the epidermal cells but without causing damage. The morphology of PH differs among *Colletotrichum* species utilizing this infection mode. In the second stage, SH are differentiated from PH. They are thinner than PH and radiate into the adjacent cells. Damage to plant tissues takes place subsequently. This infection mode is called intracellular hemibiotrophy (Reviewed by Bailey et al., 1992; Perfect et al., 1999; O'Connell et al., 2000), and is characteristic for *C. destructivum* infecting cowpea (Latunde-Dada et al., 1996). Cultivars of *Sorghum bicolor* exhibited different responses to *C. Sublineolum* infection: on susceptible cultivars, intracellular hemibiotrophy was apparently applicable, whereas on resistant ones no biotrophic period was observed before penetrated cells died (Wharton and Julian, 1996).

A few *Colletotrichum* species follow a different infection mode after penetration. During the initial period after entering the host tissues, these species spread around the host epidermal cell walls below the cuticle, resulting in the disintegration of cell walls.

With the extensive spread of the pathogens between and within the host cells as well as through the cell walls, plant tissues are heavily damaged. The biotrophic stage is almost neglectable. This infection mode is called subcuticular intramural. However, after the plants are infected successfully, a necrotrophic stage will occur, which is similar to that observed in species with intracellular hemibiotrophic infection strategies (Reviewed by Bailey et al., 1992; Perfect et al., 1999; O'Connell et al., 2000). Typically, *C. capsici* infects cotton bolls in a subcuticular intramural mode (Roberts and Snow, 1984).

Colletotrichum gloeosporioides possesses both infection modes depending upon what plants are infected. For example, this pathogen infects leaves of *Populus tremuloides* in an intracellular hemibiotrophic mode (Marks et al., 1965), whereas attacks fruits of *Carica papaya* in the subcuticular intramural mode (Chau and Alvarez, 1983). On avocado fruits, this pathogen showed that both infection strategies were applied (Coates et al., 1993).

Colletotrichum species can grow into and remain on the seeds of many hosts, and these seeds then may become a source of inoculum in the local area after planting, or spread the disease to remote places when these infected seeds are transported and planted there in the next growing season. In some hosts, only a few seeds are infected when *Colletotrichum* species infect the plants, but these seeds do not show obvious symptoms (Agrios, 2005).

2.2.3.2. Infection process of some *Colletotrichum* species

Some *Colletotrichum* species, such as *C. orbiculare* (on Nicotiana tabacum) and *C. destructivum* (on lucerne), utilize the intracellular hemibiotrophic infection strategy to invade their hosts, which is similar to *C. truncatum* on lentil, so their infection processes are described below. *Colletotrichum gloeosporioides* (on mulberry) adopted a distinct infection mode, so its infection process is also addressed.

2.2.3.2.1. Colletotrichum orbiculare on Nicotiana tabacum

Colletotrichum orbiculare (Berk. & Mont.) von Arx, one of the pathogen species that causes anthracnose, can infect *Nicotiana tabacum* cv. Xanthi (Shen et al., 2001). It was shown that at 12 h incubation time, average percentages of conidium germination and appressorium formation were 76% and 63% on the leaves, respectively. At 24 h incubation time, they were 78% and 93%, respectively. Between 24 and 48 h incubation time, the fungus grew into epidermal cells, and was characterized by the initial formation of roundish vesicles that further developed into PH. Before PH touched the cell walls, they generated branches. When PH reached a cell wall, their shape changed slightly before they penetrated through the cell wall into another cell. However, damage to these cells was not observed at this stage. The same observation was conducted in stomatal guard cells at 72 h incubation time. It was between 96 and 120 h incubation time that SH were generated. Although these SH were differentiated from PH, they were thinner and caused more damage to the cells than the latter since they invaded into other cells. That resulted in the appearance of symptoms. By 120 h incubation time, the leaf tissue partially died because of the spread and damage of SH within these tissues. At this time, the fungus had generated acervuli on the leaf surface. Investigation of plant symptoms showed that before 96 h incubation time, no symptoms appeared on the leaves, but after this time, water-soaked lesions began to form. The colour of lesions could be initially light green, and then the lesions would turn dry as time went on. It was shown that *C. orbiculare* is a hemibiotrophic pathogen on *N. tabacum* because its initial colonization did not kill host cells and the infection was symptomless (Shen et al., 2001).

2.2.3.2.2. Colletotrichum destructivum on lucerne

Colletotrichum destructivum O'Gara is also an anthracnose fungus and can infect lucerne. Conidia on lucerne leaves generated septa prior to gemination (Latunde-Dada et al., 1997). Following germination, germ tubes developed, and appressoria were differentiated by 12 h incubation time. After 24 h, infection vesicles were observed in epidermal cells, with a thin "neck" connecting the vesicle to appressorium. Vesicles grew

and lobes started to be generated from the lateral sides of vesicles and extended. As a result, at 48 h incubation time vesicles consisting of many enlarged lobes were formed and presented different shapes in the epidermal cells. These multilobed vesicles, i.e. PH, were of considerable size but did not overcome the restriction of epidermal cell walls. After 60 h, PH produced thin outgrowths. These outgrowths later elongated and turned into SH which could cross the cell walls and reach the surrounding epidermal cells. After 72 h, SH were so developed that their spread in eppidermal cells was easy to observe. At the same incubation time, acervuli began to emerge, surrounding PH in the leaf cells. By 96 h, a great number of acervuli, each with a single seta, were found on the leaves. When lucerne isolates of *C. destructivum* were applied to cowpea, it was observed that PH grew much slower than on lucerne. Even when the incubation time was doubled, the size was still smaller. Cowpea isolate could not infect lucerne (Latunde-Dada et al., 1997).

2.2.3.2.3. Colletotrichum gloeosporioides on mulberry

Germinated conidia of *C. gloeosporioides* were observed on the leaf surface of mulberry after 3 h incubation time (Kumar et al., 2001). At 9 h incubation time, the percent germination of conidia reached 60% which increased further at 12 h incubation time, when germ tubes of germinated conidia had elongated and grew directionally towards stomata. As the germ tubes continued growing, branches were differentiated after 24 h. These branches spread on the leaf surface, and were assumed to be able to perceive the location and opening of stomata. When stomata were open, germ tubes would grow towards them, or else they would change direction to locate other open stomata (Kumar et al., 2001). When branches spread above stomata, they expanded into and formed infection vesicles. Primary infection hyphae were differentiated underneath these vesicles and passed through the stomata to infect the plant tissues (Kumar et al., 2001). Sometimes infection vesicles were formed in the lumen of stomata. Because a germ tube could form several branches, each branch could enter a stomatum, thereby enhancing the infection of the plant compared to penetration of a single germ tube without branches. At 48 h incubation time, more vesicles were formed and more penetrations were observed. At 144 h incubation time, acervuli pierced the epidermis and

a large number of conidia were found in the acervuli. As a result, symptoms tended to be more visible (Kumar et al., 2001). In contrast to other pathogens of this genus, no appressoria were found on the mulberry leaves, and the penetration was through the open stomata (Kumar et al., 2001).

2.2.4. Effects of environmental conditions on Colletotrichum species and latent infection

Environmental conditions, mainly temperature and humidity, play an important role in the disease cycles caused by *Colletotrichum* species. In general, pathogens in this genus prefer high temperature and high humidity for infection. Free water, rain, or high humidity are very important for releasing conidia from acervuli and spreading them in the field, as well as for germination of conidia and penetration of host tissue (Agrios, 2005).

A study by Ningen and Cole (2004) showed that decreasing night temperature could restrict the infection of *C. gloeosporioides* on *Euonymus fortunei* resulting in lower disease ratings. Two isolates of *C. gloeosporioides* on mango, I-2 and I-4, showed different responses to temperature when forming appressoria; the temperature optimum for I-2 was 5 ºC higher than that for I-4 (Estrada et al., 2000). Using the same temperature (30ºC) and incubation time (5 h), increased relative humidity resulted in an increase in the frequency of occurrence of papaya anthracnose, caused by *C. gloeosporioides* (Duran et al., 1998). A combined experiment of temperature and wetness showed that at the same wetness level, optimal temperatures were different for the conidial germination of *C. acutatum* on strawberry leaves, formation of appressoria, and secondary conidiation. The optimal temperatures varied from 23.0 to 27.7 ºC, 17.6 to 26.5 ºC, and 21.3 to 32.7 ºC for these three processes, respectively (Leandro et al., 2003). Another experiment dealing with the influence of temperature and wetness period on watermelon anthracnose caused by *C. orbiculare* showed that at the same temperature the fungus thrived in longer wetness periods, and that infection might not occur if the time of exposition to wetness for inoculated seedlings was less than 2 h under designed temperature treatments (Monroe et al., 1997). Besides the effects of temperature and humidity, light conditions also affected the development of the *Colletotrichum* pathogens.

For example, constant light could increase sporulation of *C. gloeosporioides* on green pepper compared to 24 h darkness (Mello et al., 2004).

Latent infection of *C. acutatum*, *C. gloeosporioides,* and *C. fragariae* on strawberry fruits was investigated by King et al. (1997). During the latent period, temperature was a determinant factor. Higher temperature shortened the latent period when inoculated detached fruits were placed under different temperatures ranging from 5 to 35ºC. This study also showed that three species had different sensitivities to the lower temperatures of 5 and 10 ºC in terms of sporulation. Among the three species, *C. acutatum* was most cold tolerant (King et al., 1997). The situation seems to be different for infection of peaches by *C. gloeosporioides* and *C. acutatum* where the pathogens can also cause latent infection (Zaitlin et al., 2000). One and a half to three months prior to maturity of fruits, inoculations conducted several times caused either no symptoms on the fruits, or only small lesions. The typical symptoms were exhibited only when the fruits became ripe (Zaitlin et al., 2000). The reason for latent infections in this case is probably related to the nutritient levels. When fruits develop toward maturity, changes in the nutrient composition of the fruits may result in the development of pathogens from latent to active condition. Similar examples are *C. gloeosporioides* on avocado fruit, mango (attached fruits), and papaya (Binyamini and Schiffmann-Nadel, 1972; Daquioag and Quimio, 1979; Dickman and Alvarez, 1983). A study by Liu et al. (1995) presented an explanation based on nutrient availability as to why the two pathogens *C. musae* and *C. gloeosporioides* latently infected immature banana fruits and mangoes, respectively. It was shown that the starch content played an important role during these processes with immature fruits containing higher amounts of starch.

For *Colletotrichum* species with latent infection, it is necessary to use appropriate methods for early detection. Recently, studies showed that paraquat and ethanol immersion could be utilized to detect latent infection of *Colletotrichum* species (Rajeswari et al., 1997; Zaitlin et al., 2000; Ishikawa, 2004; MyeongHyeon et al., 2004).

2.2.5. Population structure of Colletotrichum species

Colletotrichum species have often been grouped based on the morphological characteristics of infection structures, symptoms, or virulence to the host plants. The isolates are sometimes categorized into different physiological races based on the reactions to differential varieties (Caten, 1987). A study by Ali et al. (1987) showed that when nine isolates of *C. graminicola* from sorghum were inoculated onto six differential lines of sorghum in the greenhouse and field, disease severity varied from 1.2 to 4.5 on a scale from 1 to 5. Further comparisons showed that all isolates caused higher disease ratings (2.0 to 4.5) on the first two differential lines and lower disease ratings (1.2 to 1.7) on two other lines, whereas on the remaining two lines disease severity ranged from low (1.2 to 1.7) to high (2.3 to 4.1). Based on the interaction between all isolates and the last two lines, three physiological races were identified (Ali et al., 1987). Marley et al. (2001) used different methods to identify five physiological races of *Colletotrichum sublineolum* of sorghum among 50 isolates. Initially, nine morphological groups and seven pathogenic groups were separated based on morphological variability and pathogenicity of these isolates. A typical isolate from each group was chosen and inoculated onto differential sorghum lines. It was shown that 16 isolates varied in their virulence ("the degree or measure of pathogenicity") and aggressiveness ("ability to cause severe disease"), and five physiological races were established accordingly (Marley et al., 2001).

Colletotrichum lindemuthianum, pathogen of *Phaseolus vulgaris*, is highly variable and different races were studied by researchers from different regions. In Brazil, Menezes and Dianese (1988) inoculated 12 differential cultivars with 201 isolates, and distinguished nine races, four of which were new. Alam and Rudolph (1988) found five major races in West Germany, and among which beta, delta, and lambda were more variable races because a total of seven subraces (three from beta, two from delta, and two from lambda) were identified. Other reports regarding the identification of new race(s) included: race epsilon in Ontario, Canada (Tu et al., 1984); race 73 (close to race alpha-Brazil) and race 7 (similar to race delta) in Michigan, the USA (Kelly et al., 1994); race delta-mutant in Asturias, Spain (Fernández et al., 2000). Resistance breeding against this fungus in *P. vulgaris* encountered a great challenge due to this high variability in the

pathogen.

In Australia, *Colletotrichum orbiculare* infected the weed *Xanthium spinosum* (Auld and Say, 1999). Compared to an Argentinian isolate, an isolate of *C. orbiculare* from Australia was more pathogenic to the weed, had a higher growth rate and higher yields when cultured on potato dextrose agar for colony growth and lima bean agar for conidium production (Auld and Say, 1999). *Colletotrichum graminicola* seriously threatens normal growth of turf grass, e.g. annual blue grass and creeping bent grass. It was found that the creeping bent grass isolates of *C. graminicola* could infect annual blue grass, but annual blue grass isolates could not infect creeping bent grass. It was also shown that *C. graminicola* isolates infecting the same host were genetically highly similar (Browning et al., 1999).

To distinguish races of *C. truncatum* of lentil, 1771 lentil accessions, the lentil variety Indianhead, and 50 lentil isolates with different origins were used (Buchwaldt et al., 2004). A field screening was first conducted by inoculating 1771 lentil lines with one isolate. As a result 5% of lines showed at least moderate resistance, which were further examined under controlled conditions, identifying 16 lines with resistance to the fungus. Seven of these 16 resistant lines were inoculated with the 50 *C. truncatum* isolates, and the races Ct0 and Ct1 were found based on the reaction of the isolates to seven host differentials (Buchwaldt et al., 2004).

2.3. *Colletotrichum truncatum*

2.3.1. Characteristic features

Sutton (1992) summarized the morphological characteristics of conidium, appressorium, sclerotium, seta, and the colony of *Colletotrichum truncatum* (Schwein.) Andrus and Moore. Conidia of isolates from lentil (Kaiser et al., 1998), pea (O'Connell et al., 1993), and soybean (Ford et al., 2004) had a similar shape to those described by Sutton (1992), but were longer. Observations by Ford et al. (2004) showed that conidia of lentil isolates had a different shape compared with those of other host plant isolates. Conidia of lentil isolates were elliptical, while they were falcate for soybean isolates; the

former only had one pointed end, but the latter showed two pointed ends. Beyond those differences, it was also observed that lentil isolates generated wider and shorter conidia compared to soybean isolates (Ford et al., 2004). Conidial dimensions were 18.6 × 4.0 µm. Armstrong-Cho and Banniza (2006) induced the teleomorph form of *C. truncatum*, *Glomerella truncata* sp. nov, in the laboratory. Eight oblong ascospores were observed in an ascus which was generated in a brown-black perithecium. Mean sizes of the perithecium, ascus, and ascospore were 350 × 200 µm, 90 × 8 µm, and 15.7 × 6.7 µm, respectively (Armstrong-Cho and Banniza, 2006).

2.3.2. Host range

Colletotrichum truncatum can infect many leguminous crops, such as lentil, pea, fababean, soybean, sweet pea, chickpea, and cowpea (Anderson et al., 2000; Boyette, 1991; Weidemann et al., 1988; Adebitan et al., 1996). *Colletotrichum truncatum* is also a pathogen of some weeds, e.g. hemp sesbania (Boyette , 1991), wild vetch (Bailey et al., 2003), Florida beggarweed (Cardina et al., 1988), jimsonweed, dogbane, cocklebur (Hartman et al., 1986) and scentless chamomile (Graham et al., 2006), where it is assumed to be suitable as a biocontrol agent of these weeds.

Lentil isolates of *C. truncatum* could infect fababean and slightly infect pea, but could not infect chickpea, bean, and soybean in both field and controlled conditions (Anderson et al., 2000). Experiments carried out in controlled conditions showed that lesions on pea caused by lentil isolates were much less sporulating than those on fababean, suggesting that the infection process of a lentil isolate is different on pea and fababean. When pea isolates of *C. truncatum* were applied onto lentil and fababean, similar lesions were observed that did not sporulate on either host (Anderson et al., 2000). Similar to these observations, lesions on soybean caused by an isolate from hemp sesbania did not sporulate either (Boyette, 1991). Studies by Hartman et al. (1986) showed that *C. truncatum* isolates from ten weeds, such as milkweed, velvetleaf, jimsonweed, and cocklebur, could infect soybean.

2.3.3. Epidemiology of Colletotrichum truncatum on lentil

2.3.3.1. Lentil anthracnose in Canada

Lentil anthracnose, caused by *Colletotrichum truncatum* (Schwein.) Andrus & W. D. Moore, was initially identified in Manitoba in 1986 and was found again near Winnipeg in 1987 (Morrall, 1988). In Saskatchewan, this disease was first reported from Zealandia in July 1990. During a subsequent survey, this disease was identified on two cultivars, Laird and Eston (Morrall and Pedersen, 1991). At present, anthracnose is distributed in all lentil-producing regions of western Canada (Tullu et al., 2003). Research showed that in Canada there are two races of lentil anthracnose, Ct0 and Ct1 (Buchwaldt et al., 2004). Some lentil cultivars, e.g., CDC Redberry, CDC Robin, CDC Rosetown, CDC Rouleau, and CDC Viceroy, have resistance to race Ct1, but there are no cultivars showing resistance to race Ct0 (Saskatchewan Ministry of Agriculture, 2008b).

2.3.3.2. Symptoms of lentil anthracnose

The fungus can infect leaves, stems, and pods of lentil during different growth stages. Occurrence of symptoms initially starts at the seedling stage. After plants are infected, lesions will appear on infected plant tissues. They are initially white to grayish in colour and finally become brown, and superficial lesions often enlarge and develop into deeper lesions (Morrall, 1988; Buchwaldt et al., 1996; Kaiser et al., 1998; Bailey et al., 2003). The disease often appears on lower leaves and stems, but later on it will move up, so the higher leaves and stems will become infected. During early flowering, when leaves are covered by many lesions, plants will defoliate. The disease will also spread onto surrounding plants causing disease patches to develop in fields (Bailey et al., 2003). Merging stem lesions can damage vascular tissues of stems, causing crop wilt. When infection is heavy, plants can die (Morrall, 1988; Buchwaldt et al., 1996; Kaiser et al., 1998; Bailey et al., 2003).

When the canopy closes, diseased patches turn yellow. Under favorable environment conditions, development of the disease will accelerate and the disease tends

to be heavier resulting in large patches of infected plants. Dark brown discolourations in the middle of a patch indicate dead plants. Black microsclerotia form on heavily infected stems, which explains why lentil stubble can appear black in the field after harvest (Bailey et al., 2003).

2.3.3.3. Infection process of *Colletotrichum truncatum* on lentil

Infection studies showed that conidium germination and appressorium formation happened earliest at 3 h and 6 h after inoculation (ai), respectively (Chongo et al., 2002). Following conidium germination, an appressorium was differentiated from a germ tube of the germinated conidium. It was observed that the fungus used an infection peg generated on the underside of the appressorium as the only way to penetrate epidermal cells of leaves and stems. At 24 h ai PH were found colonizing epidermal cells, and between 24 h and 72 h ai, PH expanded within the initially colonized epidermal cells and extended towards adjacent cells. The earliest symptoms appeared on infected plants at 72 h ai (Chongo et al., 2002). It was shown that there was at least a 48 h gap between initial penetration and the appearance of symptoms. During this period of time, the fungus grew further into the plant tissues, but cells were still alive. It is for these reasons that *C. truncatum* is considered to be a hemibiotrophic fungus on lentil (Chongo et al., 2002). At 144 h ai, severe infection symptoms were visible.

2.3.3.4. Disease cycle

Inoculum of lentil anthracnose mainly has four sources: dust from infected lentil plants during harvest, crop residues carrying the fungus left in the field, soil in the fields where infected lentil residues were left, and infected lentil plants (Buchwaldt et al., 1996).

Thus, lentil anthracnose is a stubble-borne, seed-borne, and wind-borne disease (Buchwaldt et al., 1996; Morrall et al., 2006; Bailey et al., 2003). Warm and wet environmental conditions are favourable for its occurrence and development (Bailey et al., 2003). During day time, 20-24ºC is most conducive for disease development (Chongo

and Bernier, 2000a). It was reported that the disease developed fastest on four to six weeks old plants (Chongo and Bernier, 2000b).

Microsclerotia on lentil residues could only maintain their infectivity for approximately one year when colonized residues were left on the soil surface, suggesting the pathogen could not tolerate adverse environment conditions, e.g. extremely low temperatures. If residues colonized by microsclerotia were buried under the soil, it was found that microsclerotia were still alive after four years (Buchwaldt et al., 1996).

In the spring the microsclerotia can be spread from the soil surface to lower leaflets and stems by splashed rain, causing the first disease symptoms. During the growing season, the conidia from these infected leaflets and stems can be spread to surrounding plants when it is raining, resulting in repeated infection cycles (Bailey et al., 2003). Wind was shown to play a role in the spread of the disease between fields by microsclerotia (Buchwaldt et al., 1996; Bailey et al., 2003). Infected faba bean and wild vetch probably can also serve as secondary hosts of lentil anthracnose (Bailey et al., 2003).

The severity of lentil anthracnose is determined by response of lentil cultivars to the fungus, virulence of predominant races, and environmental conditions (e.g., temperature, humidity, wind, and light).

2.3.4. Infection process of Colletotrichum truncatum on other legume plants

2.3.4.1. *Colletotrichum truncatum* on pea

Pea (*Pisum sativum*) is one of the hosts of *C. truncatum*, and studies by O'Connell et al. (1993) showed that after germination of conidia and the formation of appressoria of the fungus, infection pegs were produced from the appressoria before 36 h ai. The infection pegs could penetrate the wall of epidermal cells, resulting in the growth of the fungus inside the epidermal cells. Between 36 and 48 h ai, PH were observed in epidermal cells, and PH gradually enlarged in both thickness and length. At 72 h ai, they were 4.5 to 6 μm thick in diameter and had generated many branches. These PH with branches continued to grow, however, growth was restricted to the initially colonized

epidermal cell. Therefore, until 72 h ai, the fungus had caused almost no damage to epidermal cells. At 72 to 96 h ai, damage began to appear as SH emerged. These SH were only 1.5 to 3.5µm thick in diameter and appeared to be able to grow unrestricted through adjacent cells. As a result, symptoms began to appear during this period. It was observed that some SH did not branch while they were growing, and it was suggested that this may be important for SH to reach remote areas (O'Connell et al., 1993). At 120 h ai, invaded epidermal cells tended to die because their walls were damaged by SH. As the infection period was characterized by a symptomless interval starting with the penetration of epidermal cells by the fungus and ending with cells being killed, it was confirmed that *C. truncatum* on pea is a hemibiotrophic fungus. Acervuli were not observed on plant leaves. In this study, the authors also inoculated other legumes (lentil, soybean, cowpea, adzuki bean, French bean, groundnut, and Lima bean) with *C. truncatum* isolates, but symptoms were not observed. During the infection process, the fungus could generate appressoria after conidial germination, but it could not penetrate into tissues of these plants. It seems that these plants were not hosts of the *C. truncatum* isolates which can infect pea (O'Connell et al., 1993).

2.3.4.2. *Colletotrichum truncatum* on soybean

Colletotrichum truncatum can infect the whole plant of soybean including roots (Khan and Sinclair, 1992). Manandhar et al. (1985) studied the infection process of *C. truncatum* on soybean leaf tissues and observed that geminating conidia were different from ungerminated ones in which the former generated one to three septa while the latter had none. At 4 h incubation time the percentage of conidial germination reached 70%. Germ tubes developed at the different locations on the conidium: one end or both ends. Germ tubes sometimes formed from the middle of the conidium, but the probability was very small. It was found that when conidia were distributed in a small number on the leaf surface, the percentage of germination was less than that when they were located in high density. It was common that an appressorium was generated from the end of germ tubes. Interestingly, it was also observed that a germ tube could sometimes generate two appressoria. Following the formation of appressoria, infection pegs would develop under

the appressoria and penetrate leaf tissues (Manandhar et al., 1985). Besides infection pegs, germ tubes could also invade the cells before the formation of appressoria. The fungus could also go into leaf tissue through stomata (Manandhar et al., 1985).

After penetrating the cuticle and epidermis, the fungus invaded mesophyll cells within 48 h incubation time. At this time, infection hyphae were also found filling the intercellular space of the mesophyll. During this period acervuli were also generated (Manandhar et al., 1985). Petiole, leaf vein, and interveinal lamina were three locations where acervuli commonly developed. At 72 h incubation time the fungus was observed to colonize the vascular bundles of both leaves and petioles, resulting in the interruption of nutrient and water transport (Manandhar et al., 1985).

The generation of enzymes or toxic compounds by *C. truncatum* after plant tissues were invaded might play a role in damaging these tissues (Manandhar et al., 1985). The fungus was shown to prefer colonizing the wet area because wetter veinal areas were easier to be penetrated compared to drier interveinal areas (Manandhar et al., 1985).

2.4. Control of lentil anthracnose

2.4.1. Studies on the resistance of lentil to Colletotrichum truncatum

Breeding for and using resistant cultivars are economical and effective measures in controlling crop diseases. To investigate the resistance of lentil to anthracnose, and look for resistance resources that can be incorporated into new cultivars, some studies were carried out. Fifteen lentil lines and cv. Indianhead exhibited resistance to race Ct1 (Buchwaldt et al., 2004). Currently, several lentil cultivars in addition to Indianhead also show resistance to race Ct1, e.g. CDC Redberry, CDC Robin, CDC Rosetown, CDC Rouleau, and CDC Viceroy, whereas no lentil cultivars are resistant to race Ct0 (Saskatchewan Ministry of Agriculture, 2008b; Buchwaldt et al., 2004). It was shown that two wild *Lens* species *Lens ervoides* (Brign.) Grande and *L. lamottei* Czefr. had higher resistance to both races during field and greenhouse screenings (Tullu et al., 2006). Two interspecific hybrids resistant to both races have been developed, i.e. LR-59 and LR-61. The former was from a cross of lentil cv. Eston × one of *L. ervoides* accessions, and

the latter from a lentil breeding line *L. culinaris* subsp. *culinaris* 971-16 × one of *L. lamottei* accessions (Fiala, 2006).

During studying the host-pathogen interaction, it was found that *C. truncatum* developed similarly on a susceptible lentil genotype as on a resistant lentil genotype in terms of infection patterns during the first 48 h ai (Chongo et al., 2002). As time went on, the fungus developed faster on the susceptible genotype than on the resistant genotype because lesions appeared much earlier, and the number and size of lesions were higher and larger on the former (Chongo et al., 2002). At different day and night temperatures (16:12ºC, 20:16ºC, 24:20ºC, and 28:24ºC, day : night), the incidence of anthracnose on most of the resistant lentil genotypes tested was lower than that on a susceptible cultivar. Stem lesions on most resistant genotypes were smaller, and percentages of stem lesion with sporulation on all resistant genotypes were lower (Chongo and Bernier, 2000a). Higher temperatures increased the anthracnose incidence on the resistant lentil genotypes, while it did not appear to influence the fungus on a susceptible cultivar. When temperature was increased from 16:12 to 24:20ºC, the lesion size and percentage of stem lesions with sporulation on all genotypes were increased accordingly (Chongo and Bernier, 2000a). It was concluded that the following parameters could be used to screen lentil lines partially resistant to the anthracnose in the fields: incubation period, latent period, disease severity, and area under disease progress curve (AUDPC). The selected lines would be very useful in breeding for resistance to *C. truncatum* through crossing to the cultivars with partial resistance or with high yield (Chongo and Bernier, 1999a).

A major gene, *LCt-2*, and minor genes were found to determine resistance of lentil lines to *C. truncatum* and the level of resistance, respectively (Tullu et al., 2003). The molecular markers linked to the major gene, $OPEO6_{1250,}$ $UBC\text{-}704_{700}$, $EMCTTACA_{350}$, $EMCTTAGG_{375}$, and $EMCTAAAG_{175}$, were identified with the objective to simplify selection of lentil cultivars resistant to *C. truncatum* (Tullu et al., 2003). Three markers were used, two (i.e. UBC 227_{1290} and RB 18_{680}) linked to genes of resistance to *Ascochyta lentis* and one (i.e. $OPO6_{1250}$) linked to the gene resistant to *C. truncatum*, and recombinant inbred lines possessing genes resistant to these two pathogens were identified (Tar'an et al., 2003). However, the study found a lower ratio of

resistant lines and a higher ratio of susceptible lines with the marker $OPO6_{1250}$ (Tar'an et al., 2003).

2.4.2. Control of lentil anthracnose

To minimize the loss of plants caused by any species of *Colletotrichum*, integrated management measures are recommended (Waller, 1992). These measures cover a wide range from pre-seeding to post-harvest.

In regions free of the disease, anthracnose-free lentil seed is strongly recommended (Bailey et al., 2003), which can inhibit the spread of the disease significantly. Lentil cultivars with partial resistance to anthracnose, resulting in only smaller lesions and requiring a longer incubation period compared to susceptible cultivars, were believed to play an important role in restricting the spread of the disease (Chongo and Bernier, 1999a; Chongo and Bernier, 2000a). The use of these partially resistant cultivars combined with fungicide applications, e.g. chlorothalonil, was considered to deliver best results in controlling the development of anthracnose (Chongo et al., 1999b).

Infected plant residues represent a major source of primary infection and good residue management can reduce disease pressure. The time of survival of *C. truncatum* in lentil residue depended upon where the residue was located. If infected residue was exposed above ground for one year, infectivity of the pathogen began to decrease. The reason was probably that the exposed microsclerotia could not tolerate adverse environmental conditions, i.e. high temperatures which lasted a long time. If infected residue was buried underground, higher infectivity was maintained for as long as four years (Buchwaldt et al., 1996). Therefore, to reduce the risk of anthracnose, new lentil cropping should follow at least a four-year rotation (Bailey et al., 2003). Because microsclerotia in dust and lentil residue can be scattered by wind, it was determined that fields with a new lentil crop should be at least 240 m away from fields with a history of lentil anthracnose (Buchwaldt et al., 1996). To avoid the infection of lentil crops by the pathogen from volunteer lentil plants and weed (e.g. wild vetch) growing in the lentil fields, timely eradication of these hosts is very important (Bailey et al., 2003).

Before an application of fungicides, field surveys are necessary, which are conducted prior to flowering and focus on the lower leaves (Bailey et al., 2003). A study by Chongo et al. (1999b) showed that a fungicide application (chlorothalonil) played an important role in protecting lentil plants from the anthracnose. If there is a sign that infection is progressing, it is suggested to spray foliar fungicides no later than when the canopies are about to close (Bailey et al., 2003). The following options of foliar fungicides are currently available to control lentil anthracnose: BRAVO 500, DITHANE DG RAINSHIELD NT, QUADRIS, and HEADLINE EC. Each of them requires applications at an appropriate growth phase of lentil and needs optimal times to use (McVicar et al., 2006; Saskatchewan Ministry of Agriculture, 2008a).

3. Materials and Methods

3.1. Investigation of conidium germination on glass well slides

3.1.1. Selection of isolates and culture maintenance

Isolates used from lentil included CT-15 (race Ct1), CT-20 (race Ct0), CT-21 (race Ct1), CT-32 (race Ct0), CT-34 (race Ct0), and CT-35 (race Ct1). Prior to experimentation, all isolates were passed through a susceptible host.

Conidial suspensions for inoculation were prepared from 7- to 10-day old cultures growing on oatmeal agar (OMA) (30 g powdered oatmeal, 8.8 g agar (Difco, Becton, Dickinson and Company, Franklin Lakes, NJ, USA), 1 liter distilled water) and incubated in an incubator at 22°C and alternating 12 h light and 12 h dark. The cultures were washed with sterile distilled water and the resulting conidial suspensions were diluted to a final concentration of 5×10^4 conidia/mL as determined with a haemocytometer.

3.1.2. Inoculation of glass well slides

Percent conidium germination of isolates was compared using glass well slides to determine whether isolates belonging to different races had differences irrespective of physical or chemical cues provided by a host plant.

The progress of germination of conidia over time was studied in distilled water at 27ºC in an incubator for all the isolates. A droplet of 100 µL of conidium suspension was spread into a well on a well slide that was incubated in a Petri dish lined with moistened filter paper. High humidity was maintained by placing the dishes in trays with moistened filter paper and loosely covering the trays with plastic. Percent conidium germination was assessed at 2-hour interval for 20 hours. Four Petri dishes were removed at each

assessment time and percent conidium germination was calculated by examining at least 100 conidia within several fields of vision selected from left to right and from top to bottom across the well on each slide at × 100 magnification under a light microscope (Nikon Microphot FXA, Japan). Conidia were considered to have germinated when the germ tube was at least half the width of the conidium. The number of germinated conidia was expressed as a percentage of the total number of conidia counted. The experiment was conducted twice.

3.1.3. Data analyses

Statistical analyses were conducted using the SAS program (SAS Institute Inc. Cary, NC, USA). Regression analyses were conducted to determine the germination rate (slopes) for each isolate and replicate. The mixed procedure was used to compare two groups (races Ct0 and Ct1) by linear contrast using slope data and percent conidium germination data at 20 h after the conidium suspensions were spread on the well slides, respectively, considering 'isolate' as a fixed factor and 'repeat' and 'repeat × isolate' as random factors.

3.2. Effects of plant age, conidium concentration, and inoculation method on conidium germination on lentil leaves

3.2.1. Selection of isolate and inoculum preparation

Isolate CT-15 (race Ct1) was grown on OMA for 10 to 14 days. Conidia were washed from Petri plates with sterile distilled water, and three conidium suspensions of 5×10^4 (low concentration), 5×10^5 (intermediate concentration), and 1×10^6 (high concentration) conidia/mL were prepared.

3.2.2. Plant materials

Lentil cultivars CDC Robin, a small red cultivar with brown seed coat and red cotyledon (Vandenberg et al., 2002), and Eston, a small green cultivar with yellow cotyledons (Slinkard and Bhatty, 1981) were used in the experiments. The two cultivars were planted at 8 seeds per 95mm × 95mm plastic pot filled with soil-less mixture (Terra-Lite Redi-Earth®, Scotts-Sierra Horticultural Products Co., Marysville, Ohio, USA). Plants were thinned to 6 per pot two weeks after seeding. Two sets of plants were planted to obtain 3- and 6-week old plants. Ten pots per cultivar per plant age were prepared. The pots were maintained in a growth chamber at 22°C/16ºC day/night with 16 h light and 8 h dark. A complete fertilizer solution (20-20-20) (NPK+ micronutrients) was applied at a concentration of 3g per liter water two weeks after seeding and then once a week.

3.2.3. Inoculation and clearing of lentil plants

Four leaves consisting of at least four leaflets from four pots of each cultivar were removed from each, 3- and 6-week old plants. One 3- and one 6-week old leaf of each cultivar was placed in a sandwich box lined with moistened paper towel that represented one replicate. Leaflets of each leaf were point-inoculated with a droplet of 10 μL suspensions of isolate CT-15 using 5×10^4, 5×10^5, and 1×10^6 conidia/mL, respectively. The 4th leaflet was point-inoculated with the same amount of distilled water as a control. Four replicate sandwich boxes were prepared in this manner. Sandwich boxes with inoculated detached leaves were placed in an incubator at 22°C, 88~92% RH, and continuous light for 16 h.

After removing leaves for point-inoculation, a transparent plastic sheet was used to wrap around each pot. The plants in each pot were sprayed with 10 mL suspensions of the same isolate using a hand-held CO_2 powered sprayer (model RUH8210, Oxygen regulator, Uniweld, USA). To ensure that there were enough inoculated leaves, two pots of each cultivar and plant age were sprayed with each of three concentrations of conidium suspensions. Two pots were sprayed with distilled water as control. All sprayed plants

were incubated in a mist chamber at 22°C for 16 h. Pots sprayed with the same concentration of suspension were put together. The experiments were conducted twice.

After 16 h, the four detached leaflets of each treatment combination were removed from sandwich boxes and placed in a glass vial containing a mixture of 3:1 (glacial acetic acid : 95% ethanol) for at least 24 h for leaflets of 3-week old plants, and at least 48 h for the leaflets of 6-week old plants to clear the chlorophyll. To ensure that there were at least four sprayed leaflets from whole plants with sufficient conidium numbers to be used to investigate conidium germination, one leaflet from each spray-inoculated plant was picked from the mist chamber, resulting in 12 leaflets per treatment combination. Spray-inoculated leaflets of the same treatment combination were placed in glass vials containing the same mixture as above. The time of clearing was also the same as above. The mixture was changed every 12 h. Samples were then stained with Cotton Blue and light microscopy was conducted as described in section 3.1.2. Four leaflets per treatment combination were analyzed for each inoculation method. For spray-inoculation, four leaflets were selected randomly from 12 leaflets collected from the whole plants. At least 100 conidia were examined per leaflet, and percent conidium germination was scored as described in section 3.1.2.

3.2.4. Data analysis

Data were transformed using the arcsine square root transformation to stabilize the variance, followed by mixed procedure for the complete data set. Repeats of the experiment were considered a random factor, whereas plant age, inoculation method, and conidium concentration were fixed. Whenever there was a significant interaction between two parameters, further analyses were conducted. The least square adjusted means were used to compare mean percentages of conidium germination when necessary.

When investigating the effect of plant age on conidium germination based on conidium concentration, a significant interaction of plant age × inoculation method was found under high concentration, so data were further analysed for each inoculation method under this concentration. When evaluating the effect of conidium concentration on conidium germination based on inoculation method, there was a significant interaction

between plant age and conidium concentration for the spray-inoculation method, thus this required further analyses for each plant age. When investigating the effect of inoculation method on conidium germination according to conidium concentration, there was a significant interaction between plant age and inoculation method at high concentration, so further analyses were conducted for each plant age at this concentration.

3.3. Study of the infection process on detached lentil leaves

3.3.1. Selection of isolates and inoculum preparation

Three isolates of race Ct0 (CT-20, CT-30, CT-34) and three isolates of race Ct1 (CT-15, CT-21, CT-35) of *C. truncatum* were incubated for 10-14 days in OMA Petri dishes at 22°C under 12 h/12 h light/dark. Conidia were harvested and the concentration for all conidium suspensions was adjusted to 1×10^5 conidia/mL.

3.3.2. Plant materials

Seeding and maintenance of lentil plants as well as applications of fertilizer were the same as described in 3.2.2.

3.3.3. Investigation of conidium germination and appressorium formation on the surface of green lentil leaflets

Six leaflets for each cultivar at the age of three weeks were picked randomly and placed in a single Petri dish lined with moistened filter paper for inoculation with six isolates. Leaflets of the same cultivar were placed together. Three Petri dishes were prepared this way at each time, and values were averaged. The experiment was repeated six times, representing six replicates blocked over time. For each replicated set of Petri dishes, preparation and inoculation of the six isolates were conducted based on a sequence determined randomly to reduce experimental error which might be caused by different time periods between conidium preparation and inoculation for each isolate.

Using the point-inoculation method, a droplet of 10 μL of conidium suspension was added to each leaflet surface. Inoculated leaflets were incubated at 22°C and 88~92% RH under continuous light for 12 h, immediately air dried after that and stained for microscopy with Aniline Blue. Investigations on conidium germination and appressorium formation were carried out under the light microscope at × 100 magnification following the same sequence as conidium preparation and inoculation. This experiment was designed as split-plot design, where cultivar was the main-plot factor, and isolate was the sub-plot factor. Data analysis was conducted using the mixed procedure in SAS, considering isolate and cultivar as fixed factors and repeat and repeat × cultivar as random factors.

3.3.4. Investigation of the infection process into leaf tissue

For detailed cytological studies of the infection process, three replicate leaflets for each cultivar were placed in a Petri dish lined with moistened filter paper. The leaflets from the same cultivar were placed together. For each isolate five Petri dishes were prepared and incubated in an incubator at 22°C, 88~92% RH, and continuous light. One Petri dish per isolate was removed after the following incubation periods: 24, 36, 48, 60, and 72 h. The chlorophyll of leaflets was removed with the clearing solution described earlier. Leaflets inoculated with CT-15 were stained with Aniline Blue to stain fungal structures. All other leaflets were not stained, and decolorized leaflets were directly examined using a microscope (Olympus BX51 (TRF), Olympus Optical Co Ltd., Japan) with differential interference contrast (DIC) function at × 400 magnification. This avoided the problem of low contrast between the stained fungal structures and plant cells. Characteristic features of the infection process were documented by photography with a Spot Insight B/W camera (Model# 3.1.0, Diagnostic Instruments, USA).

3.3.5. Investigation of percent penetration into detached lentil leaflets

To quantify the penetration success of the isolates into lentil leaf tissue, four replicate leaflets for each cultivar were picked and placed in a Petri dish lined with moistened filter paper. The inoculation method with the six isolates was the same as described in section 3.3.3, and leaflets were incubated for 48 h in an incubator at 22°C, 88~92% RH, and continuous light. Samples were cleared as described in section 3.2.3, and the numbers of infection vesicles and PH within epidermal cells and of appressoria on the leaflet surface were counted using the DIC microscope at × 200 magnification. The percent penetration was calculated by dividing the number of infection vesicles and PH within epidermal cells by the number of observed appressoria on the leaflet surface. The experiment was analyzed as a completely randomized design using the mixed procedure of SAS.

3.3.6. Investigation of the first appearance of infection vesicles

Preparation of Petri dishes with leaflets was the same as described under section 3.3.4. The point-inoculation method was used as described in section 3.3.3. Sets of inoculated leaflets were incubated for 16, 18, 19, 20, 21, 22, 23, and 24 h in an incubator with 22°C, 88~92% RH, and continuous light. Sample preparation for microscopy using a DIC microscope was the same as described under section 3.3.4.

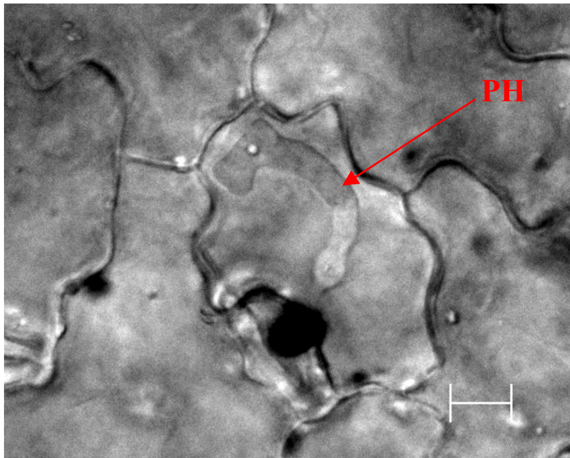
After de-staining, 87 to 391 appressoria per leaflet were evaluated for the appearance of infection vesicles (IV). The percentage of appressoria from which infection vesicles had developed was calculated for each of three leaflets for the time period when the infection vesicles were first visible.

To investigate any differences between the two races in the first appearance of infection vesicles, the slopes were first calculated for the linear graphs describing the percentage of infection vesicles over time. Slope data was then analyzed with the mixed proceduure of SAS.

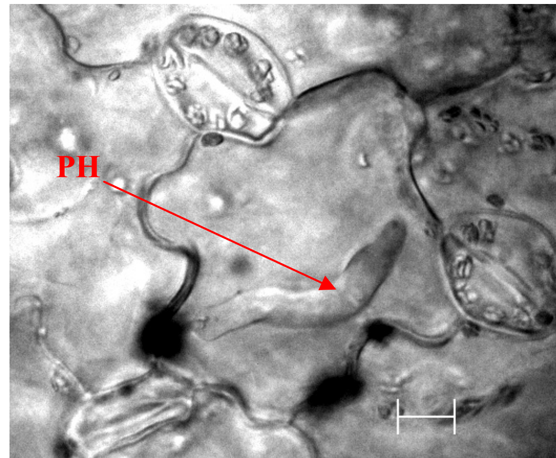
3.3.7. Investigation of the size and shape of primary hyphae

Preparation of Petri dishes with leaflets was the same as described in section 3.3.4. The point-inoculation method was used as described in section 3.3.3. Inoculated leaflets were incubated for 48 and 60 h, respectively, in an incubator at 22°C, 88~92% RH, and continuous light. Samples were cleared as described in section 3.3.4. Twenty PH per leaflet of each cultivar were observed for each isolate at each incubation time. The lengths of PH, measured from the appressorium to the end(s) of PH (for PH with up to 4 branches) or as 2 cross diameters (for more complex PH), the percentage of the area of plant epidermal cell occupied by PH, and the type (1-4, Figure 3.1) of PH based on their shapes were recorded using a DIC microscope. The experiment was analyzed as a completely randomized design. The total length of PH measurements and the percent epidermal cell area occupied by PH were analyzed using general linear model procedure of SAS. The frequency of different types of PH was analyzed using genmod procedure of SAS.

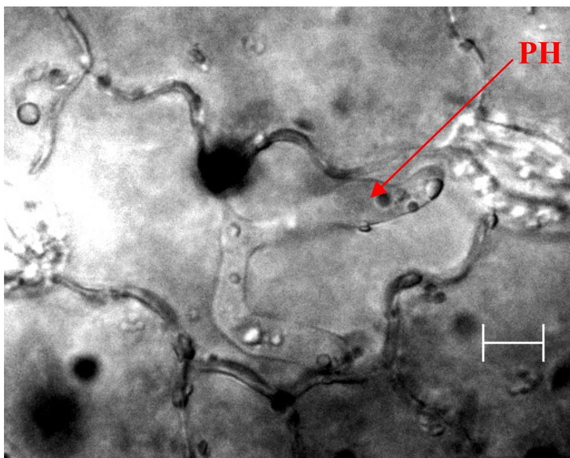
Figure 3.1 Different types of primary hyphae (PH) of *Colletotrichum truncatum* based on their shapes in the detached lentil leaves observed at 48 or 60 h postinoculation (hpi) under a DIC microscope at × 400 magnification. Type 1: A and B; Type 2: C and D; Type 3: E and F; Type 4: G and H. Bar=10μm.



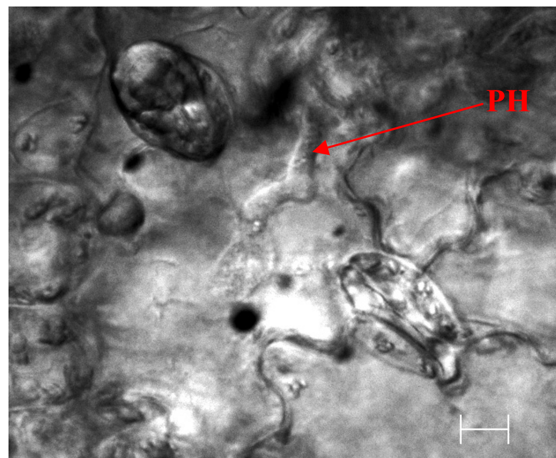
A) CT-15 (Ct1) on Eston at 48 hpi



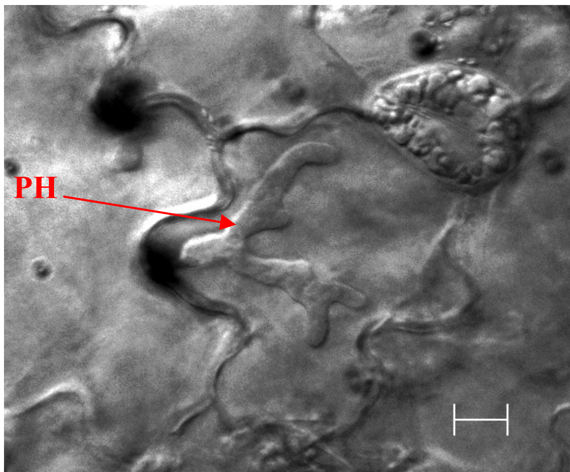
B) CT-15 on Eston at 48 hpi



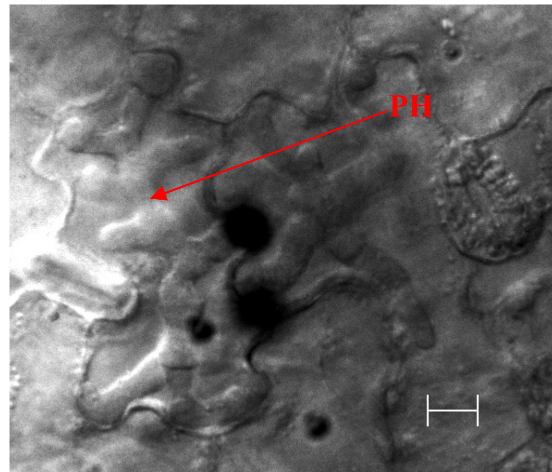
C) CT-15 on Eston at 48 hpi



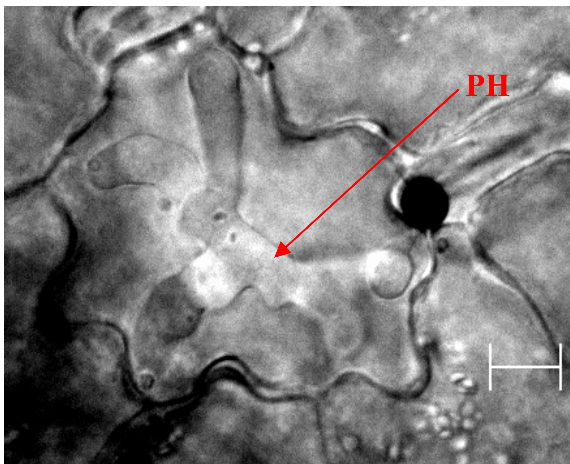
D) CT-35 on CDC Robin at 48 hpi



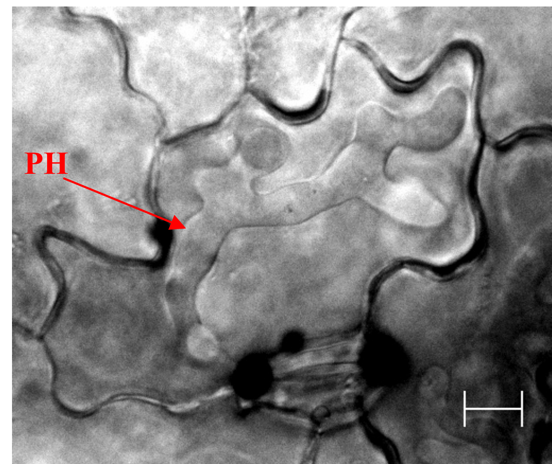
E) CT-20 on Eston at 48 hpi



F) CT-20 on Eston at 60 hpi



G) CT-20 (Ct0) on CDC Robin at 48 hpi



H) CT-15 on Eston at 48 hpi

3.3.8. Investigation of the first appearance of secondary hyphae

Following procedures as described under 3.3.4, sets of inoculated leaflets were incubated for 60, 62, 64, 66, 68, 70, and 72 h. After the leaflets were cleared, samples were investigated for the first appearance of SH, and the time when SH first appeared for each isolate on each cultivar was recorded. Observations were descriptive only.

3.4. Study of the infection process on attached lentil leaves

3.4.1. Selection of isolates and inoculum preparation

Isolates CT-34 (race Ct0) and CT-35 (race Ct1) of *C. truncatum* were used in this study. The two isolates were incubated for 10-14 days in OMA Petri dishes at 22°C under 12 h/12 h light/dark. Conidia were harvested and concentrations were adjusted to 1×10^5 conidia/mL.

3.4.2. Plant materials

Plants of lentil cultivars Eston and CDC Robin were grown in a growth chamber at 22°C/16°C day/night with 16 h/8 h light/dark for three weeks. Applications of soil and fertilizer were the same as described in section 3.2.2.

3.4.3. Inoculation of lentil plants

Before inoculation, moistened paper was placed at one bottom end of the transparent plastic containers (Rubbermaid® brand, Rubbermaid Commercial Products, Saratoga Springs, NY, USA) covering 1/3 length of the bottom. A single whole plant from each cultivar was gently removed from the pots, and the roots were wrapped with some wet soil before placing the plant horizontally on the moistened paper in the container. More moistened paper was used to cover the roots. Leaflets were fixed to the bottom of the containers with tape. A total of six containers were prepared this way, three for each isolate. Each container had one CDC Robin plant and one Eston plant. Ten leaflets per plant were inoculated. The inoculation method was the same as for detached leaflets described under 3.3.3. After inoculation, a thin plastic film and a lid were used to cover the container to maintain higher humidity. Containers were placed in an incubator at 22º C, 88-92% RH, and continuous light for 48 h. For each cultivar, six to ten leaflets inoculated with each isolate were removed and tissue samples were prepared for microscopy as described under 3.3.4. Three leaflets from each cultivar were assessed for

each isolate. Twenty PH per leaflet were evaluated for their total length, the percent area of the plant epidermal cells occupied by PH, and the type of PH, as described under 3.3.7. The experiment was analyzed as a completely randomized design. Total length of PH and the percent plant epidermal cell area occupied by PH were analyzed using the mixed procedure in SAS. Frequencies of the type of PH were analyzed with the genmod procedure in SAS.

4. Results

4.1. Investigation of conidium germination on glass well slides

In this experiment, conidium germination of three race Ct0 isolates and three race Ct1 isolates was observed on glass well slides and percentage of conidium germination was determined at 2-hour interval for 20 hours. At 2 h after the conidium suspensions were spread on the well slides, conidium germination of race Ct0 and race Ct1 isolates ranged from 0 to 3 % and 0 to 4 %, respectively. At 20 h after the conidium suspensions were spread on the well slides, conidium germination of race Ct0 and race Ct1 isolates ranged from 16 to 69 % and 15 to 76 %, respectively. Overall, percentage of conidium germination increased over time for isolates of both races, but the data from the two races did not fall into two distinct groups (Figure 4.1).

Group comparisons between race Ct0 and race Ct1 based on the slopes (rate of conidium germination) and 20 h germination data showed no significant differences between these two races (Figure 4.1, Tables 4.1 and 4.2, and Appendix 1).

Figure 4.1 Increases in the percentage of conidium germination of six isolates belonging to race Ct0 or race Ct1 of *Colletotrichum truncatum* between 2 and 20 hours after the conidium suspensions were spread on the glass well slides at 27ºC

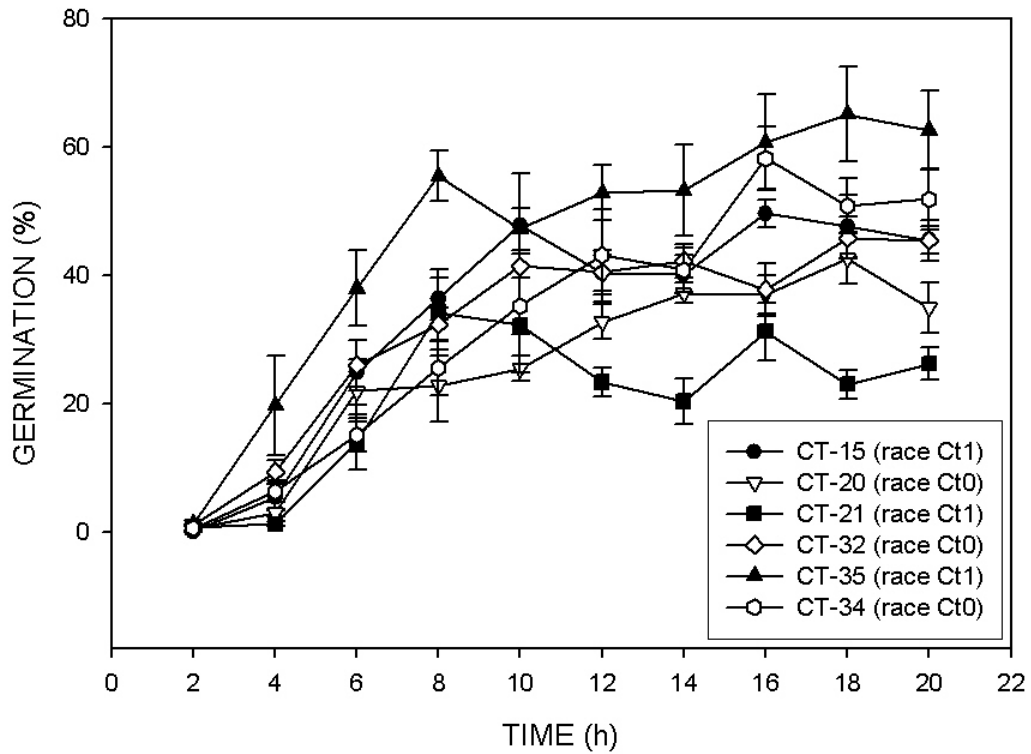


Table 4.1 Means and standard errors of percent conidium germination over time (slope) of isolates belonging to race Ct0 or race Ct1 of *Colletotrichum truncatum* between 2 and 20 hours after the conidium suspensions were spread on the glass well slides

Isolate	Race	Mean	Std Error
CT-15	Ct1	2.5189	0.1453
CT-20	Ct0	2.1580	0.1424
CT-21	Ct1	1.2652	0.1429
CT-32	Ct0	2.2492	0.1873
CT-34	Ct0	3.1576	0.2066
CT-35	Ct1	2.9742	0.3430

Table 4.2 Results of contrast analysis of two races (Ct0 and Ct1) of *Colletotrichum truncatum* based on the slopes and percent conidium germination at 20 h after the conidium suspensions were spread on the glass well slides

Source	Label	Num DF	Den DF	F Value	Pr > F
Slope	race Ct1 vs Ct0	1	25.5	2.53	0.1242
20-h data	race Ct1 vs Ct0	1	27.7	0.04	0.8415

4.2. Effects of plant age, conidium concentration, and inoculation method on conidium germination on lentil leaves

In this experiment, three- and six-week old plants of lentil cvs. Eston and CDC Robin were inoculated with CT-15, a race Ct1 isolate, using spray- and point-inoculation methods at three conidium concentrations, i.e. a low concentration with 5×10^4 conidia/mL, an intermediate concentration with 5×10^5 conidia/mL, and a high conidium concentration with 1×10^6 conidia/mL. Percentage of conidium germination in each treatment combination is shown in Table 4.3. Results of the analyses based on the entire data using the mixed procedure of SAS are shown in Appendix 2. There were significant interactions of plant age × inoculation method, inoculation method × conidium concentration, and plant age × inoculation method × conidium concentration (Appendix 2), so the experimental factors plant age, inoculation method, and conidium concentration were analysed one by one.

Table 4.3 Means and standard errors of percent conidium germination on three- and six-week old plants of lentil cvs. Eston and CDC Robin inoculated with *Colletotrichum truncatum* isolate CT-15 (race Ct1) at three concentrations using two inoculation methods

Age	Method	Cultivar	Concentration	Mean	Standard Error
3-week	Point-inoculation	Eston	1×10^6	62.50	2.8785
			5×10^5	72.25	3.2390
			5×10^4	82.50	2.7710
		CDC Robin	1×10^6	58.00	2.5774
			5×10^5	68.25	4.0872
			5×10^4	75.63	2.8781
	Spray-inoculation	Eston	1×10^6	72.75	1.3195
			5×10^5	77.00	2.2440
			5×10^4	78.63	1.9080
		CDC Robin	1×10^6	68.00	3.4434
			5×10^5	75.13	2.8249
			5×10^4	76.25	1.5324
6-week	Point-inoculation	Eston	1×10^6	81.75	2.4330
			5×10^5	87.00	1.5119
			5×10^4	93.88	0.6928
		CDC Robin	1×10^6	76.88	2.2155
			5×10^5	81.63	2.6922
			5×10^4	90.13	1.3016
	Spray-inoculation	Eston	1×10^6	79.25	2.4839
			5×10^5	87.75	0.8814
			5×10^4	91.63	1.5111
		CDC Robin	1×10^6	72.75	3.0923
			5×10^5	88.88	0.9717
			5×10^4	88.75	0.9590

Plant age

Analyses of the entire data set showed that plant age significantly affected conidium germination, but there were significant interactions for plant age × inoculation method and plant age × inoculation method × conidium concentration (Appendix 2). Data were then analysed separately for each conidium concentration. At the low conidium concentration, plant age significantly affected conidium germination, and conidium germination was significantly higher on six-week old plants than on three-week old plants (Table 4.3. and Appendix 3). At the intermediate concentration, plant age did not affect conidium germination significantly (Appendix 4), whereas at the high concentration, plant age was a marginally significant factor. However, there was a significant interaction between plant age and inoculation method at that concentration (Appendix 5), so data were further split up by inoculation method. When spray-inoculating with a high conidium concentration, there were no significant differences in conidium germination between the two plant ages (Appendix 6); when point-inoculating, conidium germination was significantly higher on six-week old plants than on three-week old plants (Appendices 7 and Table 4.3).

Inoculation method

Analyses of the entire data showed that inoculation method had no significant effect on conidium germination, but there were significant interactions for plant age × inoculation method, inoculation method × conidium concentration, and plant age × inoculation method × conidium concentration (Appendix 2). Separate analyses for each conidium concentration showed that at low and intermediate concentrations, inoculation method had no effect on conidium germination (Appendix 3 and 4); at the high concentration, there were also no significant differences between two inoculation methods in conidium germination, but there was a significant interaction of plant age and inoculation method (Appendix 5), so data were further explored by analyzing for each plant age separately. This revealed that on three-week old plants conidium germination was significantly higher when using spray-inoculation than using point-inoculation

(Appendices 8 and Table 4.3.); on six-week old plants conidium germination was not significantly different between the two inoculation methods (Appendix 9).

Conidium concentration

Analyses of the entire data showed that conidium concentration significantly affected conidium germination, but there were significant interactions for inoculation method × conidium concentration and plant age × inoculation method × conidium concentration (Appendix 2). Analyses by inoculation method showed that conidium concentration did not significantly affect conidium germination in point-inoculating (P = 0.0549) (Appendix 10). There were significant differences among three concentrations when spray-inoculating, but there was also a significant interaction between plant age and conidium concentration (Appendix 11), so further analyses were conducted for each plant age separately. It was shown that when spray-inoculating three-week old plants there were significant differences among the three concentrations (P = 0.0048) (Appendix 12). The high concentration resulted in significantly lower conidium germination than low and intermediate concentrations (P = 0.0021 and P = 0.0107, respectively), whereas there were no significant differences between low and intermediate concentrations (Appendix 13). On six-week old plants results were the same as those on three-week old plants ($P < 0.0001$) (Appendix 14), i.e. the high concentration resulted in significantly lower conidium germination than low and intermediate concentrations (both $P < 0.0001$), whereas there were no significant differences between low and intermediate concentrations (Appendix 15).

The above experiments suggested that the point-inoculation method used on the leaves of three-week old lentil plants using a conidium concentration of 5×10^5 conidia/mL was the most suitable method for further experiments. However, when the experiments were conducted on green and uncleared leaves, it was found that 5×10^5 conidia/mL resulted in a conidium concentration too high for detailed counts, suggesting that the clearing process had washed off a certain number of conidia, specifically non-

germinated conidia. Therefore, the conidium concentration was slightly reduced to 1×10^5 conidia/mL for subsequent experiments on green leaves used without clearing.

4.3. Study of the infection process on detached lentil leaves

4.3.1. Conidium germination and appressorium formation on the surface of detached green leaves

In this study, conidium germination and appressorium formation of six isolates were investigated on the surface of detached green (i.e. non-destained) leaves of CDC Robin and Eston at 12 hpi. It was shown that there was no significant interaction of isolate and cultivar (P = 0.0557) for percent conidium germination, while there was a significant interaction of isolate and cultivar (P = 0.0001) for percent appressorium formation. On Eston, conidium germination of race Ct0 isolates was significantly higher, with 68.9% germinated conidia, than that of race Ct1 isolates, with 63.5% (Figure 4.2, Table 4.4, Appendix 16). However, no significant differences in the percentage of appressorium formation were observed between the two races at that time (Fig. 4.2, Table 4.5, Appendix 17). On CDC Robin, race Ct0 isolates had significantly higher percentages of conidium germination (62.5%) and of appressorium formation (37.3%) than race Ct1 isolates (50.4% and 29.3%, respectively) at that time (Fig. 4.2, Tables 4.4 and 4.5, Appendices 16 and 17).

Figure 4.2 Percentages of conidium germination (left) and of appressorium formation (right) of three isolates of race Ct0 (CT-20, CT-30, CT-34) and three isolates of race Ct1 (CT-15, CT-21, CT-35) of *Colletotrichum truncatum* at 12 h postinoculation on the detached leaves of lentil cultivars CDC Robin and Eston

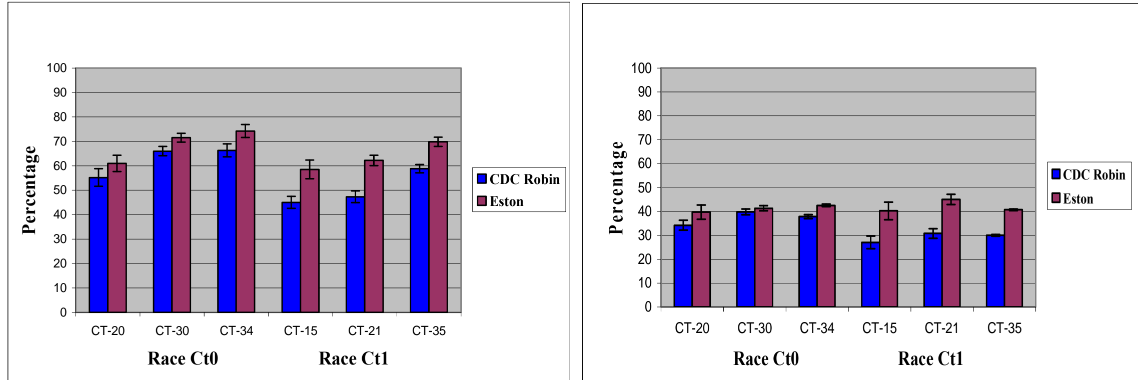


Table 4.4 Results of the contrast analysis of percent conidium germination of race Ct0 and race Ct1 of *Colletotrichum truncatum* on the detached lentil leaves at 12 h postinoculation

Label	Num DF	Den DF	F Value	Pr > F
Ct1 vs Ct0 on Eston	1	17.5	5.33	0.0334
Ct1 vs Ct0 on CDC Robin	1	14.7	31.87	<.0001

Table 4.5 Results of the contrast analysis of percent appressorium formation of race Ct0 and race Ct1 of *Colletotrichum truncatum* on the detached lentil leaves at 12 h postinoculation

Label	Num DF	Den DF	F Value	Pr > F
Ct1 vs Ct0 on Eston	1	13.4	0.21	0.6589
Ct1 vs Ct0 on CDC Robin	1	16.7	33.18	<.0001

4.3.2. Penetration into detached leaf tissue

4.3.2.1. Investigation of the infection process into leaf tissue

In this study, the infection process of six isolates into detached leaf tissue of both cultivars, CDC Robin and Eston, was observed by microscopy between 24 hpi and 72 hpi at 12-h interval.

Observations of inoculated lentil leaflets showed that following conidium germination, generation and growth of germ tubes, and appressorium formation on the leaflet surface, infection vesicles (IVs) were observed inside the epidermal cells of both cultivars under the penetration sites at 24 hpi (Fig. 4.3). In most cases the IVs appeared to be directly connected to the underside of appressoria. Sometimes appressoria were not found above IVs, probably because these appressoria were washed away during the leaf clearing process.

After IVs were formed, larger PH within epidermal cells developed between 36 and 60 hpi (Figs. 3.1 and 4.4). During this period PH expanded in size. Either a single PH extended from the appressorium or two to several lobes were developed from PH and continuously grew. As a result, PH presented different shapes (Fig. 3.1). The development of fungal infection structures (IV and PH) in specific leaflets was not synchronized as an IV was observed in one epidermal cell while PH were developing in others (Fig. 3.1E). At 36 hpi, the majority of PH was Type 1 and Type 2. It was obvious that PH showed different shapes starting at 48 hpi (Fig. 3.1). By 60 hpi, PH had developed further in size and number of branches compared to 48 hpi (Fig. 3.1F). For some isolates, e.g. CT-30, thinner SH began to develop from some PH by 60 hpi. By 72 hpi, SH became very prominent, being much thinner than PH, either developing inside a locally parasitized epidermal cell or spreading through the cell wall to adjacent cells (Fig. 4.5).

Fig. 4.3 An infection vesicle (IV) of isolate CT-35 (race Ct1) of *Colletotrichum truncatum* inside an epidermal cell on a detached leaflet of lentil cultivar CDC Robin at 24 h postinoculation under a DIC microscope at × 400 magnification. Bar=5µm.

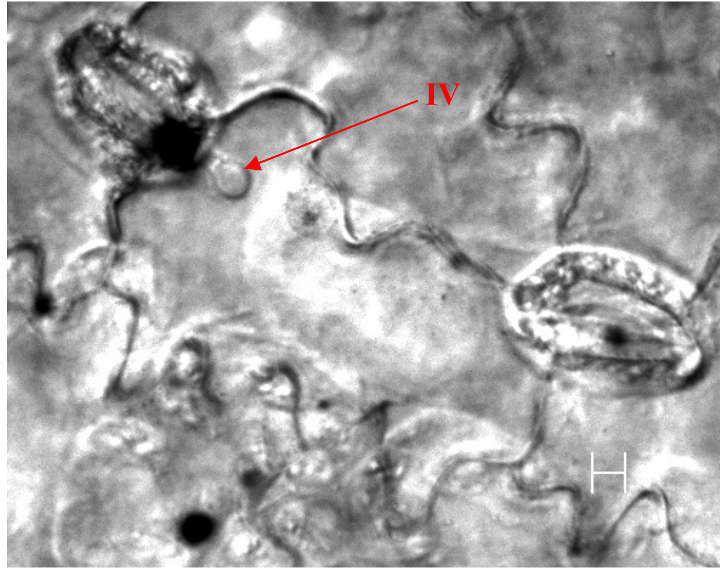


Fig. 4.4 A primary hypha (PH) of isolate CT-35 (race Ct1) of *Colletotrichum truncatum* inside an epidermal cell on a detached leaflet of lentil cultivar Eston at 36 h postinoculation under a DIC microscope at × 400 magnification. Bar=10µm.

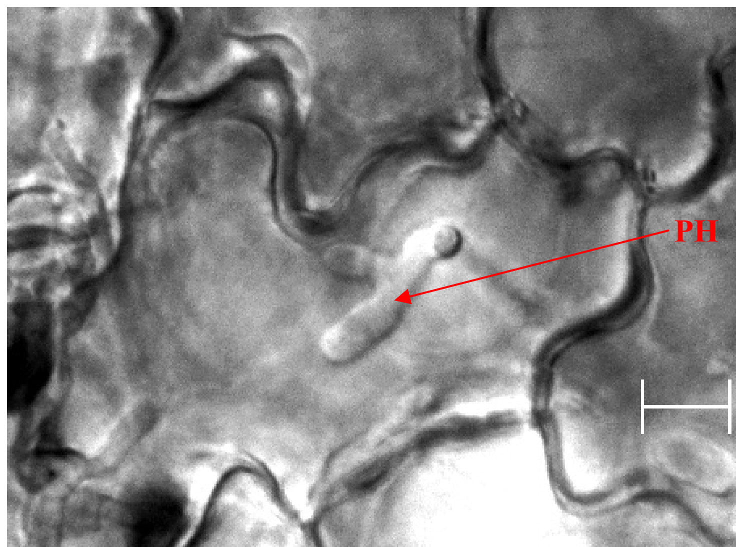
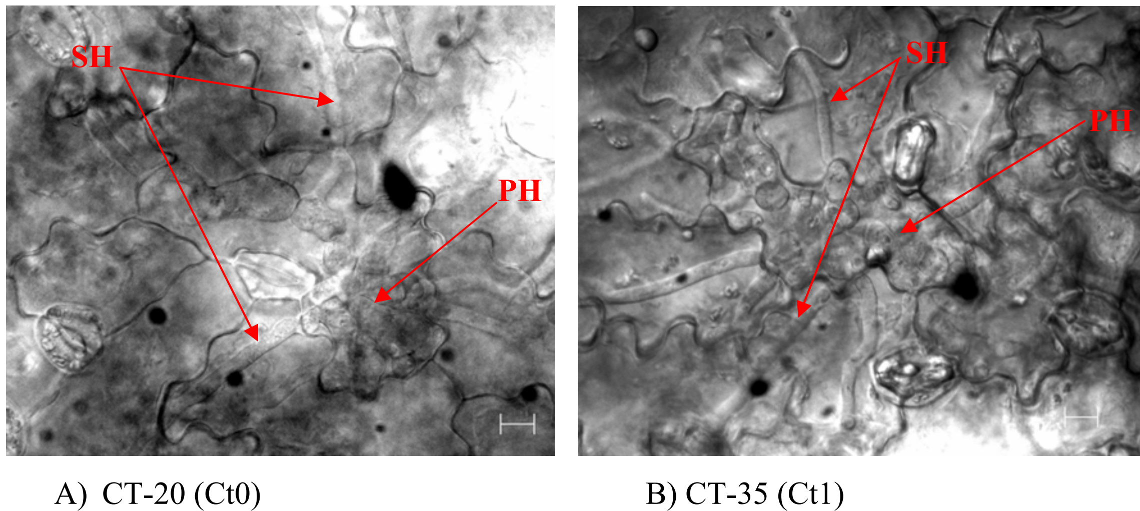


Fig. 4.5 Primary hyphae of *Colletotrichum truncatum* isolates on the detached leaflets of lentil cultivar Eston at 72 h postinoculation (PH: primary hyphae, SH: secondary hyphae) under a DIC microscope at × 400 magnification. Bar=10μm.



A) CT-20 (Ct0) B) CT-35 (Ct1)

4.3.2.2. Investigation of percent penetration of six isolates into detached lentil leaflets

In this experiment, the percentage of appressoria with IV and/or PH, i.e. percent penetration, was determined at 48 hpi. Data analyses revealed that there was no significant interaction between isolate and cultivar (P = 0.6193). Both isolate and cultivar had significant effects on penetration (P = 0.0007 and P = 0.0045, respectively; Appendix 18). Contrast analysis showed that isolates of race Ct0 had a significantly higher percentage of penetration on CDC Robin and Eston than isolates of race Ct1 (Fig. 4.6 and Table 4.6). As chlorophyll of leaflets had been removed prior to counting, it was speculated that some appressoria were removed during that process and as a result, the percent penetration may have possibly been overestimated. However, it was hypothesized that any washing-off of appressoria might happen at equal frequency on both cultivars, so relative differences should still be valid.

Fig. 4.6 Percent penetration of three isolates of race Ct0 (CT-20, CT-30, CT-34) and three isolates of race Ct1 (CT-15, CT-21, CT-35) of *Colletotrichum truncatum* into detached leaflets at 48 h postinoculation on the lentil cultivars CDC Robin and Eston

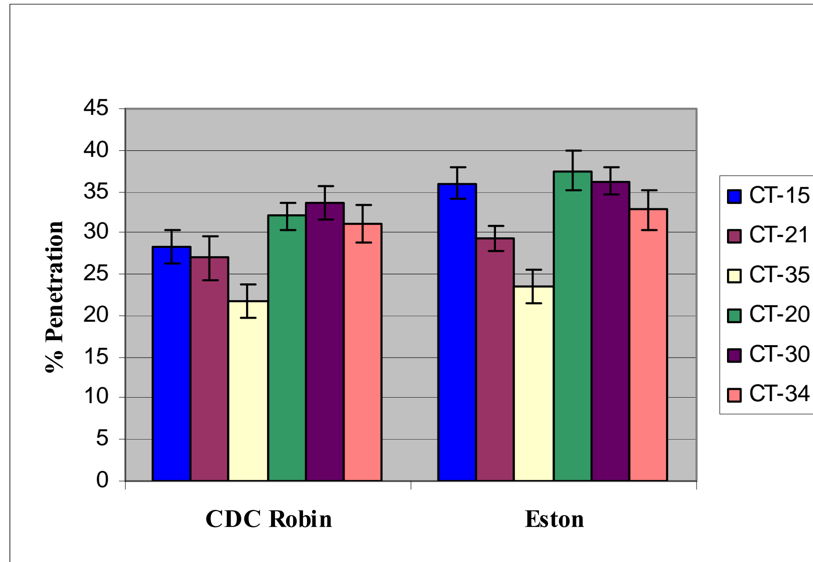


Table 4.6 Results of the contrast analysis of percent penetration of race Ct0 and race Ct1 of *Colletotrichum truncatum* on the detached leaflets of lentil cultivars CDC Robin and Eston at 48 h postinoculation

Label	Num DF	Den DF	F Value	Pr > F
Ct1 vs Ct0	1	32.6	27.26	<.0001
Ct1 vs Ct0 on Eston	1	16.3	13.30	0.0021
Ct1 vs Ct0 on CDC Robin	1	16.4	13.98	0.0017

4.3.2.3. Investigation of the first appearance of infection vesicles

In this experiment, leaflets were examined for infection vesicles on an hourly basis, and the percentage of appressoria with infection vesicles inside the epidermal cells was investigated for each of three replicate leaflets of two cultivars between 16 and 24 hpi. The time point when the average percentage of appressoria with IV on the three replicate leaflets of each cultivar inoculated with each isolate was equal or exceeding 0.5% was considered the time when infection vesicles of this isolate were first produced. The standard of 0.5% was determined because in the initial stage of IV appearance, the percentage of appressoria with IV was very low.

Results showed that on both CDC Robin and Eston, there were no significant differences between race Ct0 and race Ct1 in the first appearance of infection vesicles (Table 4.7 and Appendix 19).

Table 4.7 Results of the contrast analysis of the first appearance of infection vesicles of race Ct0 and race Ct1 of *Colletotrichum truncatum* on the detached leaflets of lentil cultivars CDC Robin and Eston

Cultivar	Label	Num DF	Den DF	F Value	Pr > F
CDC Robin	race Ct0 vs race Ct1	1	3.11	0.36	0.5876
Eston	race Ct0 vs race Ct1	1	10.1	0.12	0.7398

4.3.2.4. Investigation of the size and different types of primary hyphae

In this study, the size (including total length of PH and percentage of epidermal cell area occupied by PH) and different types of PH based on their shapes (Figure 3.1 in Materials and Methods) of the six isolates were investigated on detached leaves of lentil cultivars Eston and CDC Robin at 48 and 60 hpi.

Data analysis revealed that incubation time, cultivar, and isolate significantly affected the total length of PH (Appendix 20). Because of a significant interaction between incubation time and cultivar ($P < 0.0001$), data for 48 and 60 hpi were analysed separately (Appendix 20). At 48 hpi, isolate and cultivar significantly affected the total length of PH (Appendix 21). PH were significantly larger on Eston compared to CDC Robin, and the interaction between cultivar and isolate was non-significant (Table 4.8 and Appendix 21). In comparison, at 60 hpi, there were no significant differences among the isolates (Appendix 22); a cultivar effect was present, and PH on CDC Robin were marginally larger than those on Eston (Table 4.8 and Appendix 22). However, the interaction between isolate and cultivar was non-significant (Appendix 22). Further analyses showed that at 48 hpi, on Eston the total length of PH of race Ct0 isolates was significant higher than that of race Ct1 isolates. On CDC Robin, there were no significant differences between the two races (Tables 4.8 and 4.9). At 60 hpi, on both cultivars there were no significant differences between the two races (Table 4.10).

Table 4.8 Total length of primary hyphae of six *Colletotrichum truncatum* isolates after 48 and 60 h incubation time on the detached leaves of lentil cultivars Eston and CDC Robin (Values in brackets are standard errors of the means)

Race	Isolate	Total Length of Primary Hyphae (µm)			
		48 h		60 h	
		Eston	CDC Robin	Eston	CDC Robin
Ct0	CT-20	71.29 (6.14)	55.67 (4.73)	69.54 (0.97)	70.88 (2.77)
	CT-30	75.92 (1.00)	61.54 (2.50)	68.88 (3.92)	77.54 (1.16)
	CT-34	66.67 (3.58)	57.96 (1.79)	64.88 (1.48)	74.25 (1.67)
Ct1	CT-15	67.75 (1.09)	57.58 (0.74)	72.13 (1.76)	74.33 (0.83)
	CT-21	57.25 (1.44)	58.96 (0.33)	67.13 (2.79)	67.67 (1.70)
	CT-35	59.29 (2.09)	46.96 (1.04)	69.58 (1.05)	72.04 (2.96)

Table 4.9 Results of the contrast analysis of total length of primary hyphae of race Ct0 isolates and race Ct1 isolates of *Colletotrichum truncatum* at 48 h postinoculation on the detached leaves of the lentil cultivars Eston and CDC Robin

Cultivar	Contrast	DF	Contrast SS	Mean Square	F Value	Pr > F
Eston	race Ct0 vs race Ct1	1	8752	8752	11.12	0.0009
CDC Robin	race Ct0 vs race Ct1	1	1361	1361	2.72	0.1002

Table 4.10 Results of the contrast analysis of total length of primary hyphae of race Ct0 isolates and race Ct1 isolates of *Colletotrichum truncatum* at 60 h postinoculation on the detached leaves of the lentil cultivars Eston and CDC Robin

Cultivar	Contrast	DF	Contrast SS	Mean Square	F Value	Pr > F
Eston	race Ct0 vs race Ct1	1	307	307	0.50	0.4806
CDC Robin	race Ct0 vs race Ct1	1	744	744	1.21	0.2713

For the percentage of epidermal cell area occupied by PH, incubation time, isolate, and cultivar had significant effects. The isolate × cultivar interaction was also significant (P = 0.0140), but none of the other interactions was (Appendix 23), so data were further analyzed for each cultivar separately. On Eston, there were no significant differences in the percentage of epidermal cell area occupied by PH among the isolates or between the races at both 48 and 60 h incubation times (Appendix 24 and Table 4.12). The percentage of epidermal cell area occupied by PH was significantly affected by incubation time, and was higher at 60 hpi than at 48 hpi (Table 4.11 and Appendix 24). However, on CDC Robin, isolate and incubation time had significant effects (Appendix 25). Race Ct0 isolates occupied significantly more space in epidermal cells than race Ct1 isolates at both (48 and 60 h) incubation times (Tables 4.11 and 4.12), and the percentage of epidermal cell area occupied by PH was significantly higher at 60 hpi than at 48 hpi (Table 4.11 and Appendix 25). Further analyses showed that at 48 and 60 hpi, the percentage of epidermal cell area occupied by PH of race Ct0 isolates was not significantly differenct from race Ct1 isolates on Eston, but on CDC Robin, race Ct0 isolates covered significantly more space than race Ct1 isolates (Tables 4.11, 4.13, and 4.14).

Table 4.11 Percentage of epidermal cell area occupied by primary hyphae of six *Colletotrichum truncatum* isolates after 48 and 60 h incubation time on the detached leaves of lentil cultivars CDC Robin and Eston (Values in brackets are standard errors of the means).

Race	Isolate	Percent Epidermal Cell Area (%)			
		48 h		60 h	
		Eston	CDC Robin	Eston	CDC Robin
Ct0	CT-20	15.37 (0.55)	14.73 (0.75)	20.42 (1.06)	22.27 (0.65)
	CT-30	16.22 (0.62)	15.57 (0.56)	21.23 (0.73)	22.93 (0.49)
	CT-34	15.82 (0.47)	15.20 (0.51)	22.25 (0.86)	24.12 (1.02)
Ct1	CT-15	15.37 (0.51)	12.08 (0.55)	21.95 (0.82)	18.32 (1.16)
	CT-21	14.95 (0.43)	11.52 (0.57)	22.92 (1.26)	17.37 (0.72)
	CT-35	14.50 (0.46)	11.23 (0.62)	21.07 (0.54)	19.17 (1.05)

Table 4.12 Results of the contrast analysis of percent epidermal cell area occupied by primary hyphae of race Ct0 isolates and race Ct1 isolates of *Colletotrichum truncatum* at both 48 h and 60 h incubation time on the detached leaves of the lentil cultivars Eston and CDC Robin

Cultivar	Contrast	DF	Contrast SS	Mean Square	F Value	Pr > F
Eston	race Ct0 vs race Ct1	1	1.51	1.51	0.01	0.9107
CDC Robin	race Ct0 vs race Ct1	1	3158	3158	29.71	< .0001

Table 4.13 Results of the contrast analysis of percent epidermal cell area occupied by primary hyphae of race Ct0 isolates and race Ct1 isolates of *Colletotrichum truncatum* at 48 h postinoculation on the detached leaves of the lentil cultivars Eston and CDC Robin

Cultivar	Contrast	DF	Contrast SS	Mean Square	F Value	Pr > F
Eston	race Ct0 vs race Ct1	1	66.7	66.7	0.89	0.3463
CDC Robin	race Ct0 vs race Ct1	1	1138	1138	22.39	< .0001

Table 4.14 Results of the contrast analysis of percent epidermal cell area occupied by primary hyphae of race Ct0 isolates and race Ct1 isolates of *Colletotrichum truncatum* at 60 h postinoculation on the detached leaves of the lentil cultivars Eston and CDC Robin

Cultivar	Contrast	DF	Contrast SS	Mean Square	F Value	Pr > F
Eston	race Ct0 vs race Ct1	1	41.3	41.3	0.25	0.6171
CDC Robin	race Ct0 vs race Ct1	1	2093	2093	12.93	0.0004

At 48 and 60 h incubation time, there were no significant differences in the frequency of different types of PH between the two races on Eston and CDC Robin (Tables 4.15 and 4.16). Isolates of both races generated types 1, 2, and 4 of PH at similar frequencies in the leaf epidermal cells of Eston and CDC Robin at each incubation time (Table 4.15 and Appendices 26-29). However, at 48 h incubation time isolates of race Ct0 had generated more type 3 of PH compared to isolates of race Ct1 on CDC Robin, but this difference was not evident at 60 h incubation time (Table 4.15 and Appendices 27 and 29).

Table 4.15 Frequency of different types of primary hyphae (PH, based on shape) of six *Colletotrichum truncatum* isolates after 48 and 60 h incubation time on the detached leaves of lentil cultivars CDC Robin and Eston (Values in brackets are standard errors of the means)

Time (h)	Race	Isolate	Type of PH							
			1		2		3		4	
			Eston	CDC Robin	Eston	CDC Robin	Eston	CDC Robin	Eston	CDC Robin
48	Ct0	CT-20	7.0 (0.58)	7.7 (0.88)	4.3 (0.33)	5.7 (0.66)	3.3 (0.88)	3.3 (0.88)	5.3 (0.33)	3.3 (0.33)
		CT-30	4.3 (0.88)	7.0 (0.58)	6.7 (0.88)	3.0 (0.58)	5.7 (0.88)	7.7 (0.88)	3.3 (0.88)	2.3 (0.33)
		CT-34	5.3 (1.45)	7.7 (1.20)	5.0 (2.52)	4.3 (0.88)	6.7 (0.88)	5.3 (1.85)	3.0 (0.58)	2.7 (0.33)
	Ct1	CT-15	7.3 (1.45)	8.3 (1.20)	6.7 (0.88)	5.3 (1.20)	3.3 (0.88)	3.3 (0.88)	2.7 (0.33)	3.0 (0.58)
		CT-21	6.3 (0.88)	6.7 (1.20)	6.0 (0.58)	7.0 (1.53)	5.3 (0.88)	2.3 (0.33)	2.3 (0.88)	4.0 (0.58)
		CT-35	6.0 (0.58)	7.3 (0.88)	7.3 (0.88)	6.3 (0.88)	3.7 (0.88)	3.0 (1.53)	3.0 (1.00)	3.3 (0.88)
60	Ct0	CT-20	4.3 (0.33)	3.0 (0.58)	5.7 (0.88)	4.7 (1.20)	7.7 (0.88)	10.0 (0.58)	2.3 (0.33)	2.3 (0.33)
		CT-30	5.3 (0.88)	3.3 (0.33)	5.0 (0.58)	3.3 (0.88)	7.7 (0.33)	11.3 (0.88)	2.0 (0)	2.0 (0)
		CT-34	5.0 (0.58)	4.0 (0.58)	4.7 (1.20)	4.0 (1.15)	8.3 (0.88)	9.7 (0.88)	2.0 (0.58)	2.3 (0.33)
	Ct1	CT-15	4.0 (0.58)	4.0 (1.15)	5.0 (1.15)	3.3 (0.88)	7.7 (0.88)	9.0 (0.58)	3.3 (0.33)	3.7 (0.88)
		CT-21	5.0 (0.58)	3.0 (0.58)	4.0 (0.58)	3.7 (1.20)	8.7 (1.20)	10.3 (1.20)	2.3 (0.33)	3.0 (0.58)
		CT-35	4.3 (0.88)	3.3 (0.88)	5.0 (0.58)	6.3 (1.20)	8.0 (0.58)	8.0 (0.58)	2.7 (0.33)	2.3 (0.33)

Table 4.16 Results of the contrast analysis of frequency of different types of primary hyphae (PH, based on shape) of race Ct0 isolates and race Ct1 isolates of *Colletotrichum truncatum* after 48 and 60 h incubation time on the detached leaves of lentil cultivars Eston and CDC Robin

Source	DF	48 h			60 h		
		Chi-Square	**Pr > ChiSq**	**Type**	**Chi-Square**	**Pr > ChiSq**	**Type**
race Ct0 *vs*. race Ct1 on Eston	1	0.43	0.5101	LR	0.05	0.8220	LR
race Ct0 *vs*. race Ct1 on CDC Robin	1	0.00	0.9722	LR	0.23	0.6337	LR

As only the infection process of CT-34 (race Ct0) and CT-35 (race Ct1) after 48 h incubation was studied on attached leaves (Chapter 4.4), comparisons of the total length of PH and of percent epidermal cell area occupied by PH of these two isolates on detached leaves were conducted to facilitate the comparison of detached and attached leaf inoculation assays. It was shown that there were no significant differences between the two isolates on the detached leaves of Eston, but CT-34 had significantly larger PH than CT-35 on the detached leaves of CDC Robin (Appendices 30-33).

4.3.2.5. Investigation of the first appearance of secondary hyphae

In this experiment, the first appearance of SH was investigated for each isolate on two cultivars at 60, 62, 64, 66, 68, 70, and 72 hpi. The time point when the average percentage of PH with SH on three leaflets for each isolate was equal or exceeding 5% was considered the time when SH of this isolate were first produced.

Results showed that on Eston SH were produced at 60 hpi when inoculated with CT-30, CT-34, and CT-35, and at 62 hpi when inoculated with CT-15, CT-20, and CT-21. On CDC Robin, SH were produced at 60 hpi after inoculated with CT-30, at 62 hpi when inoculated with CT-15, CT-20, and CT-34, and at 64 hpi when inoculated with CT-21 and CT-35.

4.4. Study of the infection process on attached lentil leaves

In this study, leaflets growing on intact plants of the lentil cultivars CDC Robin and Eston were fixed with tape at the bottom of plastic containers, inoculated by point-inoculation with CT-34 (race Ct0) and CT-35 (race Ct1), and incubated for 48 h. The total length of PH, the percent area of plant epidermal cell occupied by PH, and the type of PH were investigated.

The total length of PH of CT-34 and CT-35 varied from 12.5 to 115 µm and 7.5 to 60 µm on CDC Robin, respectively, and from 12.5 to 100 µm and 7.5 to 72.5 µm on Eston. The percent plant epidermal cell area occupied by PH of these two isolates ranged from 3 to 26% and 1 to 18% on CDC Robin, respectively, and from 3 to 25% and 1 to 20% on Eston. Analyses of the data showed that the interaction term between isolate and cultivar was not significant for the total length of PH (P = 0.1489), nor for the percent plant epidermal cell area occupied by PH (P = 0.1501). The total length of PH and percent plant epidermal cell area occupied by PH of CT-34 were significantly higher than those of CT-35 on the attached leaflets of both cultivars (Tables 4.17, 4.18, and 4.19).

Table 4.17 Means of total length (Ltotal (µm)) of primary hyphae and means of percent plant epidermal cell area occupied by primary hyphae (Percentage) of two isolates (CT-34 and CT-35) of *Colletotrichum truncatum* at 48 h incubation time on the attached leaves of lentil cultivars CDC Robin and Eston (Values in brackets are standard errors of the means)

Race	Isolate	Ltotal (µm)		Percentage	
		Eston	CDC Robin	Eston	CDC Robin
Ct0	CT-34	45.79(1.00)a	47.38(0.57)a	10.48(0.28)a	10.85(0.47)a
Ct1	CT-35	29.21(1.31)b	23.54(1.17)b	6.90(0.54)b	5.18(0.27)b

Note: Different letters in the same column indicate significant differences.

Table 4.18 Comparisons of least squares means of total length of primary hyphae of *Colletotrichum truncatum* isolates CT-34 (race Ct0) and CT-35 (race Ct1) inoculated onto attached leaflets of lentil cultivars CDC Robin and Eston at 48 h incubation time

Cultivar	Effect	iso	iso	Estimate	Standard Error	DF	t Value	Pr > |t|
CDC Robin	iso	CT-34	CT-35	23.83	3.46	92.9	6.88	<.0001
Eston	iso	CT-34	CT-35	16.58	3.61	107	4.59	<.0001

Table 4.19 Comparisons of least squares means of percent plant epidermal cell area occupied by primary hyphae of *Colletotrichum truncatum* isolates CT-34 (race Ct0) and CT-35 (race Ct1) inoculated onto attached leaflets of lentil cultivars CDC Robin and Eston at 48 h incubation time

Cultivar	Effect	iso	iso	Estimate	Standard Error	DF	t Value	Pr > |t|
CDC Robin	iso	CT-34	CT-35	5.67	0.97	97.5	5.81	<.0001
Eston	iso	CT-34	CT-35	3.58	1.06	115	3.37	0.0010

Four types of PH were identified and the frequency of each type was counted for each isolate on the attached leaves of Eston and CDC Robin (Table 4.20). Because of significant interaction between isolate and type of PH (*P* = 0.0011), the frequency of each PH type was analyzed individually. For types 1 and 4, there were significant differences between CT-34 and CT-35. However, no such differences were found for types 2 and 3 (Table 4.21). Since the research objective of this study was to investigate whether there were significant differences in the infection progress between the two isolates on CDC Robin and there were no such differences on Eston, the frequency of each PH type was further analyzed individually for both isolates on each cultivar. The frequency of types 1, 2, and 3 of PH did not significantly differ between CT-34 and CT-35 on either Eston or CDC Robin. CT-34 had significantly more type 4 of PH than CT-35 on Eston. However, on CDC Robin there were no significant differences in the frequency of type 4 of PH between the two isolates (Tables 4.20 and 4.22).

Table 4.20 Frequency of the different types of primary hyphae (PH, based on shape) of two isolates (CT-34 and CT-35) of *Colletotrichum truncatum* at 48 h incubation time on the attached leaves of lentil cultivars CDC Robin and Eston (Values in brackets are standard errors of the means)

Cultivar	Type of PH	Isolate	
		CT-34	CT-35
Eston	1	8.0 (1.00) a	12.7 (0.88) a
	2	4.3 (0.33) a	3.7 (1.20) a
	3	1.7 (0.88) a	1.3 (0.33) a
	4	6.0 (1.00) a	2.3 (0.66) b
CDC Robin	1	8.0 (1.00) a	12.3 (0.66) a
	2	5.3 (0.33) a	5.7 (1.20) a
	3	3.0 (0.58) a	0.7 (0.33) a
	4	3.7 (0.66) a	1.3 (0.33) a

Note: Different letters in the same row indicate significant differences.

Table 4.21 Comparison of the frequency of different types of primary hyphae (PH, based on shape) of two isolates (CT-34 and CT-35) of *Colletotrichum truncatum* at 48 h incubation time

PH Type	Effect	iso	iso	Estimate	Standard Error	DF	t Value	Pr > |t|
1	iso	CT-34	CT-35	-0.4258	0.1837	1	5.37	0.0205
2	iso	CT-34	CT-35	0.0532	0.2689	1	0.04	0.8431
3	iso	CT-34	CT-35	0.8382	0.5373	1	2.43	0.1188
4	iso	CT-34	CT-35	0.9495	0.3683	1	6.65	0.0099

Table 4.22 Comparison of the frequency of different types of primary hyphae (PH, based on shape) of two isolates (CT-34 and CT-35) of *Colletotrichum truncatum* at 48 h incubation time on the attached leaves of lentil cultivars CDC Robin (CR) and Eston (E)

PH Type	Cul	Effect	iso	iso	Estimate	Standard Error	DF	Chi-Square	Pr > ChiSq
1	CR	iso	CT-34	CT-35	-0.4329	0.2621	1	2.73	0.0986
2	CR	iso	CT-34	CT-35	-0.0606	0.3483	1	0.03	0.8618
3	CR	iso	CT-34	CT-35	1.2528	0.8018	1	2.44	0.1182
4	CR	iso	CT-34	CT-35	1.0116	0.5839	1	3.00	0.0832
1	E	iso	CT-34	CT-35	-0.4187	0.2575	1	2.64	0.1040
2	E	iso	CT-34	CT-35	0.1671	0.4097	1	0.17	0.6834
3	E	iso	CT-34	CT-35	0.5108	0.7303	1	0.49	0.4843
4	E	iso	CT-34	CT-35	0.8873	0.4491	1	3.90	0.0482

5. Discussion

5.1. Investigation of conidium germination on glass well slides

Conidium germination is the first stage for the fungus to infect plant tissues. Isolates with higher percentage of conidium germination contribute to greater number of appressorium and possibly more penetration, and could thus present a greater threat to plant growth. Suitable temperature, light, and sufficient humidity and nutrients are all essential conditions for conidium germination. In this experiment, distilled water was used, and the purpose was to examine germination of the six isolates of *Colletotrichum truncatum* on inert glass well slides. The objective was to determine whether there were significant differences in the percentage of conidium germination between isolates of the two races irrespective of potential cues given by the host plant surface. The results showed no significant differences in the percentage of conidium germination between the two races on inert glass slides. Since glass slides provide limited cues to the fungus to encourage development, the results indicated that there were no inherent differences between the two races in conidium germination. Therefore, any differences in germination on lentil plants caused by the two races of *C. truncatum* should be the result of their specific interaction.

It was shown that conidia of the six isolates began to germinate after 2 h of incubation. This was somewhat less than the minimum incubation time observed by Chongo et al. (2002), who determined that conidial germination started after 3-6 h of incubation. Generally one germ tube was formed from a conidium, but there were also two germ tubes observed on some conidia, which was similar to the description by Chongo et al. (2002) who described up to 3 germ tubes per conidium. However, no conidia with three germ tubes were detected in this study.

In some cases, appressoria were formed shortly after conidial germination which had been observed earlier by Chongo et al. (2002). In this case, germ tubes were not

visible because appressoria were located very close to conidia. These conidia with short germ tubes and very early appressorium development may penetrate hosts more quickly. Conidia with more than one appressorium also may have more penetration attempts.

In this experiment, conidia were suspended in water and incubated under high humidity to make sure that the pathogen was provided with a conducive condition for conidium germination. Studies by Bradley et al. (2003) showed that *Stemphylium* sp. had much higher conidial germination in free water than at 100% relative humidity (RH) when infecting clover species. Similar results were shown by Green and Bailey (2000), who worked on the factors affecting infection of *Alternaria cirsinoxia* on leaves of Canada thistle. They found that *A. cirsinoxia* preferred free water for conidial germination and appressorium formation. Studies by Fernando et al. (2000) found different results, i.e. ≥ 96% RH resulted in higher conidium germination during a study on the interaction between *C. acutatum* and *Hevea brasiliensis*, and it was unnecessary to use free water. However, studies by Stojanovic et al. (1999) showed even if RH was almost 100%, *in vitro* conidial germination of *C. gloeosporioides* only reached 2.5% after 24 h.

Some researchers used the glass slides in various studies to investigate conidium germination and formation of other infection structures, e.g. appressoria. Studies by Sheikholeslami et al. (2005) showed that old conidia of *Erysiphe betae* had lower germination compared to fresh conidia on glass slides. Studies by Li et al. (2005) showed that conidia of *E. pulchra* germinated on glass slides at the same time as on dogwood leaves, but the formation of appressoria was much later.

5.2. Effects of plant age, conidium concentration, and inoculation method on conidium germination on lentil leaves

In this experiment, the race Ct1 isolate CT-15 was used to optimize the inoculation protocol with regard to lentil plant age, conidium concentration, and the inoculation method. The objective was to develop a protocol to be used for further experiments using multiple isolates of *C. truncatum*. In this study, three parameters were investigated and compared in one experiment. The advantage of such an experimental design was to create the same experimental conditions for the purpose of minimizing the experimental error and to save time in carrying out the experiments. However, the disadvantage was the complexicity of data analyses, particularly interpretation of interactions among different parameters.

At the intermediate concentration of 5×10^5 conidia/mL, there were no significant differences in conidium germination between the two plant ages. At the same time, inoculation method had no significant effect. Three-week old lentil plants would be a better option for studying the infection of *C. truncatum* compared to six-week old plants since time spent in managing the plants would be shortened in half, thus saving time and speeding up the research process. At the low concentration of 5×10^4 conidia/mL, there were significant differences in conidium germination between the two plant ages. However, at the high concentration of 1×10^6 conidia/mL, the things tend to be more complicated because of an interaction of plant age and inoculation method.

The results also showed that inoculation with the low (5×10^4 conidia/mL) and intermediate (5×10^5 conidia/mL) pathogen concentrations by either point-inoculation or spray-inoculation had no significant effect on conidium germination. When the spray-inoculation method was used, more labour was required compared to the point-inoculation method because whole plants were inoculated. This involved wrapping pots in transparent plastic sheets, moving them into a mist chamber after inoculation with a sprayer and finally moving them onto a normal phytotron bench. In contrast, for point-inoculation, detached leaves were placed into a sandwich box, inoculated with conidium suspensions using a pipetter, and incubated in an incubator. Furthermore, the inoculated area was easy to find on point-inoculated leaflets and thus the observation of germinating

conidia was conducted faster than on spray-inoculated leaflets where conidia were distributed over a larger area. Savings in time and space would favour the use of the point-inoculation method. Therefore, point-inoculation would be chosen at a low or an intermediate concentration.

In terms of the effect of conidium concentration on germination, it was shown that when point-inoculating, there were no significant differences between intermediate (5×10^5 conidia/mL) and high concentrations (1×10^6 conidia/mL) ($P = 0.1464$) or between intermediate and low (5×10^4 conidia/mL) concentrations ($P = 0.0925$), but conidium germination was significantly lower at high concentration than at low concentration ($P = 0.0258$). It was also shown that when point-inoculating, plant age had no effect on conidium germination. Therefore, an intermediate concentration with 5×10^5 conidia/mL was considered the best option among the three concentrations when point-inoculating.

It is worthy mentioning here that the point-inoculation study involved detached leaves, whereas spray-inoculation was done on whole plants with attached leaves.

Investigating resistance of wheat to *Fusarium graminearum*, Miedaner et al. (2003) showed that inoculating wheat heads with a spray method was better compared to inoculating florets using a point-inoculation method. Similarly, McCallum and Tekauz (2002) reported that spraying entire spikes of barley with *F. graminearum* had a higher inoculation efficiency and was better in identifying resistance than injecting single florets. A study by Warham (1990) also showed that a spray-inoculation method produced better inoculation results compared to other inoculation methods when investigating the interaction of wheat with *Tilletia indica*. The above researchers preferred to use the spray-inoculation method. However, they did not do microscopy studies, which may be the reason why they did not choose the point-inoculation method.

A study by Chongo and Bernier (2000) showed that increasing conidium concentrations for spray-inoculation of lentil plants and favourable growth stages (i.e. 4-6 weeks after planting) could enhance the development of *C. truncatum* (2000). Increased conidium concentration shortened the incubation and latent periods, while lesion numbers were raised and disease severity was higher when using a high concentration of 1×10^5 conidia/mL (Chongo and Bernier, 2000). In contrast, higher conidium concentration (10^6 conidia/mL) of *C. gloeosporioides* did not result in higher severity of anthracnose on

Stylosanthes scabra (Chakraborty, 1990). The current study suggests that higher conidium concentration inhibited conidium germination compared to lower concentration.

Lentil plants of 4-6 weeks were found to be more susceptible to *C. truncatum* (Chongo and Bernier, 2000). This is consistent with the results of the current study where six-week old plants were found to have higher conidium germination than three-week old plants at three concentrations when both inoculation methods were used (Table 4.3). In particular, conidium germination was significantly higher on six-week old plants than on three-week old plants at 5×10^4 conidia/mL under both inoculation methods, and also at 1×10^6 conidia/mL when point-inoculating (Table 4.3). Higher conidium germination may result in higher number of appressorium and higher infection. A study on the interaction between *Ascochyta rabiei* and chickpea showed that as partially resistant chickpea plants grew older, they became more susceptible (Chongo and Gossen, 2001). A similar result was reported by Makowski (1993) regarding the interaction of mallow/velvetleaf and *C. gloeosporioides* f. sp. *malvae*. In contrast, a study on muskmelon showed that the earlier seedlings were inoculated with *F. oxysporum* f. sp. *melonis*, the more disease developed (Latin and Snell, 1986). As the bean cutivar Prelude grew from 8 to 14 days of age, its resistance to *C. lindemuthianum* was getting stronger (Bigirimana and Höfte, 2001). Based on the results and discussion above, it is clear that an evaluation of inoculation methods requires investigating many factors, such as level of resistance of plant, plant growth stage, conidium concentration, environmental condition, and experimental design. The results of the current study suggest that the point-inoculation method on three-week old plants using a conidium concentration of 5×10^5 conidia /mL is the optimum option for further experiments.

5.3. Study of the infection process on detached lentil leaves

5.3.1. Conidium germination and appressorium formation on detached green leaf surface

The research hypothesis for this study was that isolates of the two races would not differ in terms of conidium germination and appressorium formation on Eston, but that isolates of race Ct0 would have significantly higher conidium germination and appressorium formation than isolates of race Ct1 on CDC Robin. This study showed that on Eston, conidium germination of race Ct0 isolates was significantly higher than that of race Ct1 isolates at 12 hpi; but no significant differences in appressorium formation were observed between the two races at that time. On CDC Robin, race Ct0 isolates had significantly higher percentages of conidium germination and appressorium formation than race Ct1 isolates at 12 hpi. Differences in the percentage of appressorium formation therefore support the research hypothesis, but data on percentage of conidium germination did not. Results suggest that a higher percentage of conidium germination was not automatically correlated with a higher percentage of appressorium formation as on the leaflet surface of Eston race Ct0 isolates had significantly higher conidium germination than race Ct1 isolates, but there were no significant differences in appressorium formation between the two races. Similar to observations on well slides, it was observed here that some conidia germinated and generated long germ tubes, at the end of which appressoria were eventually differentiated. In contrast, other conidia generated appressoria shortly after they germinated, thus had only very short germ tubes. Compared to conidium germination, appressorium formation is more closely related to the infection process of *C. truncatum* because only under the appressorium will a penetration peg be formed to pierce the cell wall of epidermise, so that the fungus can enter the leaflet tissue. When more penetration pegs are formed, it is predicted that more parts of the plant will be parasitized by the pathogen, and plants will face more damage. The results for appressorium formation showed that CDC Robin delayed or restricted appressorium formation of race Ct1 isolates while Eston did not. This may explain why there tends to be less disease on CDC Robin after inoculation with race Ct1 isolates

compared to that on Eston, whereas similar levels of disease occur for race Ct0 isolates on both cultivars.

Other researchers have investigated the infection process and other characteristics of plant pathogens by looking at conidium germination and appressorium formation. Mould et al. (1991) studied the infection process of two races of *C. trifolii* on *Medicago sativa*, and found that race identity did not influence conidium germination and appressorial development. A study by Daykin and Milholland (1984) showed that the initial infection process by *C. gloeosporioides*, including conidium germination and appressorium formation, on susceptible and resistant cultivars of muscadine grape was similar. Comparisons of percentages of conidium germination and of appressorium formation of *C. orbiculare* at 12 and 24 hpi on two *Nicotiana* species by Shen et al. (2001) showed that except for the percentage of germination at 24 hpi, significant differences between the two *Nicotiana* species were observed. In two different hosts, tobacco and round-leaved mallow, *C. orbiculare* had different percentage of conidium germination and different number of appressorium (Shen et al., 2001). Jahromi et al. (2002) investigated the difference in percent appressorium formation of *Rhynchosporium alismatis* on *Damasonium minus* (a rice weed) at two temperatures, 25 and 30ºC, and found that under a higher temperature *R. alismatis* produced significantly lower appressoria on leaves than under a lower temperature. Some researchers used other parameters in studies of the infection process. Morin et al. (1996) found that conidium germinated at similar time and pre-penetration structures had no big differences in morphology when studying the pre-penetration phase of *C. gloeosporioides* f. sp. *malvae* on the leaves of five different weed species. Auld and Say (1999) compared different *C. orbiculare* isolates on the same weed species using conidium production, colony diameter, and other factors. The results of my study suggest that conidium germination and appressorium formation are important components in the *C. truncatum* infection process. . The study of conidium germination and appressorium formation is simple and fast. Meanwhile, this method is less ambiguous compared to the timing and morphological description of infection structures, or measurement of colony diameter. The results of this part of my study supported those by Shen et al. (2001) showing that investigations of

conidium germination and of appressorium formation can be used to identify the reaction of isolates from different races of the fungus to different hosts or cultivars.

5.3.2. Penetration into detached leaf tissue

Based on observation until 72 hpi in this study, isolates from both races followed the typical infection process on both lentil cultivars, i.e. conidium germination, appressorium formation, penetration into plant tissue, and appearances and growth of IV, PH, and SH. This infection process is similar to that described for *C. truncatum* on pea (O'Connell et al., 1993), *C. orbiculare* on tobacco (Shen et al., 2001), *C. destructivum* on lucerne (Latunde-Dada et al., 1997), and *C. sublineolum* on susceptible *Sorghum bicolour* cultivar (Wharton and Julian, 1996). The differences are in the timing of IV, PH, and SH appearances. In the study by Khan and Hsiang (2003), SH of *C. graminicola* were not found on four turfgrass species before 168 hpi. *Colletotrichum gloeosporioides* penetrated into mulberry leaf through the open stomata without formation of appressoria (Kumar et al., 2001). *Colletotrichum truncatum* on soybean showed a different infection process as hyphae initially spread below the cuticle after penetration into the plant tissue, after which the pathogen spread into the cell wall (Manandhar et al., 1985). It was observed that at the same incubation time on the same leaflet, some conidia could germinate, and others could not; penetration could be successful from some appressoria, but not from others. These may be related to different sensitivity of the infection structures to the plant tissue and environmental conditions. In this study PH of *C. truncatum* were not observed to extend towards the surrounding cells in the lentil leaves. The production of SH sometimes was not triggered when PH had occupied the entire epidermal cells, but occurred, for example, when PH covered 20 to 30% of the epidermal cell area. This could be the result from the interaction of the fungus and plant, i.e. specific signalling between the fungus and the host, and/or changes in environmental conditions for the PH. Observations clearly showed that SH were much thinner, spread faster than PH, and crossed cell walls. It has been suggested that this may be resulted from a change of surface features of hyphal cells during the switch from the biotrophic (PH) to the

necrotrophic stage (SH). Extracellular matrixes and glycoproteins found on PH are not detected on the surface of SH (Perfect et al., 2001).

For the experiment studying the percent penetration of six isolates into detached lentil leaflets, the research hypothesis was that on Eston there would be no significant differences in the percent penetration between race Ct0 isolates and race Ct1 isolates, but on CDC Robin percent penetration of race Ct0 isolates would be significantly higher than that of race Ct1 isolates. This study showed that race Ct0 isolates had a significantly higher percentage of penetration than race Ct1 isolates on both cultivars. These results do not support the research hypothesis. Khan and Hsiang (2003) found that on one turfgrass species, not only did conidia of *C. graminicola* germinate quicker, but also the penetration pore was generated faster and hyphae spread more widely within epidermal cells compared to three other turfgrass species. When investigating the size of PH at 48 and 60 h incubation time on the detached leaves of lentil cultivars Eston and CDC Robin, the research hypothesis was that on Eston there would be no significant differences between the isolates of race Ct0 and isolates of race Ct1 in the size of PH, while on CDC Robin the size of PH of race Ct0 isolates would be significantly larger than that of race Ct1 isolates. Studying the percent epidermal cell area occupied by PH at both incubation times (48 and 60 hpi), as well as the percentage of epidermal cell area occupied by PH at 48 and 60 hpi each showed that on Eston there were no significant differences between the two races. However, on CDC Robin PH of race Ct0 isolates occupied significantly more space than PH of race Ct1 isolates. These results support the research hypothesis. The comparison of CT-34 (race Ct0) and CT-35 (race Ct1) in terms of the total length of PH and plant epidermal cell area occupied by PH on the detached leaves also supported the research hypothesis. On Eston there were no significant differences between two isolates, but CT-34 had significantly longer PH than CT-35 on CDC Robin. Compared to the length of PH, the measurement of the percentage of epidermal cell area occupied by PH appears to be a better approach to investigate these infection structures because the measurement of length, as explained in section 3.3.6, was based on one to three measurements depending on the type of PH. For PH type 2 to 4, two or three measurements would still not reflect the complete size accurately, but measuring each branch would have been impractical. In contrast, estimating the cell area occupied by PH

more clearly reflected the size of PH, in particular as PH of *C. truncatum* are restricted to one epidermal cell. An epidermal cell with more area occupied by a PH would be expected to be more affected because more nutrients would be withdrawn from the host cells.

Results of this study showed that on CDC Robin the development of race Ct1 isolates was restricted compared to race Ct0 isolates as IV and SH of race Ct1 isolates appeared later in epidermal cells. Studies by Chongo et al. (2002) showed that symptoms appeared eight to eleven days earlier on the susceptible lentil cultivar Eston than on the resistant lentil line PI 320937 after inoculation with *C. truncatum* isolates. PI 320937 limited swelling of hyphae and production of lesions compared to Eston after inoculation with an isolate of moderate virulence and one of high virulence (Chongo et al., 2002). In my studies, as no hypersensitive reaction was observed on CDC Robin after inoculation with race Ct1 isolates, the term “incompatibility” is not appropriate for describing the interaction between race Ct1 isolates and CDC Robin. On Eston, development of race Ct1 isolates was not restricted as on CDC Robin and isolates of this race developed similarly to race Ct0 isolates in terms of percent epidermal cell area occupied by PH. Based on these observations, race Ct0 and race Ct1 do not appear to be classical physiological races, but may represent aggressive races as reviewed by Caten (1987), or some intermediate forms.

5.4. Study of the infection process on attached lentil leaves

In this study, attached leaves from whole plants of both lentil cultivars Eston and CDC Robin were point-inoculated with one race Ct0 isolate (CT-34) and one race Ct1 isolate (CT-35). My research hypothesis was that on Eston there would be no significant differences in the size of PH between these two isolates, but on CDC Robin PH of CT-34 would be significantly larger than that of CT-35. This was shown on the detached leaves in chapter 4.3. The objective here was to determine whether this was also true on the attached leaves. Therefore the point inoculation method was used to compare the results of this experiment with those from the previous experiments on detached leaves, and also to make sure that each leaflet received the same amount of conidium suspension. To inoculate leaflets effectively, lentil plants were placed horizontally at the bottom of plastic containers so that the leaflets could be easily fixed with the tape.

Based on previous experiments on detached leaves, 48 h of incubation time was considered to be optimum to observe the development of PH inside plant epidermal cells. At this time, SH had not yet emerged from PH, so it was easier to measure the length of PH and estimate the percent cell area occupied by PH.

Previous experiments showed that on detached leaflets of CDC Robin, the total length of PH and the percentage of plant epidermal cell area occupied by PH were significantly higher for CT-34 than for CT-35, whereas there were no significant differences in these two parameters between the two isolates on Eston. On the attached leaves of CDC Robin, CT-34 also had higher values for these measurements for PH compared to CT-35, but in contrast to the results on the detached leaves, these high PH values were also found on the attached leaves of Eston. This suggests that isolates of *C. truncatum* developed differently on the detached and attached leaflets. Compared to the observations of CT-34 and CT-35 on detached leaves, the types of PH of these two isolates were similar on the attached leaves, but the size of PH was smaller than that in detached tissues, indicating that the development of PH was slower on attached leaves. In other words, the attached leaves had different sensitivity to *C. truncatum* compared to detached leaves.

One of the differences between detached leaflets and attached leaflets is that the former loses the supply of water and mineral nutrients from the roots during the period of incubation. As a result, detached leaflets lose increasingly more water and probably start to experience plasmolysis of cells, which may have an influence on the infection process as well. Studies by Liu et al. (2007) suggested that senescence in detached leaves could be one important reason for the differences between detached and attached leaves of Arabidopsis after being infected by *Colletotrichum* spp. They found that *C. linicola* could not penetrate attached leaves of Arabidopsis, but was able to invade detached leaves of the plant (Liu et al., 2007). Wei et al. (1997) showed that senescence played a role in speeding up the switch from the biotrophic to the necrotrophic phase of infection when investigating the interaction between round-leaved mallow and *C. gloeosporioides* f. sp. *malvae*. They suggested that this might be resulted from disorder in detoxification and repairing systems in older plant tissues (Wei et al., 1997). The influence of senescence was also shown in the interaction between *C. gloeosporioides* and citrus where healthy flower tissues escaped infection by the fungus but senescent petals were easily penetrated (Zulfiqar et al., 1996). In my study, *C. truncatum* could penetrate the attached leaflets of lentil although PH developed more slowly compared to the detached leaflets.

When studying the infection process of pathogens on the plants, finding a suitable inoculation method is vital as it influences the time requirements of experiments and the quality of data. When spraying the whole plants, it is difficult to ensure that individual leaflets receive the same amount of conidium suspension, in particular when leaflets only need to be inoculated with a small amount. There is an obvious problem after spraying the whole plant that the conidium suspension can drip from higher leaves onto lower leaves, resulting in different conidium concentrations. Whether the point-inoculation method is feasible for inoculation of attached leaves depends on kind of plants. For example, Arabidopsis leaves grow along the soil surface and can easily be inoculated with point-inoculation method. Lentil plants grow erectly, so it is necessary to find a suitable method to fix the leaves in a horizontal position as was done in this study. Based on my experiments, preparing lentil plants as described here for the whole plant assay is time consuming, especially when a large number of leaves need to be inoculated.

Using detached leaves has many advantages compared to experiments on whole plants. First of all, using detached leaves is highly efficient. When inoculating many leaves, this method saves much time. In addition, detached leaves can be inoculated by more than one fungus to compare the responses of the plant to them simultaneously. Inoculation of detached leaves does not encourage the induced resistance on the whole plant (Herath et al., 2001). Secondly, use of detached leaves can save resources (Tedford, et al., 1990). After the leaves are excised from the plants, plants can continue to grow, other leaves and plant tissues can be used in different experiments, if possible, seeds can also be harvested. Finally, using detached leaves can save space and labour. Detached leaves after inoculation can be incubated in smaller containers and incubators under specific conditions. Even though the use of detached leaves has the above advantages, it can not completely replace the use of attached leaves from the whole plants because attached leaves are different from detached leaves in their physiology due to senescence and different levels of water and nutrient supplies. It is therefore important to verify the detached-leaf data with the whole-plant assay for the selected parameters.

This study does not support the research hypothesis and contradicts the findings on detached leaflets that were inoculated with the same isolates. On attached leaves the total length of PH and percent plant epidermal cell area occupied by PH between the race Ct1 isolate and race Ct0 isolate were different on both CDC Robin and Eston, but on detached leaves the difference between the isolates was only found on CDC Robin. CDC Robin has race Ct1-resistance which may dominate any senescence effect on detached CDC Robin leaves, whereas that senescence effect on detached Eston leaves facilitates infection by both isolates with the result that no observable differences between the race Ct1 isolate and race Ct0 isolate can be seen.

This study showed that PH of two isolates, each from one of two races of *C. truncatum*, developed differently in terms of length and area of the epidermal cell occupied by this structure at 48 h incubation time on attached leaflets of both cultivars. CT-34 had significantly longer PH and occupied significantly more area in plant epidermal cells in both cultivars than CT-35. This suggests that the two isolates are different in aggressiveness and may represent aggressive races rather than physiological races as described by Caten (1987). However, the leaves of lentil lines had quantitatively

different reactions to ascospore-derived isolates of CT-21 (race Ct1) × CT-30 (race Ct0). Stems were more resistant than leaves. Specifically, the reaction of stem of CDC Robin to those isolates fell into two major groups and showed a binomial distribution that could suggest major resistance genes regulate resistance in stems (Cohen-Skali et al., 2008).

6. Summary and Conclusions

Investigation of conidium germination of two races (Ct0 and Ct1) of *Colletotrichum truncatum* on inert glass well slides showed that there were no significant differences between race Ct0 and race Ct1, indicating that race Ct0 was not inherently different from race Ct1 in conidium germination. Therefore, any differences in conidium germination in subsequent experiments could be attributed to specific interactions between the two races and lentil cultivars with different resistance.

The study on effects of plant age, conidium concentration, and inoculation method on conidium germination on lentil leaves determined parameters for a protocol for infection studies of *C. truncatum* on lentil. It was shown that the point-inoculation method on destained leaves of three-week old lentil plants using a conidium concentration of 5×10^5 conidia/mL was most suitable for further experiments. Since 5×10^5 conidia/mL resulted in a large number of conidia on green leaves, a conidium concentration of 1×10^5 conidia/mL was used in the following experiments.

At 12 hpi, conidium germination of race Ct0 isolates was significantly higher on the surface of detached green leaves of Eston than that of race Ct1 isolates. However, there were no significant differences between the two races in the percentage of appressorium formation. At that time, on CDC Robin race Ct0 isolates had significantly higher percentages of conidium germination and appressorium formation than race Ct1 isolates.

Investigation of the infection process of isolates of the two races into leaf tissues of lentil cultivars CDC Robin and Eston showed that IVs were observed inside the epidermal cells under the penetration sites at 24 hpi. During the period of 36 to 60 hpi, PH began to be generated and expanded in size. Since the development of fungal infection structures in leaves was not synchronized, different types of PH were observed at 36, 48, 60, and 72 hpi. Thinner SH of some isolates (e.g. CT-30) were observed

initially to develop from some PH at 60 hpi. By 72 hpi, SH became very prominent, spread at a locally parasitized epidermal cell, even passed through the cell wall and reached to the surrounding cells.

Investigation of percent penetration of the isolates of two races into detached lentil leaflets at 48 hpi showed that isolates of race Ct0 had a significantly higher penetration frequency than isolates of race Ct1 on CDC Robin and Eston. With regard to the first appearance of infection vesicles, the study showed that on both CDC Robin and Eston, there were no significant differences between isolates of both races.

At 48 hpi, race Ct0 isolates had significantly longer PH than race Ct1 isolates on detached leaves of Eston; at 48 hpi on detached leaves of CDC Robin and at 60 hpi on detached leaves of both cultivars, there were no significant differences between the two races. There were also no significant differences between the two races in the percentage of epidermal cell area occupied by PH at 48 and 60 hpi on Eston, but on CDC Robin, PH of race Ct0 isolates occupied significantly more space than those of race Ct1 isolates at both incubation times.

The frequencies of types 1, 2 and 4 of PH were similar among isolates of both races in leaf epidermal cells of Eston and CDC Robin at 48 and 60 hpi. At 48 hpi more type 3 of PH was generated by isolates of race Ct0 than by isolates of race Ct1 on CDC Robin, but this difference was not found at 48 hpi on Eston and at 60 hpi on both cultivars.

Investigation of the infection process on attached lentil leaves was carried out using two isolates CT-34 (race Ct0) and CT-35 (race Ct1) on CDC Robin and Eston at 48 hpi. Results showed that CT-34 had significantly longer PH and a significantly higher percentage of plant epidermal cell area occupied by PH than CT-35 on both cultivars. For the frequency of different types of PH, only type 4 of PH was significantly more frequent with CT-34 than with CT-35 on Eston; for this type on CDC Robin and types 1, 2, and 3 of PH on both cultivars, there were no significant differences between the two isolates.

To conclude, this study indicates that the two races have different virulence which were evident in conidium germination, appressorium formation, and growth of primary infection hyphae. Race Ct0 is more virulent than race Ct1, irrespective of whether it infects Eston or CDC Robin. The development of primary infection hyphae of race Ct1

was clearly inhibited and/or delayed during the infection process on the leaves of the resistant lentil cultivar CDC Robin.

Future studies should be focused on testing more isolates of both races on attached lentil leaves to confirm the differences observed in the infection process on detached and attached leaves. Such experiments should also include a wider range of incubation times as were done on detached leaves. Current ongoing research looking at the inheritance of virulence suggests that different stem reactions to the two races are more dramatic than leaf reactions (Cohen-Skali, 2008). Therefore, studies of the infection process should also be extended to lentil stems.

7. References

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8. Appendices

Appendix 1 ANOVA table for the slopes and percent conidium germination at 20 h after the conidium suspensions were spread on the well slides of three isolates of race Ct0 and three isolates of race Ct1 of *Colletotrichum truncatum*

Source	Effect	Num DF	Den DF	F Value	Pr > F
Slope	iso	5	11.6	14.87	0.0001
20-h data	iso	5	11.5	12.14	0.0003

Appendix 2 Results from a mixed model analysis of percent conidium germination on three- and six-week old plants of lentil cvs. Eston and CDC Robin inoculated with *Colletotrichum truncatum* CT-15 (race Ct1) at three conidium concentrations using spray- and point-inoculation methods

Effect	Num DF	Den DF	F Value	Pr > F
age	1	1.57	43.17	0.0396
inoc	1	157	3.66	0.0574
age*inoc	1	157	6.20	0.0138
cul	1	157	20.01	<.0001
age*cul	1	157	0.07	0.7848
inoc*cul	1	157	1.61	0.2065
age*inoc*cul	1	157	0.01	0.9093
con	2	2.23	26.03	0.0284
age*con	2	157	1.25	0.2884
inoc*con	2	157	5.55	0.0047
age*inoc*con	2	157	4.39	0.0140
cul*con	2	157	0.80	0.4531
age*cul*con	2	157	0.14	0.8736
inoc*cul*con	2	157	0.74	0.4796
age*inoc*cul*con	2	157	0.55	0.5776

Note: age=plant age, inoc=inoculation method, cul=cultivar, con=concentration

Appendix 3 Results from a mixed model analysis of percent conidium germination on three- and six-week old plants of lentil cvs. Eston and CDC Robin inoculated with 5 × 10^4 conidia/mL of *Colletotrichum truncatum* CT-15 (race Ct1) isolate using two inoculation methods

Effect	Num DF	Den DF	F Value	Pr > F
age	1	51.2	109.82	<.0001
inoc	1	51.2	2.74	0.1040
age*inoc	1	51.2	0.04	0.8368
cul	1	51.2	11.55	0.0013
age*cul	1	51.2	0.00	0.9451
inoc*cul	1	51.2	1.07	0.3061
age*inoc*cul	1	51.2	0.36	0.5531

Note: age=plant age, inoc=inoculation method, cul=cultivar

Appendix 4 Results from a mixed model analysis of percent conidium germination on three- and six-week old plants of lentil cvs. Eston and CDC Robin inoculated with 5 × 10^5 conidia/mL of *Colletotrichum truncatum* CT-15 (race Ct1) isolate using two inoculation methods

Effect	Num DF	Den DF	F Value	Pr > F
age	1	1.01	20.48	0.1360
inoc	1	1	3.03	0.3320
age*inoc	1	47.6	0.08	0.7752
cul	1	1.07	2.12	0.3715
age*cul	1	47.6	0.03	0.8709
inoc*cul	1	47.6	2.45	0.1241
age*inoc*cul	1	47.6	0.91	0.3459

Note: age=plant age, inoc=inoculation method, cul=cultivar

Appendix 5 Results from a mixed model analysis of percent conidium germination on three- and six-week old plants of lentil cvs. Eston and CDC Robin inoculated with 1 × 10^6 conidia/mL of *Colletotrichum truncatum* CT-15 (race Ct1) isolate at three concentrations using two inoculation methods

Effect	Num DF	Den DF	F Value	Pr > F
age	1	2.01	19.18	0.0481
inoc	1	53.9	2.70	0.1062
age*inoc	1	53.9	12.92	0.0007
cul	1	53.9	8.33	0.0056
age*cul	1	53.9	0.28	0.6014
inoc*cul	1	53.9	0.04	0.8437
age*inoc*cul	1	53.9	0.02	0.8894

Note: age=plant age, inoc=inoculation method, cul=cultivar

Appendix 6 Results from a mixed model analysis of percent conidium germination on three- and six-week old plants of lentil cvs. Eston and CDC Robin spray-inoculated with 1 × 10^6 conidia/mL of *Colletotrichum truncatum* CT-15 (race Ct1) isolate

Effect	Num DF	Den DF	F Value	Pr > F
age	1	2	4.04	0.1823
cul	1	26	4.43	0.0452
age*cul	1	26	0.21	0.6537

Note: age=plant age, cul=cultivar

Appendix 7 Results from a mixed model analysis of percent conidium germination on three- and six-week old plants of lentil cvs. Eston and CDC Robin point-inoculated with 1×10^6 conidia/mL of *Colletotrichum truncatum* CT-15 (race Ct1) isolate

Effect	Num DF	Den DF	F Value	Pr > F
age	1	2	40.63	0.0237
cul	1	26	3.66	0.0669
age*cul	1	26	0.08	0.7858

Note: age=plant age, cul=cultivar

Appendix 8 Results from a mixed model analysis of percent conidium germination on three-week old plants of lentil cvs. Eston and CDC Robin inoculated with 1×10^6 conidia/mL of *Colletotrichum truncatum* CT-15 (race Ct1) isolate using two inoculation methods

Effect	Num DF	Den DF	F Value	Pr > F
inoc	1	27	15.77	0.0005
cul	1	27	3.20	0.0847
inoc*cul	1	27	0.00	0.9650

Note: inoc=inoculation method, cul=cultivar

Appendix 9 Results from a mixed model analysis of percent conidium germination on six-week old plants of lentil cvs. Eston and CDC Robin inoculated with 1×10^6 conidia/mL of *Colletotrichum truncatum* CT-15 (race Ct1) isolate using two inoculation methods

Effect	Num DF	Den DF	F Value	Pr > F
inoc	1	26.8	1.69	0.2048
cul	1	26.8	5.17	0.0313
inoc*cul	1	26.8	0.05	0.8237

Note: inoc=inoculation method, cul=cultivar

Appendix 10 Results from a mixed model analysis of percent conidium germination on three- and six-week old plants of lentil cvs. Eston and CDC Robin point-inoculated with *Colletotrichum truncatum* CT-15 (race Ct1) isolate at three concentrations

Effect	Num DF	Den DF	F Value	Pr > F
age	1	1.1	103.54	0.0503
cul	1	1.24	5.95	0.2066
age*cul	1	78.9	0.07	0.7992
con	2	2.57	11.04	0.0549
age*con	2	78.9	0.56	0.5737
cul*con	2	78.9	0.17	0.8406
age*cul*con	2	78.9	0.17	0.8414

Note: age=plant age, cul=cultivar, con=concentration

Appendix 11 Results from a mixed model analysis of percent conidium germination on three- and six-week old plants of lentil cvs. Eston and CDC Robin spray-inoculated with *Colletotrichum truncatum* CT-15 (race Ct1) isolate at three concentrations

Effect	Num DF	Den DF	F Value	Pr > F
age	1	2	27.09	0.0350
cul	1	82	6.39	0.0134
age*cul	1	82	0.02	0.9002
con	2	82	36.15	<.0001
age*con	2	82	6.21	0.0031
cul*con	2	82	1.66	0.1969
age*cul*con	2	82	0.60	0.5486

Note: age=plant age, cul=cultivar, con=concentration

Appendix 12 Results from a mixed model analysis of percent conidium germination on three-week old plants of lentil cvs. Eston and CDC Robin spray-inoculated with *Colletotrichum truncatum* CT-15 (race Ct1) isolate at three concentrations

Effect	Num DF	Den DF	F Value	Pr > F
cul	1	41	2.84	0.0996
con	2	41	6.10	0.0048
cul*con	2	41	0.17	0.8409

Note: cul=cultivar, con=concentration

Appendix 13 Results of the contrast analysis of conidium germination at three conidium concentrations when spray-inoculating three-week old plants of lentil cvs. Eston and CDC Robin with *Colletotrichum truncatum* CT-15 (race Ct1) isolate

Effect	con	con	Estimate	Standard Error	DF	t Value	Pr > |t|
con	$1*10^6$	$5*10^4$	-0.0803	0.0245	41	-3.29	0.0021
con	$1*10^6$	$5*10^5$	-0.0654	0.0245	41	-2.67	0.0107
con	$5*10^4$	$5*10^5$	0.0150	0.0245	41	0.61	0.5440

Note: con=concentration

Appendix 14 Results from a mixed model analysis of percent conidium germination on six-week old plants of lentil cvs. Eston and CDC Robin spray-inoculated with *Colletotrichum truncatum* CT-15 (race Ct1) isolate at three concentrations

Effect	Num DF	Den DF	F Value	Pr > F
cul	1	42	3.64	0.0634
con	2	42	37.38	<.0001
cul*con	2	42	2.16	0.1284

Note: cul=cultivar, con=concentration

Appendix 15 Results of the contrast analysis of conidium germination at three conidium concentrations when spray-inoculating six-week old plants of lentil cvs. Eston and CDC Robin with *Colletotrichum truncatum* CT-15 (race Ct1) isolate

Effect	con	con	Estimate	Standard Error	DF	t Value	Pr > |t|
con	$1*10^6$	$5*10^4$	-0.1930	0.0239	42	-8.09	<.0001
con	$1*10^6$	$5*10^5$	-0.1597	0.0239	42	-6.69	<.0001
con	$5*10^4$	$5*10^5$	0.0333	0.0239	42	1.39	0.1704

Note: con=concentration

Appendix 16 Results from a mixed model analysis of percent conidium germination of six isolates representing two races Ct0 and Ct1 of *Colletotrichum truncatum* on the detached lentil leaves at 12 h postinoculation

Effect	Num DF	Den DF	F Value	Pr > F
iso	5	14.2	27.77	<.0001
cul	1	29.8	37.84	<.0001
iso*cul	5	14.2	2.84	0.0557

Note: iso=isolate, cul=cultivar

Appendix 17 Results from a mixed model analysis of percent appressorium formation of six isolates representing two races Ct0 and Ct1 of *Colletotrichum truncatum* on the detached lentil leaves at 12 h postinoculation

Effect	Num DF	Den DF	F Value	Pr > F
iso	5	9.13	37.98	<.0001
cul	1	25.3	53.76	<.0001
iso*cul	5	9.13	19.52	0.0001

Note: iso=isolate, cul=cultivar

Appendix 18 Results from a mixed model analysis of percent penetration of six isolates representing two races Ct0 and Ct1 of *Colletotrichum truncatum* on the detached leaflets of lentil cultivars CDC Robin and Eston at 48 h postinoculation

Effect	Num DF	Den DF	F Value	Pr > F
iso	5	10.1	11.18	0.0007
cul	1	32.6	9.29	0.0045
iso*cul	5	10.1	0.73	0.6193

Note: iso=isolate, cul=cultivar

Appendix 19 Results from a mixed model analysis of the first appearance of infection vesicles of six isolates representing two races Ct0 and Ct1 of *Colletotrichum truncatum* on the detached leaflets of lentil cultivars CDC Robin and Eston

Cultivar	Effect	Num DF	Den DF	F Value	Pr > F
CDC Robin	iso	5	2.06	0.09	0.9862
Eston	iso	5	4.03	0.37	0.8487

Appendix 20 ANOVA table for the total length of primary hyphae of six isolates representing race Ct0 and race Ct1 of *Colletotrichum truncatum* at both incubation times (48 and 60 h) on the detached leaves of both lentil cultivars, Eston and CDC Robin

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
h	1	31360	31360	49.84	<.0001
iso	5	13436	2687	4.27	0.0007
h*iso	5	6359	1272	2.02	0.0730
cul	1	3048	3048	4.84	0.0279
h*cul	1	17675	17675	28.09	<.0001
iso*cul	5	3000	600	0.95	0.4452
h*iso*cul	5	5107	1021	1.62	0.1507
Error	1416	890955	629		
Corrected Total	1439	970941			

Note: iso=isolate, cul=cultivar, h=incubation time

Appendix 21 ANOVA table for the total length of primary hyphae of six isolates representing two races Ct0 and Ct1 of *Colletotrichum truncatum* at 48 h postinoculation on the detached leaves of lentil cultivars Eston and CDC Robin

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
iso	5	16778	3356	5.21	0.0001
cul	1	17701	17701	27.48	<.0001
iso*cul	5	5849	1170	1.82	0.1074
Error	708	456018	644		
Corrected Total	719	496346			

Note: iso=isolate, cul=cultivar

Appendix 22 ANOVA table for the total length of primary hyphae of six isolates representing two races Ct0 and Ct1 of *Colletotrichum truncatum* at 60 h postinoculation on the detached leaves of lentil cultivars Eston and CDC Robin

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
iso	5	3018	603.5	0.98	0.4275
cul	1	3022	3021.7	4.92	0.0269
iso*cul	5	2258	451.6	0.74	0.5972
Error	708	434938	614.3		
Corrected Total	719	443235			

Note: iso=isolate, cul=cultivar

Appendix 23 ANOVA table for the percentage of epidermal cell area occupied by primary hyphae of six isolates representing two races Ct0 and Ct1 of *Colletotrichum truncatum* at both (48 and 60 h) incubation times on the detached leaves of both lentil cultivars, Eston and CDC Robin

Source	DF	Type III SS	Mean Square	F Value	Pr > F
h	1	16585.3	16585.3	146.50	<.0001
iso	5	1838.3	367.7	3.25	0.0064
h*iso	5	105.6	21.1	0.19	0.9677
cul	1	770.0	770.0	6.80	0.0092
h*cul	1	96.6	96.6	0.85	0.3557
iso*cul	5	1620.7	324.1	2.86	0.0140
h*iso*cul	5	268.3	53.7	0.47	0.7959
Error	1416	160299.9	113.2		
Corrected Total	1439	181584.7			

Note: h=incubation time, iso=isolate, cul=cultivar

Appendix 24 ANOVA table for the percentage of epidermal cell area occupied by primary hyphae of six isolates representing two races Ct0 and Ct1 of *Colletotrichum truncatum* at both (48 and 60 h) incubation times on the detached leaves of lentil cultivar Eston

Source	DF	Type III SS	Mean Square	F Value	Pr > F
iso	5	171.8	34.4	0.29	0.9208
h	1	7075.1	7075.1	58.91	<.0001
h*iso	5	184.5	36.9	0.31	0.9086
Error	708	85028.6	120.1		
Corrected Total	719	92460.0			

Note: iso=isolate, h=incubation time

Appendix 25 ANOVA table for the percentage of epidermal cell area occupied by primary hyphae of six isolates representing two races Ct0 and Ct1 of *Colletotrichum truncatum* at both (48 and 60 h) incubation times on the detached leaves of lentil cultivar CDC Robin

Source	DF	Type III SS	Mean Square	F Value	Pr > F
iso	5	3287	657.5	6.18	<.0001
h	1	9607	9606.8	90.36	<.0001
h*iso	5	189	37.9	0.36	0.8783
Error	708	75271	106.3		
Corrected Total	719	88355			

Note: iso=isolate, h=incubation time

Appendix 26 Contrast analysis of frequency of different types of primary hyphae (PH, based on shape) of six isolates representing race Ct0 and race Ct1 of *Colletotrichum truncatum* on the detached leaves of lentil cultivar Eston at 48 h incubation time

Contrast	DF	Chi-Square	Pr > ChiSq	Type
race Ct1 vs race Ct0 (type 1)	1	0.87	0.3503	LR
race Ct1 vs race Ct0 (type 2)	1	0.21	0.6476	LR
race Ct1 vs race Ct0 (type 3)	1	0.94	0.3318	LR
race Ct1 vs race Ct0 (type 4)	1	1.71	0.1909	LR

Appendix 27 Contrast analysis of frequency of different types of primary hyphae (PH, based on shape) of six isolates representing race Ct0 and race Ct1 of *Colletotrichum truncatum* on the detached leaves of lentil cultivar CDC Robin at 48 h incubation time

Contrast	DF	Chi-Square	Pr > ChiSq	Type
race Ct1 vs race Ct0 (type 1)	1	0.00	0.9849	LR
race Ct1 vs race Ct0 (type 2)	1	3.40	0.0653	LR
race Ct1 vs race Ct0 (type 3)	1	5.76	0.0164	LR
race Ct1 vs race Ct0 (type 4)	1	0.66	0.4182	LR

Appendix 28 Contrast analysis of frequency of different types of primary hyphae (PH, based on shape) of six isolates representing race Ct0 and race Ct1 of *Colletotrichum truncatum* on the detached leaves of lentil cultivar Eston at 60 h incubation time

Contrast	DF	Chi-Square	Pr > ChiSq	Type
race1 vs race0 (type 1)	1	0.19	0.6618	LR
race1 vs race0 (type 2)	1	0.19	0.6639	LR
race1 vs race0 (type 3)	1	0.03	0.8702	LR
race1 vs race0 (type 4)	1	0.76	0.3828	LR

Appendix 29 Contrast analysis of frequency of different types of primary hyphae (PH, based on shape) of six isolates representing race Ct0 and race Ct1 of *Colletotrichum truncatum* on the detached leaves of lentil cultivar CDC Robin at 60 h incubation time

Contrast	DF	Chi-Square	Pr > ChiSq	Type
race1 vs race0 (type 1)	1	0.00	1.0000	LR
race1 vs race0 (type 2)	1	0.10	0.7571	LR
race1 vs race0 (type 3)	1	0.72	0.3959	LR
race1 vs race0 (type 4)	1	0.93	0.3348	LR

Appendix 30 ANOVA table for the total length of primary hyphae of *Colletotrichum truncatum* isolates CT-34 (race Ct0) and CT-35 (race Ct1) at 48 h incubation time on the detached leaves of lentil cultivar Eston

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Isolate	1	1632	1632	2.56	0.1125
Error	118	75322	638		
Corrected Total	119	76954			

Appendix 31 ANOVA table for the total length of primary hyphae of *Colletotrichum truncatum* isolates CT-34 (race Ct0) and CT-35 (race Ct1) at 48 h incubation time on the detached leaves of lentil cultivar CDC Robin

Source	**DF**	**Sum of Squares**	**Mean Square**	**F Value**	**Pr > F**
Isolate	1	3630	3630	9.07	0.0032
Error	118	47207	400		
Corrected Total	119	50837			

Appendix 32 ANOVA table for the percentage of plant epidermal cell area occupied by primary hyphae of *Colletotrichum truncatum* isolates CT-34 (race Ct0) and CT-35 (race Ct1) at 48 h incubation time on the detached leaves of lentil cultivar Eston

Source	**DF**	**Sum of Squares**	**Mean Square**	**F Value**	**Pr > F**
Isolate	1	52	52.0	0.76	0.3852
Error	118	8078	68.5		
Corrected Total	119	8130			

Appendix 33 ANOVA table for the percentage of plant epidermal cell area occupied by primary hyphae of *Colletotrichum truncatum* isolates CT-34 (race Ct0) and CT-35 (race Ct1) at 48 h incubation time on the detached leaves of lentil cultivar CDC Robin

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Isolate	1	472	472	8.58	0.0041
Error	118	6494	55		
Corrected Total	119	6966			