EPIDEMIOLOGY OF *COLLETOTRICHUM ACUTATUM*, CAUSE OF ANTHRACNOSE ON HIGHBUSH BLUEBERRY, IN BRITISH COLUMBIA

by

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ABSTRACT

Blueberry anthracnose reduces yield and post-harvest quality of blueberries in British Columbia. Isolates recovered from diseased fruit during 2002-2004 were identified as *Colletotrichum acutatum* using colony morphology, growth rate, and PCR. Leaf-wetness and temperature data indicated that plants exposed to prevailing environmental field conditions and natural inoculum in 2001-2003 required 10 hr of leaf wetness at 11°C for successful fruit infection. Three peaks of infection occurred; at blossoming, mature green berry stage, and ripening. Artificially inoculated plants in 2004 were found to be susceptible throughout the season, indicating that inoculum availability and weather conditions primarily affected disease. The pathogen overwintered mostly in flower buds and infection in May-July of the preceding season resulted in the greatest inoculum recovery. Two fungal biocontrol agents, *Gliocladium catenulatum* and *Trichoderma harzianum*, reduced anthracnose by up to 45% when applied thrice in 2003-2004. Infection requirements and related epidemiological data support effective management of this disease.

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TABLE OF CONTENTS

Approval ii

Abstract iii

Acknowledgements iv

Table of contents v

List of figures vii

List of tables ix

Chapter 1. Introduction 1

1.1 Blueberries 1

1.1.1 *Vaccinium corymbosum* cultivation 2

1.1.2 Propagation and development 3

1.1.3 Crop and industry value 4

1.2 Blueberry anthracnose 5

1.2.1 Causal organisms 6

1.2.2 Symptomology 7

1.2.3 Sporulation 8

1.2.4 Industry economic losses 8

1.2.5 Current control options in BC 10

1.3 Epidemiology 11

1.3.1 Pathogen identification 11

1.3.2 Infection requirements 13

1.3.3 Pathogen overwintering 14

1.4 Latent infections and post-harvest disease 15

1.4.1 Physiological mechanisms 17

1.4.2 Latent infections causing blueberry anthracnose 18

1.5 Biological control 19

1.5.1 Post-harvest diseases 20

1.5.2 Pre-harvest management 23

1.5.3 Related research 23

1.6 Objectives of research 25

1.6.1 Potential biological control products tested 25

Chapter 2. Materials and methods 26

2.1 Fungal isolations 26

2.2 Anthracnose survey 26

2.3 Identification of *Colletotrichum* 27

2.4 Infection of blueberry and apple fruit 30

2.5 Establishing period of susceptibility of blueberry plants 31

2.5.1 Trap plants and natural inoculum 31
2.5.2 Artificial inoculation of plants 32
2.6 Pathogen overwintering and perennial persistence 33
2.7 Biological control experiments 34

Chapter 3. Results and discussion 36
3.1 Fungal isolations 36
3.2 Anthracnose survey 36
3.3 Identification of *Colletotrichum* 40
3.4 Infection of blueberry and apple fruit 45
3.5 Establishing period of susceptibility of blueberry plants 45
3.5.1 Trap plants and natural inoculum 45
3.5.2 Artificial inoculation of plants 48
3.5.3 Post-harvest disease 51
3.6 Pathogen overwintering and perennial persistence 54
3.7 Biological control experiments 56

Chapter 4. Summary and future research 59
4.1 Application of research results 59
4.2 Directions for future research 60

REFERENCES 63

LIST OF FIGURES

Figure 1.1. Spores of *Colletotrichum acutatum* from an isolate collected from a commercial blueberry field in BC in 2001 (MacDonald et al., 2001) at 400x magnification. 9

Figure 1.2. The spent fruit truss (T) where berries were held the previous year (arrows), and newly emerging flower bud on Highbush Blueberry 'Bluecrop' 16

Figure 3.1. Recovery of *Colletotrichum* from blueberry tissues collected at different plant growth stages during 2002 and 2003. Samples were collected from a commercial field, placed under moist conditions and assessed for presence of the pathogen. 37

Figure 3.2. Frequency of recovery of *Colletotrichum* from harvested fruit in seven commercial fields sampled in 2003 and 2004. Fruit were incubated under moist conditions for 2 weeks and assessed for pathogen presence. 38

Figure 3.3 Development of anthracnose and growth of *Colletotrichum acutatum*. a-d); Tissue samples collected from blueberry plants, a) fruit trusses and vegetative buds, b) blossoms, c) ripe berries, d) green berries. e-g); Visible spore masses of *C. acutatum* on diseased fruit. h); Comparison of *C. acutatum* and *C. gloeosporioides* colonies growing on PDA plates. i-j); Disease symptoms on immature green berries inoculated with a spore suspension *in vitro*. k); Apples inoculated with *Colletorichum* isolates displaying orange spore masses and decayed tissue. 42

Figure 3.4. Comparison of colony diameter of *Colletotrichum acutatum* (*C.a.*) and *C. gloeosporioides* (*C.g.*) after 14 days on PDA at 28°C. Isolate designations as in Table 2.1. Isolates designated BC were collected from blueberry fields in British Columbia during 2002-2004. 43

Figure 3.5. Identification of *Colletotrichum acutatum* and *C. gloeosporioides* using species-specific primers. The CaInt2+ITS4 primer set identifies *C. acutatum,* and CgInt+ITS4 identifies *C. gloeosporioides*. Corresponding fragments are 480 and 450 bp in size, respectively. Positive controls are isolates CR01, CR02, MI01, MI02, NS01, IS01, IS02, FL01, FL02 (*C.a.*) and NS02, IS03, IS04, FL03, FL04, SL01 (*C.g.*). Water was included as a negative control (H_2O). 44

Figure 3.6. Infection of blueberry fruit with *Colletotrichum acutatum* at different temperatures. Ripe fruit were inoculated with a spore

suspension, incubated at specified temperatures for 14 days, and then rated for sporulation. 46

Figure 3.7. Infection of apple fruit with three isolates of *Colletotrichum acuatum* (BC08, CR02, MI01) and one of *C. gloeosporioides* (IS04) at different temperatures. Apples were wounded with an 8mm cork-borer, inoculated with PDA plugs containing fungal mycelium, and incubated at specified temperatures for 7 days. Resulting radial growth from the inoculation site was measured. 47

Figure 3.8. Incidence of fruit infection from natural inoculum sources of 2-year old blueberry trap plants in 2001, 2002, and 2003, corresponding to different growth stages; EBL-early blossom; FBL-full blossom; GBR-early green berry; MGR-mature green berry; BLB-ripe berry. Numbers within graphs represent number of infection periods (IP); 10 hours of leaf wetness at temperatures ≥10°C, for peak weeks. 49

Figure 3.9. Infection of blueberry fruit resulting from artificial inoculation of blueberry plants at weekly intervals during 2004. Corresponding growth stages are indicated; EBL-early blossom; FBL-full blossom; GBR-early green berry; MGR-mature green berry; BLB-ripe berry. 52

Figure 3.10. Recovery of *Colletotrichum* after overwintering, from blueberry plants artificially inoculated at weekly intervals during 2004. Plants were sampled twice; once in December 2004 and then again in February 2005. 55

Figure 3.11. Effect of *Gliocladium catenulatum* and *Trichoderma harzianum* on fruit infection at harvest over 3 years. In 2002, potted plants were used, while in 2003 and 2004, applications were made to plants in commercial blueberry fields. 3 applications of the biological control agents were made each year. Bars labeled with the same letter are not statistically different according to an ANOVA comparison of means. 57

LIST OF TABLES

Table 1.1. Examples of recent successful biological control of post-harvest diseases by pre-harvest application (see Ippolito and Nigro [2000] for referenced literature).....22

Table 2.1. Sources of reference isolates of *Colletotrichum acutatum* and *C. gloeosporioides* used in this study. 28

Table 3.1. Responses collected as part of the 2003 anthracnose survey. 39

Table 3.2. Environmental monitoring data (temperature and leaf wetness duration) during field exposure of trap plants to natural conditions over 3 years. Corresponding growth stages are indicated for infection periods (IP), which are defined as 10 hours of leaf wetness at the indicated temperatures (10° or 12°C). 50

Table 3.3. Environmental monitoring data (temperature and leaf wetness duration) for artificially inoculated blueberry plants exposed to natural conditions in 2004. Corresponding growth stages are indicated for infection periods (IP), which were defined as 10 hours of leaf wetness with simultaneous temperatures of 12°C or higher. 53

CHAPTER 1. INTRODUCTION

1.1 Blueberries

Blueberry belongs to the Ericaceae family, which includes familiar garden plants such as rhododendrons, azaleas, heaths, heathers, as well as other plants such as salal, cranberries and huckleberries. Blueberry belongs to the genus *Vaccinium* and has been cultivated for centuries in North America, originally by native Indians, and then adopted by settlers arriving from England in the 17th century (Anonymous, 2005a). In fact, blueberries are one of the few native fruits of North America. The natives named the plant 'star berry' in reference to the calyx (blossom-end) of the berry which resembles a five-point star. They used the fruit for many purposes, including as medicine, dye colouring, or meat flavouring, and for consumption as fresh fruit, juice or tea.

In North America, blueberries are currently enjoying great demand due to their positive health benefits, which include antioxidant activity and high vitamin content, convenient consumption capability either as fresh fruit or processed goods, and great taste. They are either sold seasonally as fresh fruit, or processed into a variety of products, including jams, syrups, cereals, baked items, juices, yogurts, ice creams, and wines. A variety of industry groups promote the marketing and research of blueberry products in the major growing regions of North America, including the US Highbush Blueberry Council, and the BC Blueberry Council.

1.1.1 *Vaccinium corymbosum* cultivation

The genus *Vaccinium* contains about 400 species that are divided into various groups, including *Cyanococcus* (blueberries), *Oxycoccus* (cranberries), *Vitis-idaea* (lingonberries), and *Myrtillus* (billberries). Their taxonomy is difficult to discern due to considerable hybridization and introgression (Lyrene et al., 2003). The highbush blueberry, *V. corymbosum* L. is in the *Cyanococcus* group and is a tetraploid species (2n=4x=48), where polyploidy is thought to have occurred due to the formation of unreduced gametes. Its taxonomy has also been difficult to resolve due to its complex polyploid origins, but it is believed to have multiple origins. *Vaccinium* species are native to all continents except Antarctica and Australia (Ballington, 2001).

There are three commercially important species of blueberry; *Vaccinium corymbosum* L. (Northern highbush), *V. ashei* Reade. (Southern rabbiteye), and *V. angustifolium* Ait. (Lowbush or 'wild' blueberries). The primary species grown in British Columbia is highbush, which is acclimatized to the cooler climates of the northern temperate zone. It has an abundance of large, sweet fruit, and grows in a variety of regions, including wetlands and drier upland wooded slopes. Its geographic range stretches through the mid-western, eastern, and central USA, as well as along the Pacific coast from southern Canada to the northern parts of USA. Although still highly concentrated in North America, highbush blueberries are now cultivated in Europe, Australia, Chile and New Zealand (Alcock, 2005).

All species of *Vaccinium* are acidophilic, preferring acidic soil conditions such as swamps or hummocks. Highbush plants have the growth aspect of a deciduous woody bush and can reach 3-4 m in height, but are usually pruned to 1.8-2.5 m in cultivation.

They are winter-hardy; their flower buds are able to withstand -30°C before damage occurs (Anonymous, 2005b), and require 650-1,000 chilling hrs for normal leaf and bloom development.

Being a woody perennial, highbush blueberry plants produce shoots primarily from the base of the plant, which have a determinant, sympodial growth pattern with periodic apical abortions. Depending on cultivar, species, and vigour, buds adjacent to the aborted tips can then either remain dormant, or enlarge and initiate new shoot extension. Root systems are fine and fibrous, and begin growing before bloom. Root growth continues well into fall, reaching a depth of 50-80 cm, and they are invaded by mycorrhizal fungi (Eck, 1988).

1.1.2 Propagation and development

Commercial propagation of highbush blueberries is primarily via stem or rhizome cuttings, or via micropropagation using small plant parts aseptically grown under tissue culture conditions. Fields are usually planted in naturally acidic wetlands, where the soil has a high organic matter content (20-50%), and a pH range of 4.5-5.2. Domesticated honeybees are used to enhance pollination rates, also aiding in synchrony of fruit ripening. Irrigation is commonly used, either as overhead-sprays, or via drip-lines. Regular pruning is done to promote light penetration of the canopy, modify growth habit, and affect the ratio of flower to vegetative buds.

Blueberry plants develop through several phases in the growing season, including budding in early spring, blossoming in late spring, and then forming green berries that slowly mature through summer, before ripening into blue berries that are ready for

harvest. Depending on the cultivar, blueberries are harvested earlier in the season (e.g. 'Duke'), mid-season (e.g. 'Bluecrop'), or later (e.g. 'Elliott' and 'Jersey'). Fruit is harvested either by hand, especially if destined for the fresh market, or increasingly by machine, either for the fresh or processed market. Since all berries on a plant do not ripen simultaneously, fields are usually harvested two or three times. After harvesting, fruit is processed (cleaned, de-stemmed, and sorted) and either frozen or packed fresh for sale.

1.1.3 Crop and industry value

The BC highbush blueberry industry is a rapidly growing segment of the province's agricultural production. BC was the largest producer of fresh blueberries in 2004, and the second largest producer of processed blueberries in the world (Alcock, 2005; Sjulin, 2003) with approximate production of 27.2 million kg from 4562 ha under cultivation (BC Blueberry Council), valued at $69 million in 2004. The 2002 crop value was estimated at $30 million, with growth projected to be over 10% per year over the next 5 years. The US Highbush Blueberry Council reports that commercial cultivation of blueberries during 2000-2002 in the US was annually valued at US $178 million, with 124 million kg production, of which 65% was processed into storable products.

Planted acreage has also been steadily increasing in both Canada and the US. Statistics Canada reports that the blueberry industry value in BC has grown by more than 270% in the years spanning 1993-2002. In 2002, combined production of the eight US states that commercially produce blueberries (Michigan, New Jersey, North Carolina, Oregon, Washington, Florida, Georgia, and Maine) totalled 110 million kg, worth US $202 million, from 27,882 ha (Gianessi and Reigner, 2004). Strik and Yarborough (2005) propose that planted area under blueberry cultivation in North America will increase by

14% in the US and 22% in Canada over the next 5 years. Projected growth over 10 years is even more dramatic, at 31% in the US and 26% in Canada.

Production of blueberries in eastern Canada is primarily from Ontario, Nova Scotia and includes both highbush and lowbush (wild) varieties. In 2004, Ontario production, from a combined acreage of 164 ha, was 617,000 kg and valued at an estimated $2.4 million.

1.2 Blueberry anthracnose

A number of fungal diseases affect blueberry, of which anthracnose (ripe-rot) is a major problem that reduces yield and severely affects post-harvest fruit quality (Polashock et al., 2005). It has been reported in all major blueberry-growing regions, including New Jersey (Stretch, 1967), North Carolina (Daykin and Milholland, 1984), Michigan (Hartung et al., 1981), Mississippi (Smith et al., 1996) and BC (MacDonald, 1998). It can affect all three commercially important *Vaccinium* species (highbush, rabbiteye, and lowbush).

The disease primarily affects fruit, causing berry softening and production of orange-coloured spore masses. Affected fruit, which drop prematurely from the plant, are unpalatable and unsaleable. Further loss occurs through post-harvest infection in storage bins. Often the infection does not appear until several days after harvest. Fruit harvested for the fresh market may look sound when packed, but be rejected at the wholesale or retail level. A rejected shipment can be very costly for the grower/shipper and can have long-term implications for the reputation of BC blueberries.

Anthracnose was first reported in BC in the early 1990's (MacDonald, 1998), and the disease has been steadily increasing in severity and now occurs in many blueberry-producing areas in BC.

1.2.1 Causal organisms

Blueberry anthracnose occurs in many regions of the world (Polashock et al., 2005) and the causal organisms are reported to be two species of *Colletotrichum; C. acutatum* Simmonds and *C. gloeosporioides* Penz. The genus *Colletotrichum* includes fungal pathogens of many field crops and stored fruits (Jeffries et al., 1990; Waller, 1992). Previous research in the eastern USA originally identified *C. gloeosporioides* as the primary cause of blueberry anthracnose (Cappellini et al., 1972; Daykin and Milolland, 1984). However, recent research has shown that *C. acutatum* is also prevalent in blueberry fields, and that both species can cause anthracnose (Bristow and Windom, 2000; DeMarsay and Oudemans, 2003), with *C. acutatum* being the primary agent. The two species can be distinguished using morphological criteria, such as growth rates on agar media, and spore shape and size (Smith, 1990). However, molecular techniques are considered a more reliable method (Freeman and Katan, 1997; Vinnere et al., 2002) since morphological characteristics can overlap considerably between the two species.

C. acutatum and *C. gloeosporioides* are imperfect anamorphic fungi (subdivision Deuteromycotina, form-class Deuteromycetes, form-subclass Coelomycetidae, form-order Melanconiales, form-family Melanconiaceae), and do not generally produce their teleomorph in culture, although these have been described to belong to the genus *Glomerella* (subdivision Ascomycotina); *G. acutata* and *G. cingulata*, respectively (Sutton, 1992; Latunde-Dada, 2001). The relationship between *Colletotrichum* and

Glomerella has been demonstrated many times (Bryson et al., 1992), and no teleomorphic stage other than *Glomerella* has been associated with *Colletotrichum*. The *Colletotrichum* genus continues to be revised and clarified using molecular taxonomic techniques, with various species complexes and subspecific groups under consideration. Molecular techniques that have been employed include Polymerase Chain Reaction (PCR) with species-specific primers (Brown et al., 1996), Random Amplified Polymorphic DNA (RAPD) (Denoyes-Rothan et al., 2003; Kuramae-Izioka et al., 1997; Sreenivasaprasad et al., 1992), Restriction Fragment Length Polymorphisms (RFLP) (Guerber et al., 2003), and Arbitrarily-primed PCR (ap-PCR) (Freeman et al., 2001).

1.2.2 Symptomology

Colletotrichum, and its teleomorph *Glomerella,* are considered major plant pathogens worldwide. They have been implicated in economically damaging plant diseases of crops in tropical, subtropical and temperate regions throughout the world (Freeman et al., 1998; Holliday, 1980). Typical disease symptoms, referred to as anthracnoses, consist of sunken necrotic tissues with the production of orange conidial masses. Although these diseases can affect developing and mature plant tissues, the most significant economic loss occurs when the fruiting stage is attacked. This can occur either pre-harvest, on developing fruit in the field, or post-harvest, on stored mature fruit. The ability to cause latent or quiescent infections has made *Colletotrichum* a major post-harvest pathogen that is recognized worldwide (Freeman et al., 1998). Although other symptoms such as shoot blight and leaf spot have been reported on blueberry (Yoshida and Tsukiboshi, 2002), damage to fruit caused by fungal growth and sporulation is of greater concern to growers.

1.2.3 Sporulation

C. acutatum produces straight, cylindrical, fusiform conidia (8.5-16.5 × 2.5-4 μm) with attenuated or pointed ends (Figure 1.1) in rose, salmon-pink or orange masses produced in acervuli. Conidiophores are hyaline, septate, smooth, and infrequently branched. The conidiogenous cells are phialidic, hyaline, smooth, and cylindrical. The spore mass is produced in a sticky, slimy matrix which facilitates dispersal by raindrop splashes and wind-driven rain. Spore-trapping studies in blueberry fields in Michigan (Wharton et al., 2002), Washington (Bristow and Windom, 2000), and in BC (MacDonald et al., 2001) have shown major peaks of sporulation occurring in the field; one earlier in the season just before and during bloom, another during green fruit stage as reported by Hartung et al. (1981), and a final peak later in the season at fruit ripening.

1.2.4 Industry economic losses

Processed fruit with even a moderate level of anthracnose infection results in down-graded value at the packing house, at great cost to the grower. In 2000, many BC growers had a large portion of their fruit down-graded from grade A to puree or juice stock (MacDonald, 2001). This meant a 25% or 55% reduction in price, respectively. Some individual growers lost over $200,000 because a large percentage of their harvested fruit was in the lower grades. A report by the BC Ministry of Agriculture, Food and Fisheries in 2002 estimated industry-wide losses due to blueberry anthracnose to be as follows: $2 million due to yield losses, $1.5 million due to returned fruit claims, and $2.5 million due to down-graded fruit, for a total loss of $6 million that year. Gianessi and Reigner (2004) estimated in 2002 that 30% of US acreage was infected by anthracnose which can cause severe loss in wet seasons.

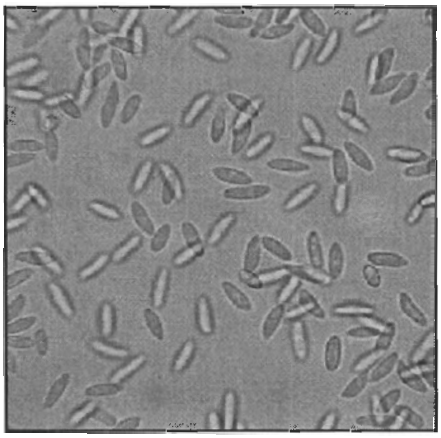

Figure 1.1. **Spores of *Colletotrichum acutatum* from an isolate collected from a commercial blueberry field in BC in 2001 (MacDonald et al., 2001) at 400x magnification.**

1.2.5 Current control options in BC

Currently, growers rely on a combination of cultural and chemical control methods for managing anthracnose (Anonymous, 2005b). Pruning the canopy increases air circulation, which promotes a drier environment after irrigation. The use of overhead irrigation is recommended only early in the morning so the plants can dry off during the day. Registered chemical controls are currently applied on a regular spray-schedule and include Bravo (active ingredient: chlorothalonil) applications at green tip, pink bud, and petal fall plant-growth stages to provide protection against early infection. Because no other fungicides were registered in Canada for the control of anthracnose in 2001, an emergency registration of Quadris (active ingredient: azoxystrobin) was obtained. In subsequent years, a similar strobilurin fungicide, Cabrio (active ingredient: pyraclostrobin) has been registered and successfully used. Both are locally systemic fungicides and can be applied 3-4 times per season. Captan (active ingredient: N-(trichloromethylthio)cyclohex-4-ene-1,2-dicarboximide) is also used as a protectant fungicide for continued foliar protection through the growing season. Growers are encouraged to move harvested berries off the field quickly, and store them at low temperatures, near 0°C if possible. Studies have found that storing berries at temperatures greater then this promotes decay rapidly (Ballinger et al., 1978; Ceponis and Cappellini, 1983; Eck, 1988). Since spores can be moved between fields on shared machinery and storage-bins, minimizing this movement can aid in preventing spread of this pathogen. Appropriate sanitation measures such as cleaning pruners, harvest machinery and storage bins can also prevent pathogen spread.

1.3 Epidemiology

Epidemiology refers to the study of disease development, spread, and factors affecting these. Plant pathologists rely on the disease triangle, consisting of the host, pathogen and environment, to describe the various factors that affect disease development (Agrios, 1997), although time is often considered a fourth factor, leading to the concept of a disease tetrahedron (pyramid). Therefore, to effectively manage blueberry anthracnose in BC, it is essential to identify its causal organisms, and then investigate host and environmental factors that increase infection probability. An integrated approach utilizing a combination of control tactics can then be used to reduce inoculum levels in the field and thereby mitigate losses due to disease.

1.3.1 Pathogen identification

To allow selection of an appropriate management strategy or tactic, essential information about the causal organism must be known, and therefore, rapid detection and identification are essential pre-requisites to any good disease management program (Schaad and Frederick, 2002). Traditional methods for distinguishing *Colletotrichum* species have relied on morphological differences such as colony characteristics, size and shape of conidia, optimal growth temperature, growth rate, presence or absence of setae, and existence of the teleomorph *Glomerella* (Gunnell and Gubler, 1992; Smith and Black, 1992; Sutton, 1992; von Arx, 1957). However, these methods may not be completely reliable for this genus, due to considerable plasticity in morphological traits, for example in response to environmental influences (Freeman et al., 1998). Nirenberg et al. (2002) found that conidia shape and size varied in response to the culture conditions under which several *Colletotrichum* species were grown. Differentiation based on host

range or host of origin is also unreliable since certain taxa, including *C. gloeosporioides, C. dematium, C. acutatum, C. graminicola* and others, infect a broad range of host plants. Other diagnostic techniques have included the use of vegetative compatibility groups (Freeman and Katan, 1997; Freeman et al., 2000; Guerber et al., 2003;) and benomyl sensitivity (Kuramae-Izioka et al., 1997; Brown et al., 1996; Bernstein et al., 1995); specifically, *C. gloeosporioides* is generally highly sensitive to benomyl, while *C. acutatum* is relatively insensitive.

Hartung et al. (1981) identified the causal organism of blueberry anthracnose as *C. gloeosporioides* and studied its epidemiology in Michigan highbush blueberry fields. In North Carolina, Daykin and Milholland (1984) described the infection process of *C. gloeosporioides* on blueberry as including a latent phase of subcuticular hyphae. Appresoria produced penetration hyphae which infected the berry and then remained quiescent until a later stage. Infection could occur during any phase of fruit formation, even early green berry. When fruit ripened sufficiently, the fungus would resume growth, invading the rest of the berry, resulting in the fruit-rot symptom. Polashock et al. (2005) identified a similar infection strategy for *C. acutatum,* implying that the causal organism might have been misidentified in earlier studies. In studying anthracnose of lupins, Talhinhas et al. (2002) describe a similar case where the pathogenic organism was originally described as *C. gloesporioides* in the past, but is now described as *C. acutatum*, based on species-specific molecular identification techniques. The latent period after infection, occuring either as subcuticular hyphae or germinated appresoria (Prusky, 1996), makes this disease especially problematic, since latently infected fruit do not show

visible disease symptoms until harvest, after which it is too late to implement control measures, except for post-harvest low-temperature storage.

1.3.2 Infection requirements

"An understanding of the processes that determine successful pathogenesis is a prerequisite for detailed biochemical and molecular work and is increasingly relevant to the practical sides of plant pathology" (Bailey et al., 1992). The infection process starts with conidial attachment and subsequent penetration of host tissues, and given an ample source of inoculum, environmental factors that are conducive to this process must be understood in an effort to discover a weakness in the pathogen life cycle that could be exploited for potential disease mitigation. Water availability and temperature are essential factors that play a role in all parts of the disease cycle, including sporulation and dispersal (Waller, 1992); however, their role in conidial germination and host infection will ultimately dictate whether pathogenesis will proceed at all. For these reasons, it is essential to determine the environmental conditions that promote anthracnose infections, provided host tissues are susceptible and inoculum is available.

Previous studies on *C. gloeosporioides* have indicated that a minimum of 12 hours of continuous leaf wetness, with concurrent temperatures at 15°C or higher, are required for successful infection of berries (Hartung et al., 1981). Infection is thought to occur at any stage during fruit development, from flower bud emergence to fruit ripening (Daykin and Milholland, 1984; Hartung et al., 1981). The infection process can be separated into several stages, including: 1) conidial deposition on the plant surface; 2) conidial attachment to the plant surface; 3) conidial germination; 4) appresoria production; 5) plant epidermis penetration; 6) growth and colonization of plant tissue;

and 7) production of acervuli and sporulation (Jefferies et al., 1990; Prusky et al., 2000). This infection process, and subsequent fungal development, relies on the two important microclimatic parameters, namely moisture and temperature (Duthie, 1997).

Lesions caused by *Colletotrichum* species, when examined by microscopy, reveal hyphae present throughout tissues where cells are dead and discoloured, with extensive degradation of the host cell walls (Bailey et al., 1992). Hyphae can be present inside cells (intracellular), within cell walls, and in intercellular spaces; this widespread growth and tissue destruction makes *Colletotrichum* an extremely effective pathogen.

1.3.3 Pathogen overwintering

The extent to which a pathogen can overwinter in the field has a profound effect on its ability to remain as a disease problem in succeeding years. *C. acutatum* is reported to overwinter within dead twigs, spent fruit trusses, and within newly-formed floral buds (Daykin and Milholland, 1984; DeMarsay and Oudemans, 2002; 2003; 2004; Ehlenfeldt and Stretch, 2003; Hartung et al., 1981). Inoculum, in the form of splash-dispersed spores, can be produced on these tissues in early spring during wet conditions (DeMarsay and Oudemans, 2004; Wharton and Diéguez-Uribeondo, 2004). Wharton and Diéguez-Uribeondo (2004) reported that as flower buds broke dormancy, the fungus grew out of the buds and colonized the surrounding stem tissue, causing necrotic black lesions up to 2 cm in size. Sporulation on these tissues occurred after about 7 days. Sticky *C. acutatum* conidia are produced in acervuli on infected tissue surfaces. They are water-borne and spread by raindrop splashes, so dispersal and inoculum spread is usually highest during wet periods. Mummified fruit on the ground may also be an inoculum source (Freeman et al., 2002) although its importance for overwintering of the pathogen is not known.

In the field, *C. acutatum* sporulates during periods of extended wetness, and these conidia are dispersed by raindrops splashing onto susceptible tissues (Caruso and Ramsdell, 1995). Secondary conidiation from newly infected tissues, such as ripe berries, promotes a rapid build-up of inoculum. Peak spore dispersal coincides with flowering and early fruit development stages with a second peak occurring at fruit maturity (Hartung et al., 1981; Wharton et al., 2002). This ensures successful infection of ripening fruit, as well as a carryover of inoculum to susceptible tissues for overwintering until the next season, such as vegetative and flowering buds, as well as twigs, trusses and stems (Figure 1.2). To what extent these various tissues are important for successful overwintering in BC fields is not currently known.

1.4 Latent infections and post-harvest disease

Latent infection has been defined as the state in which the host plant is infected by a pathogen, but does not show any symptoms (Agrios, 1997; Dennis, 1983). It is a dormant host-pathogen relationship that can change into an active one after some time (Verhoeff, 1974). Another term often used interchangeably is 'quiescent infection', which refers to the inhibition of the pathogen's development through physiological conditions imposed by the host, until some stage of maturation is reached (Swinburne, 1983). There is some debate about the distinction of these two terms, but generally, they are used to describe the existence of a dormant period between the discrete steps of the pathogen landing on the host, germinating, and continuing its pathogenic relationship with the host. This dormant period can be short (days) or long (months), and usually breaks around the same time as fruit ripening. That a pathogen can infect a host, and

Figure 1.2. **The spent fruit truss (T) where berries were held the previous year (arrows), and newly emerging flower bud on Highbush Blueberry 'Bluecrop'.**

'wait' until an opportune time to continue its infection process, is a fascinating component of host-pathogen co-evolution.

1.4.1 Physiological mechanisms

Verhoeff (1974) suggested 3 possible physiological mechanisms for the occurrence of latent infections leading to post-harvest disease, any of which could be involved simultaneously to varying degrees in a given host-pathogen relationship:

1. The presence of toxic compounds or fungal-enzyme inhibitors in unripe fruit may prevent further growth of the pathogen, once infection has occurred. As fruit ripens, these compounds lessen in concentration, allowing the pathogen to resume growth. A correlation has been found between fruit ripening and the reduction of tannins and other phenolic compounds in some crops (Verhoeff, 1974).
2. As fruit ripens, starches are converted into simple sugars such as glucose and fructose, which might reduce limitations on pathogenic growth depending on the pathogen's specific requirements.
3. Unripe fruit cell walls may resist the pathogen's pectolytic enzymes; however, upon ripening, cell wall compounds loosen allowing enzymatic degradation.

Once fruit has been harvested, two forms of deterioration are accelerated; natural senescence and microbial deterioration. These two processes are inextricably bound since, as harvested crops deteriorate through senescence, their resistance to microbial degradation generally decreases (Wilson and Wisniewski, 1994). Therefore, factors that accelerate senescence and favour microbial growth will promote post-harvest disease. For example, due to the increased reliance on mechanical harvest techniques, fruit is usually

relatively more bruised or wounded, thereby allowing a fungal pathogen easier access for successful infection (Ceponis and Cappellini, 1978; Ippolito and Nigro, 2000).

This degradation is not only a problem for growers, but one that persists through the entire distribution chain, thereby affecting the availability and price of the produce. It can also restrict the ability to service distant markets. In our increasingly global markets fuelled by consumers' desire to enjoy fruits all year round, this can be a major setback in terms of profitability. This is further exacerbated by the fact that latent infections are usually symptomless until fruit-ripening, which occurs close to harvest. By the time the grower realises the disease problem, it is usually too late and the fruit has already been substantially infected. Furthermore, under inadequate storage and transportation conditions, losses due to disease such as anthracnose can increase. It is important to note that these losses occur after a large investment of energy and money in harvesting, storing and transporting the crop has already been incurred.

1.4.2 Latent infections causing blueberry anthracnose

In the case of blueberry anthracnose, Daykin and Milholland (1984) were one of the first to examine the latent infection process. They found that the fungal spores are able to infect fruit at all stages of development. Once the spores have germinated, they produce appresoria and, a week later, penetration hyphae. After this step, no further fungal growth is observed, with no discernible reaction in the epidermal layer of the fruit either. Only when the fruit starts ripening does fungal growth resume, with infection hyphae enlarging, invading the rest of the fruit, and causing the familiar 'depressed' fruit-rot symptom. In this way, the fungus infects the fruit early on in the growing season, but remains undetected, until the fruit starts ripening, close to harvest. Once the fruit has been

harvested, the fungus can resume its growth, possibly due to reduced resistance in the detached berry. Even more serious is the fact that any spores produced at this stage can easily spread to other berries located close by in storage containers. This causes a large amount of post-harvest disease, since storage conditions are conducive to rapid dissemination of inoculum. Eck (1988) describes that a study of blueberry samples in New York (USA) stores revealed 10% of the fruit was defective due to post-harvest fungal decay. Ceponis and Cappellini (1983) suggested that extending blueberry shelf life to allow for transoceanic surface shipments to Europe would open up a large, profitable market for North American growers.

Colletotrichum causes latent infections in other fruits as well, including banana (Peres et al., 2002), papaya (Dickman and Alvarez, 1983), citrus (Ippolito and Nigro, 2000), and peach (Zaitlin et al., 2000). Other commercially important crops that are affected by latent diseases include pear (Sugar and Spotts, 1999), grape (Erincik et al., 2001), prune (Luo and Michailides, 2001), plum (Northover and Cerkauskas, 1998), tomato (Silveira and Michereff, 2000), cherry (Dugan, 1997), and mango (Prakash, 1996).

1.5 Biological control

Currently, the primary means of controlling many fungal post-harvest diseases is through the use of fungicides (Panneton et al., 2001; Ippolito & Nigro, 2000). However, due to growing concerns over potential toxicity, environmental pollution, resistance development and unavailability of registered fungicides, ecologically safer alternatives such as hot-water treatment, controlled storage atmospheres, and biological control are rapidly becoming an important part of integrated disease management programs.

Additionally, if growers wish to market their produce internationally, they have to abide by the destination country's import and regulatory laws concerning fungicide residues. This can restrict the choice of chemical control options available to growers.

In comparison to the option of using synthetically derived chemicals, biological control has the advantage of being safer for application in the environment, having less of an impact on non-target organisms and surrounding water bodies. Typically, biological control can provide self-sustaining, broad scale control of the target pest, requiring fewer applications than comparable chemical controls. The general modes of action for biological control agents include (Baker and Cook, 1982):

1. Antibiosis. The antagonist produces a toxin against the pathogen.
2. Competition. The antagonist occupies the same ecological niche as the pathogen, maybe competing for the same nutrients or space.
3. Direct interaction. There is some sort of parasitic relationship where the antagonist directly attacks the pathogen, through toxins or hydrolytic enzymes.
4. Resistance induction. The antagonist elicits a resistance mechanism in the host, thereby reducing or preventing pathogen infection.

Other techniques being considered for management of post-harvest diseases in fruit crops include the use of controlled storage-atmospheres, hot water, organic acid, and calcium treatment as well as wax-coating to prevent spoilage.

1.5.1 Post-harvest diseases

Since storage conditions can be manipulated relatively easily and remain constant, and the target crop is concentrated and exposed, the application of biological agents in

storage is more precise and economical than in the field (Wilson and Wisniewski, 1994). Thus, there is greater confidence in correct application and retention of the biological control agent. Sugar and Spotts (1999) studied control of post-harvest blue-mould decay in pear fruit, caused by *Penicillium expansum*, destined for long-term storage, using four unidentified laboratory-grown yeasts and two registered biological control products; Aspire (active agent *Candida oleophila*) and Bio-Save 110 (active agent *Pseudomonas syringae* strain ESC-11). They concluded that their laboratory-grown yeasts demonstrated excellent potential as biological control agents, offering even greater protection than the two registered biological control products. They also tried combining the use of Aspire with 100 µg/ml of the fungicide thiabendazole, which resulted in the same amount of protection as when the fungicide was used alone at its maximum label rate of 569 µg/ml. This represents a vast reduction in fungicide used, while achieving the same level of disease reduction. Ippolito and Nigro (2000) have listed many other successful examples of biological control used to reduce post-harvest diseases in various crops, including grape, strawberry, apple, cherry and pear (Table 1.1). There are no reports for blueberry.

In the case of blueberry anthracnose, the spent fruit truss also potentially provides an opportunity for biological control of the succeeding year's inoculum levels. After harvest, the wood dies down to the topmost bud, usually a fruit bud, because there are no growing points left on the spent fruit truss. This provides an environment for both saprophytic and pathogenic fungi to colonize. Since *Colletotrichum* establishes its

Table 1.1. Examples of recent successful biological control of post-harvest diseases by pre-harvest application (see Ippolito and Nigro [2000] for referenced literature).

Crop	Disease	Antagonist
Grape	Botrytis rot	*Pichia guillermondii*
Avocado	Anthracnose, Stem-end rot, Dothiorella-Colletotrichum fruit rot	*Bacillus subtilis*
Strawberry	Botrytis, Rhizopus fruit rot	*Aureobasidium pullalans* *Candida oleophila*
Apple	Blue mould	*Aureobasidium pullalans*
	Botrytis rot	*Rhodotorula glutinis*
	Bull's-eye rot	*Bacillus subtilis*
Sweet cherry	Botrytis rot, Brown rot	*Aureobasidium pullalans*
Strawberry	Botrytis rot	*Aureobasidium pullalans*
Table grape	Botrytis rot	*Aureobasidium pullalans*
Wine and table grape	Botrytis, Rhizopus, and Aspergillus rot	Yeasts and yeast-like fungi
Apple	Blue mould	*Candida sake*
Apple	Blue mould	*Aureobasidium pullalans*
	Botrytis rot	*Cryptococcus laurentii*, *Rhodotorula glutinis*
Table grape	Botrytis rot	*Aureobasidium pullalans*
Pear	Botrytis rot, Blue mould, Side rot	*Cryptococcus infirmominiatus*, *Cryptococcus laurentii*, *Rhodotorula glutinis*

primary inoculum during bloom and early fruit set (Wharton et al., 2002), the potential to introduce a biological control agent that competes for infection sites and/or nutrients on the spent fruit truss exists (Figure 1.2).

1.5.2 Pre-harvest management

Pre-harvest management can have a great effect on the incidence of post-harvest disease caused by latent infections, since by definition, latent infections are caused by pathogens infecting the plant at a stage prior to harvest, and only becoming a problem after the crop has been harvested for storage or transport. Therefore, any management steps that can reduce or displace this initial infection will result in a reduction of post-harvest disease. Ippolito and Nigro (2000) state that field application of biological control agents enables early colonization of fruit surfaces, thereby protecting the fruit from later infection by the pathogen. This is especially true in systems where the competition mechanism of biological control is operational. They cite an example of 2 biological control agents, *Aureobasidium pullulans* and *Candida oleophila,* used against *Botrytis cinerea* storage-rot on strawberries. They found the antagonists were more effective when applied in the field prior to harvest, compared to immediately after. Pre-harvest application allows the antagonists to colonise susceptible tissue, such as senescing floral parts and fresh wounds, before the arrival of the pathogen, thereby providing better control against the possibility of latent infection.

1.5.3 Related research

Biological control agents that have been tested for efficacy against *C. acutatum* include *Bacillus subtilis* and *Candida oleophila* (Wharton and Diéguez-Uribeondo,

2004). *Bacillus subtilis*, formulated as Serenade, has also demonstrated promising control of *Monilinia vaccinii-corymbosi*, the causal pathogen of mummy-berry in blueberry (Scherm, 2004). Freeman et al. (2004) also demonstrated significant reduction of anthracnose and gray mould in strawberry, caused by *C. acutatum* and *Botrytis cinerea,* respectively, using various *Trichoderma* isolates. Previous research has shown that *G. catenulatum* is an effective biological control of root pathogens such as *Fusarium oxysporum* and *Pythium aphanidermatum* on cucumber (Punja and Utkhede, 2003; Punja and Yip, 2003; Rose et al., 2003). Competition for infection sites was proposed as one possible mechanism of action (Punja and Utkhede, 2003). A similar biological agent, *Gliocladium roseum* (*Clonostachys rosea*) has been studied for its ability to reduce *B. cinerea* infection and disease severity in a variety of crops, including strawberries, raspberries, and various greenhouse flowers and vegetables (Sutton et al., 1997). Biological control agents could be used in combination with chemical controls to further reduce severity of blueberry anthracnose in the field, as has been demonstrated in integrated control of *Verticillium dahliae* infection of potato using the biological control agent *Trichoderma harzianum* and the fungicide captan (Ordentlich et al., 1990). Similarly, Larena et al. (2005) reported a three-fold reduction in the amount of fungicide necessary in controlling post-harvest brown rot, caused by *Monilinia* spp., in peach orchards, when combined with applications of a biological control agent, *Epicoccum nigrum*. Currently, control of blueberry anthracnose in BC requires scheduled and repeated use of chemical fungicides with a recommendation of a post-harvest low-temperature storage period (Anonymous, 2005b). Cultural practices such as pruning

improve air circulation within the canopy and reduce humidity, but do not substantially reduce infection and overwintering of inoculum (Bristow and Windom, 2000).

1.6 Objectives of research

Little is known about the epidemiology of blueberry anthracnose in BC, and despite its prevalence and damage, there have been no published reports regarding the species involved or specific infection requirements. This information is critical in developing disease management approaches in BC. Therefore, the objectives of this research study were to:

1. Identify the causal agent(s) of anthracnose using fungal growth-rate comparisons and PCR amplification with species-specific primers.
2. Survey commercial fields in BC for distribution and severity of anthracnose.
3. Determine periods of host susceptibility and effects of environmental factors, specifically temperature and leaf-wetness, on infection.
4. Investigate the pathogen's overwintering capability.
5. Investigate potential biological controls as part of a disease management strategy.

1.6.1 Potential biological control products tested

Products with the potential for registration were chosen for this study; *Gliocladium catenulatum*, formulated as Prestop (Verdera Oy, Finland), and *Trichoderma harzianum*, formulated as PlantShield (BioWorks, Inc., Fairport, NY, USA), A similar product named RootShield (active ingredient *Trichoderma harzianum* Rifai strain KRL-AG2) is currently registered in Canada for application on greenhouse crops for protection against root diseases caused by *Pythium*, *Rhizoctonia* and *Fusarium*.

CHAPTER 2. MATERIALS AND METHODS

2.1 Fungal isolations

Isolations were initiated in spring 2002, from a commercial blueberry field (16 ha size) located in Abbotsford, BC, with a prior history of anthracnose incidence. Tissue samples (50-100) comprised of overwintering fruit trusses, leaf buds, blossoms, green and ripe berries (Figure 3.3 a-d) were collected each week during the growing season (May-August) from arbitrarily selected plants along a few selected rows of the field. Tissues were incubated in plastic containers lined with moistened paper towels, or placed onto 1.5% water agar (Difco Laboratories, Detroit, MI, USA) in 100 x 15 mm Petri dishes, and incubated at room temperature (21-23°C) for 2-3 weeks. Any visible fungal growth was transferred to potato dextrose agar (PDA, Difco Laboratories, Detroit, MI, USA) and colonies were initially identified to genus level using morphological criteria (Vinnere et al., 2002). Sampling was repeated in the same field in 2003.

2.2 Anthracnose survey

A previous survey for anthracnose in the Fraser Valley of BC, conducted in 1998 by the BC Ministry of Agriculture, Food and Fisheries (MacDonald, 1998), showed that anthracnose was present in most blueberry-growing regions. To continue this previous survey, and to characterize the fungi causing anthracnose in BC, seven commercial fields located in the Fraser Valley of BC, as well as berries from a prominent packing and processing plant in Abbotsford, BC, were sampled during 2003-2004.

In August 2003, seven commercial blueberry fields located in the Fraser Valley of BC were sampled by arbitrarily collecting approximately 1000 ripe berries from plants in each field. Rows in each field were chosen using a random number generator, and berries from randomly chosen plants along each row were hand-picked and temporarily stored in plastic bags. These were then dispensed into clean commercial-size cardboard egg trays (one berry per compartment), moistened with sterile distilled water using a spray-bottle, and covered with black polyethylene bags for 2 weeks. The number of berries infected with *Colletotrichum*, as determined by visible orange spore masses (Figure 3.3 e-g), was assessed for each sample after 2 weeks. For each field sampled, spray records and information regarding cultural practices was requested from the growers in an attempt to correlate management practices with disease incidence. It was hoped that this information would prove valuable in determining potential grower practices that might mitigate the severity of the disease. This survey was repeated in 2004 in the same fields.

All fungal isolates collected through the repeated-field sampling and field survey were identified in subsequent experiments (growth rate and PCR). For the comparative studies described below, a number of standard reference isolates of *C. acutatum* and *C. gloeosporioides* were obtained from a range of different hosts and geographic locations (Table 2.1). All isolates were maintained on PDA plates over the duration of the studies, as well as stored on PDA slants at 4°C for the long-term.

2.3 Identification of *Colletotrichum*

All field isolates collected during 2002-2004 (total of 80) were identified to the genus *Colletotrichum* by morphological comparisons to reference cultures (Table 2.1), which included comparison of colony morphology, colony growth rate, colony colour,

Table 2.1. **Sources of reference isolates of *Colletotrichum acutatum* and *C. gloeosporioides* used in this study.**

Code	Host of origin	Geographic origin	Source
C. acutatum			
IS 01	Strawberry	Israel	S. Freeman
IS 02	Anemone	Israel	S. Freeman
MI 01	Cranberry	Michigan, USA	A. Schilder
MI 02	Cranberry	Michigan, USA	A. Schilder
CR 01	Cranberry	BC, Canada	A. Lévesque
CR 02	Cranberry	BC, Canada	A. Lévesque
FL 01	Strawberry	Florida, USA	J. Mertely
FL 02	Strawberry	Florida, USA	J. Mertely
NS 02	Blueberry	Nova Scotia, Canada	P. Hildebrand
NJ 01	Blueberry	New Jersey, USA	A. DeMarsay
NJ 02	Blueberry	New Jersey, USA	A. DeMarsay
C. gloeosporioides			
SL 01	Salal	BC, Canada	J. Elmhirst
IS 03	Strawberry	Israel	S. Freeman
IS 04	Avocado	Israel	S. Freeman
NS 01	Mallow	Nova Scotia, Canada	P. Hildebrand
FL 03	Citrus	Florida, USA	J. Mertely
FL 04	Strawberry	Florida, USA	J. Mertely

sporodochia production, and spore morphology. For growth rate comparisons, isolates were grown on PDA at 28°C for 2 weeks in three replicates. Colony diameter of all isolates was compared to those of confirmed control isolates of *C. gloeosporioides* (n=6) and *C. acutatum* (n=8). The experiment was repeated three times and data analyzed by ANOVA followed by a Tukey HSD multiple comparisons test in Jmp In 4 (SAS Software Inc., Cary, NC, USA).

For molecular identification, the previously published species-specific primer sequences CgInt (GGC CTC CCG CCT CCG GGC GG) and CaInt2 (GGG GAA GCC TCT CGC GG) along with the conserved ITS4 sequence (TCC TCC GCT TAT TGA TAT GC) based on ribosomal DNA ITS regions (Freeman et al., 1998; Peres et al., 2002) were used. To extract DNA, fungal colonies were grown on PDA for 1 week at room temperature, and approximately 40 mg of tissue (mycelia and spores) was scraped from the plates and used in the DNeasy Plant Kit (Qiagen Inc., Valencia, CA, USA) following manufacturer's instructions. Extracted DNA was then subjected to PCR amplification reactions using the two paired sets of primers; CgInt+ITS4 to identify *C. gloeosporioides*, or CaInt2+ITS4 to identify *C. acutatum*. Isolates were run twice in each reaction, once with each primer pair to ensure an identifying amplicon was only produced with one of the primer pairs. Control reference isolates (Table 2.1) were included in each reaction as positive controls, while PCR reactions with sterile distilled water instead of template DNA served as negative controls. Amplifications were carried out using a REDTaq ReadyMix PCR Reaction Mix (Sigma-Aldrich, Oakville, ON, Canada) with 4 µl extracted DNA (10-50 ng), 10 µl of the ReadyMix, 5.2 µl water, and 0.4 µl of each primer. Temperature cycling parameters consisted of a denaturing step at 94°C for 5 min,

followed by 25 cycles at 94°C for 45 sec, 59°C for 30 sec, and 72°C for 1 min. The amplified products were separated by electrophoresis through a 2% agarose gel containing 1x Tris-acetate-EDTA (TAE) buffer. Gels were photographed on a UV transilluminator after ethidium bromide staining. Isolates were assigned to the species for which a positive amplification with that species' primer-pair was obtained. PCR reactions were repeated twice for all field collected isolates (total of 80) to identify them to species level.

2.4 Infection of blueberry and apple fruit

To investigate the pathogenicity of some of the *Colletotrichum* isolates, ripe blueberry and apple fruit 'Golden Delicious' purchased from a retail store were inoculated with several isolates of *C. acutatum* (BC08, CR02, MI01) and *C. gloeosporioides* (IS04) under laboratory conditions. For blueberry trials, replicated sets of Petri dishes containing 12 berries each were sprayed with a spore suspension of 10^5 spores/ml using a spore haemocytometer (Fisher Scientific, Pittsburgh, PA, USA) obtained from a 7-day old fungal colony on PDA, and incubated at different temperatures (5, 7, 12, 15, 20, 25, and 30°C) for 2 weeks. Control dishes of berries were sprayed with sterile distilled water. At the end of the incubation period, berries were visually assessed for sporulation and rated as healthy or diseased. This experiment was repeated with both unripe green fruit as well as ripe blueberries, as each became available during the growing season. The same isolates were used to inoculate apples using 8-mm diameter mycelial plugs taken from a 5-day old fungal colony on PDA. Plugs were placed into holes cut with a cork-hole borer on two sides of 4 replicated fruit. Control apples were inoculated with sterile PDA plugs in a similar fashion. The apples were then placed on

moistened paper towels inside plastic containers and incubated at three different temperatures (10, 15, and 20°C). Fungal growth from the inoculation site was assessed after 1 week by measuring the diameter of the lesion developing around the inoculation site. Both trials were repeated three times and data analyzed using an ANOVA comparison of means in Jmp In 4 (SAS Software Inc., Cary, NC, USA).

2.5 Establishing period of susceptibility of blueberry plants

2.5.1 Trap plants and natural inoculum

During the growing seasons of 2001, 2002 and 2003, trap plants were placed in a commercial blueberry field located in Abbotsford, BC to determine the resulting level of fruit infection, and hence disease incidence on plants at harvest. This field had a prior history of fruit infection at harvest of 20-30% (MacDonald et al., 2001). Trap plants consisted of 2-year old 'Bluecrop' plants, which were placed within a single row, under the canopy of 15-year old established blueberry plants (about one trap-plant every 3 m), for a period of 1 week, for exposure to the prevailing environmental conditions and field inoculum. A total of 8 trap plants were set out each week over a period of 14 weeks starting at early blossom period in each year (dates varied according to particular growing season). Throughout the growing season, the temperature and leaf wetness were continuously recorded every hour using a Field Monitor data logger (Sensor Instruments Inc., Concord, NH, USA). Plant growth phases (early blossom, full blossom, early green berry, mature green berry and ripe blueberry) were visually counted each week and expressed as percentages. After the 1-week exposure, trap plants were transferred to a confined growing area that was isolated from any adjacent blueberry fields and provided with irrigation as needed. A control set of 8 trap plants was left in this area without any

field-exposure. Plants were grown until fruit had matured and all the berries were then harvested from each week's set of 8 trap plants. The fruit were bulked to produce a composite sample, each arising from a 1-week period of field exposure. The fruit were then incubated under moist conditions for 2 weeks in cardboard egg trays, as described previously, and assessed for percent infection. The data from each of the 3 years was analyzed separately using a correlative analysis in Jmp In 4 (SAS Software Inc., Cary, NC, USA).

2.5.2 Artificial inoculation of plants

An isolate identified as *C. acutatum*, collected in 2002, was used in this trial. The fungus was grown on PDA for 1 week to provide a source of spore-suspension inoculum. A total of 136, 2-year old blueberry plants 'Bluecrop' grown in 4-litre containers, were purchased from a commercial grower in Abbotsford, BC. At a field location in Langley, BC, isolated from any adjacent blueberry fields, 17 groups of 8 plants each were randomly placed onto plastic mulch, with approximately 3 m distance between groups. At weekly intervals, beginning on April 19, 2004, a group of plants was inoculated to run-off with a spore suspension of *C. acutatum* (10^5 spores/ml) generally between 08:00 am to 11:00 am. The plants were placed inside a misted humid chamber for 18 hours (average relative humidity of 95%) and then returned to the field. This ensured adequate moisture available for potential infection, as well as prevented spray-drift inoculation of nearby plants. A control set of plants was sprayed with water instead of spore-suspension inoculum. Growth stage of the plants was recorded each week. The environmental conditions (temperature and leaf wetness duration) adjacent to the plants were recorded using a Field Monitor data logger (Sensor Instruments Co., Inc., Concord, NH, USA).

The plants were irrigated with overhead sprinklers for 6 hr/day. The fruit were harvested over a 3-week period (beginning in mid-July) and all fruit from the group of 8 plants were bulked to form a composite sample. The experiment was terminated on August 1, 2004, when all plants had mature fruit. The degree of fruit infection was assessed after incubation under moist conditions as described previously and data compared via ANOVA in Jmp In 4 (SAS Software Inc., Cary, NC, USA). The plants were maintained in the field for subsequent studies on pathogen overwintering.

2.6 Pathogen overwintering and perennial persistence

In late October 2003 and 2004, after the blueberry field plants had entered dormancy, three replicated plastic mesh bags containing inoculated diseased blueberries were placed in a commercial blueberry field in Abbotsford, BC. Bags were constructed using 1 mm plastic mesh to approximately 10 x 20 cm in size. Berries were inoculated with a spore suspension of *C. acutatum* at a concentration of 10^5 spores/ml. The bags were filled with approximately 100 berries, tied with fishing line and placed on the soil surface within three randomly chosen rows. The bags were retrieved in late February 2004 and 2005. The decomposed berries were placed on moistened paper towels inside plastic containers and incubated at room temperature. Resulting fungal growth was transferred to PDA and examined for presence of *Colletotrichum*.

To determine the extent of pathogen persistence on plant tissues, samples of approximately 30 of each of spent fruit trusses and twigs, vegetative buds, and floral buds were collected in December 2004 and then again in February 2005 from the groups of artificially inoculated plants previously used to study periods of infection susceptibility. Tissue samples were surface-sterilized for 30 sec in 70% EtOH, followed by 1 min in 1%

NaOCl, and finally rinsed twice with sterile distilled water. Samples were then placed on 1% water agar plates, and any resulting fungal growth after 6 days of incubation at 21-23°C was transferred to PDA and examined for the presence of *Colletotrichum*. Tissue samples containing *Colletotrichum* were counted and converted into percent of total sampled for each tissue type.

2.7 Biological control experiments

In 2002, a replicated experiment involving potted blueberry plants artificially inoculated with spore-suspensions of *C. acutatum* was conducted to determine if the two commercial biological control agents, *Gliocladium catenulatum* formulated as Prestop and *Trichoderma harzianum* formulated as PlantShield, could reduce disease severity. Groups of 10 plants each were sprayed first with an aqueous suspension of biological control agents using the recommended rate of 1% w/v, and then 48 hr later, spray-inoculated with a spore suspension of *C. acutatum* (10^5 ml). Negative control plants were sprayed with water alone, while positive control plants were sprayed with inoculum alone. These sprays were applied to drench, using approximately 500 ml per group of 10 plants. Three applications of the biological control agent and spore-suspension inoculum were made over the growing season to coincide with plant growth phases of 50% bloom, 50% green berry, and 100% ripe berry (pre-harvest).

This experiment was repeated in 2003 and 2004 using field plants in two commercial fields in Abbotsford, BC with a prior incidence of anthracnose. A research permit to allow field-application of the two biological control products was obtained from Health Canada (Ottawa, Canada). Biological control agents were sprayed onto groups of 4 plants, replicated in 5 blocks along one field row, with the treatments randomized

within each block. Control plants were sprayed with water and natural field disease provided the inoculum source. Approximately 4-5 buffer plants were left between each treatment within a block, and also between blocks. The experimental plot was not included in the grower's regular chemical spray program for disease management. Biological control agents were applied at 50% blossom, early green berry, and ripe blueberry (pre-harvest) growth stages. In both trials, approximately 500 fruit were harvested from each plant at the end of the season, incubated under moist conditions for 2 weeks in cardboard egg trays as described previously, and assessed for percent berries infected. The data were analysed using an ANOVA comparison of means in Jmp In 4 (SAS Software Inc., Cary, NC, USA) to determine if there was a significant reduction in disease incidence due to the biological control agents.

CHAPTER 3. RESULTS AND DISCUSSION

3.1 Fungal isolations

Tissue samples (buds, blossoms, green and ripe berries) collected from a commercial field during May-August in 2002 and 2003 yielded *Colletotrichum* early in the season (May) in both years, coinciding with blossoming, and in July-August, coinciding with mature green-ripe berry stages (Figure 3.1). A number of other fungi, including *Trichoderma, Alternaria, Penicillium, Botrytis,* and *Fusarium* were also recovered at varying levels through the season.

3.2 Anthracnose survey

From the fields surveyed in 2003 and 2004, there was a range of fruit infection at harvest (Figure 3.2), indicating the presence of *Colletotrichum* in four of the seven fields sampled. Disease severity was greater in 2003 compared to 2004 in most fields, likely because of more rainfall during the 2003 growing-season. These results indicated that anthracnose was present in all growing regions in BC and varied in severity among different fields, most likely due to prior disease occurrence in a particular field, leading to overwintering inoculum persistence.

Relevant details and characteristic management practices recorded as part of the survey are shown in Table 3.1 for each of these fields. Most of the management practices do not correlate well with disease levels, except for disease history. Since this disease depends on splash-dispersal and near-contact for successful spread of inoculum, it is

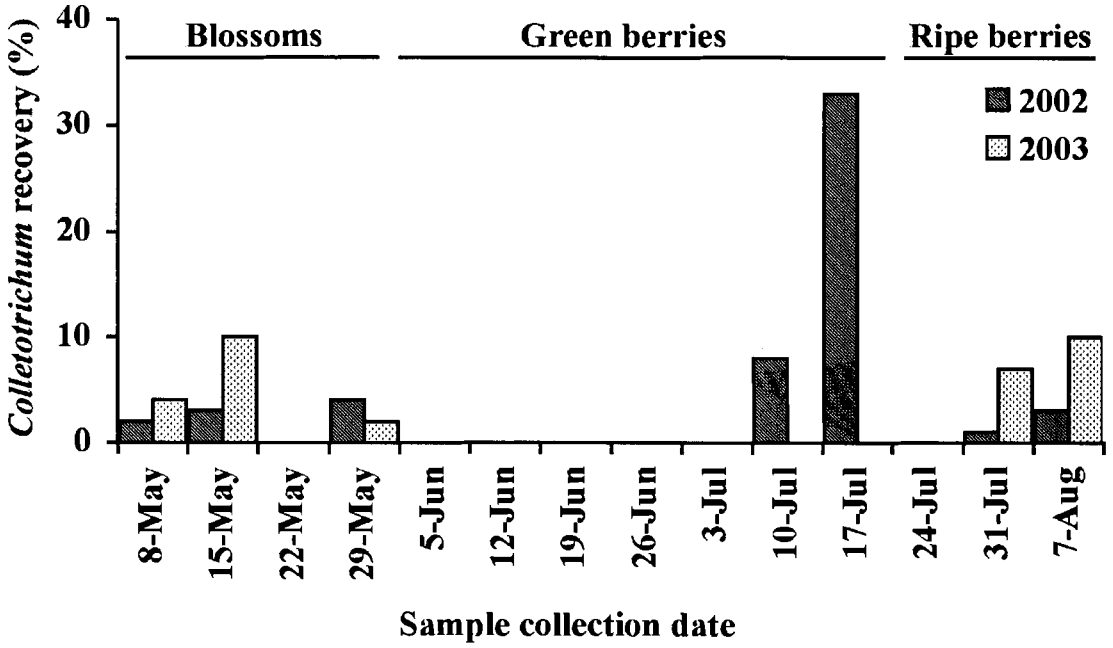

Figure 3.1. **Recovery of *Colletotrichum* from blueberry tissues collected at different plant growth stages during 2002 and 2003. Samples were collected from a commercial field, placed under moist conditions and assessed for presence of the pathogen.**

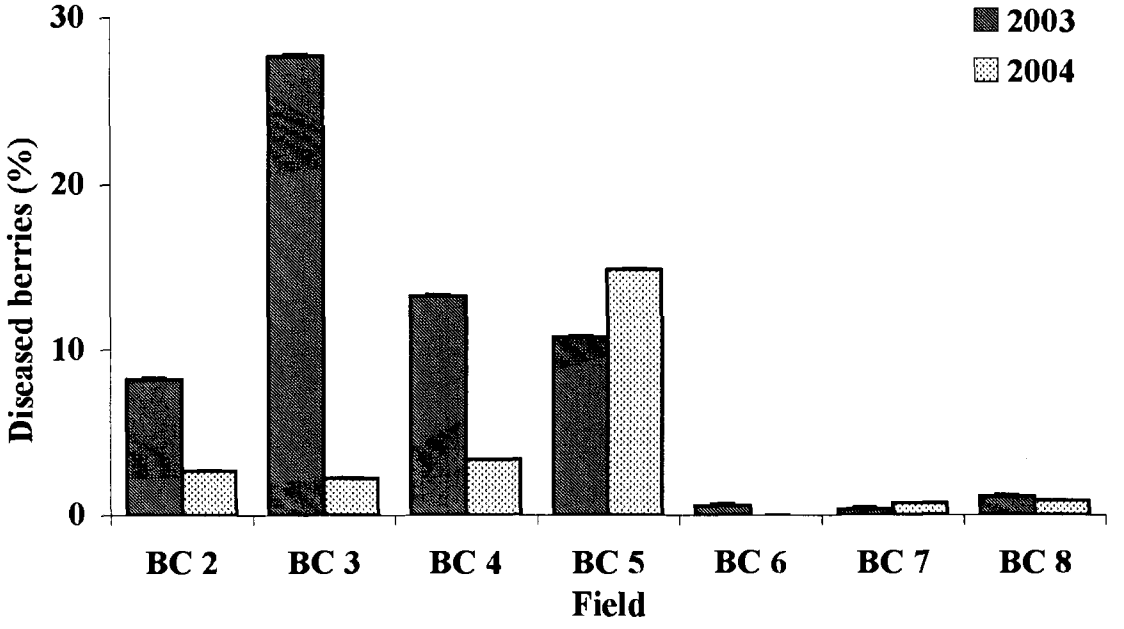

Figure 3.2. **Frequency of recovery of *Colletotrichum* from harvested fruit in seven commercial fields sampled in 2003 and 2004. Fruit were incubated under moist conditions for 2 weeks and assessed for pathogen presence.**

Table 3.1. Responses collected as part of the 2003 anthracnose survey.

	BC 2	BC 3	BC 4	BC 5	BC 6	BC 7	BC 8
Location	Matsqui	Matsqui	Matsqui	Abbotsford	Abbotsford	Cloverdale	Cloverdale
Fruit quality	Excellent	Excellent	Excellent	Good	Excellent	Good	Good
Harvest method	Machine	Machine	Hand	Both	Hand	Hand	Hand
# Cabrio	2	2	1	2	2	–	2
Age of plants	24 yrs	14 yrs	13 yrs	25 yrs	14 yrs	18 yrs	14 yrs
Disease history	No	Yes	No	Yes	No	No	No
Pruning	Yes	Vigorous	Yes	Vigorous	Vigorous	Yes	Yes
Row spacing	Standard	Standard	Standard	Narrow	Standard	Standard	Standard
Sawdust	None	None	Yes	Yes	Yes	None	None
Irrigation	Drip	Overhead	None	Overhead	None	None	Drip

logical to conclude that a clean field would remain clean, provided no other source of inoculum is brought into the field, such as shared equipment or sprayer tank-mixture water. This requirement of a wet environment for disease spread implies that grower practices that promote a drier plant canopy should potentially lower disease levels in the field. Therefore, maintaining well-pruned bushes, with larger inter-row spacing should be considered. Fields that used overhead irrigation (BC 3 and 5) had relatively higher levels of disease (more than 10%). This suggests that drip-irrigation would be better than overhead-irrigation in reducing leaf-wetness duration and subsequent infection, as well as splash-dispersal of inoculum. If overhead irrigation must be used, due to cost or other practical restrictions, irrigating earlier in the morning would allow water on the leaves to evaporate before temperatures reach the range at which anthracnose can infect.

Even though all growers reported excellent fruit quality at their respective packing-houses, the incubation period experiments show that the fungus was present on the berries, and could easily damage otherwise healthy-looking fruit given appropriate environmental conditions. Due to the hot, dry summers in BC in 2003 and 2004, field infection and fruit damage was relatively low in most fields. This may have further implications in terms of lowering inoculum levels in the field for subsequent seasons. Regardless, growers should be aware that disease levels in their field could potentially be higher, under cooler and wetter summers.

3.3 Identification of *Colletotrichum*

All 80 *Colletotrichum* isolates collected in this study, as well as the standard reference isolates, were examined for colony morphology, colony growth rate at 28°C,

colony colour, sporodochia production, and spore morphology on PDA plates. In general, *C. acutatum* colonies appeared bright-pink to orange in colour when viewed from the underside, while *C. gloeosporioides* produced gray to brown colonies (Figure 3.3 h). The spores of both species were oblong in shape, aseptate, hyaline, and generally pointed at one or both ends (Figure 1.1). There was considerable overlap in spore morphology between these two species, making it difficult to distinguish them based on spore shape and size alone. The growth rate characteristics of these two species indicated that *C. gloeosporioides* was consistently faster growing than *C. acutatum* (Figure 3.4) at 28°C ($p<0.0001$). All isolates from BC were tentatively identified as *C. acutatum* based on these results. PCR analysis using species-specific primers (Figure 3.5) confirmed their identity. A band at 480 bp was obtained for *C. acutatum* and one at 450 bp for *C. gloeosporioides*, as expected.

These results demonstrate that blueberry anthracnose in BC is caused primarily by *C. acutatum*, similar to other major highbush blueberry producing regions of the world (Wharton and Diéguez-Uribeondo, 2004). Field surveys conducted over two years indicated that only *C. acutatum* was present. Although *C. gloeosporioides* was previously reported to be the causal agent of blueberry anthracnose (Cappellini et al., 1972; Daykin and Milholland 1984; Hartung, 1981), a re-classification of isolates of *Colletotrichum* has taken place (Latunde-Dada, 2001; Wharton and Diéguez-Uribeondo, 2004), implying that the causal organism might have been initially misidentified in previous studies. These two species are phenotypically quite similar and share similar morphological and growth features, and are therefore best distinguished using molecular techniques (Freeman et al., 1998; Freeman et al., 2000; Talhinhas et al., 2002; Wharton and Diéguez-Uribeondo,

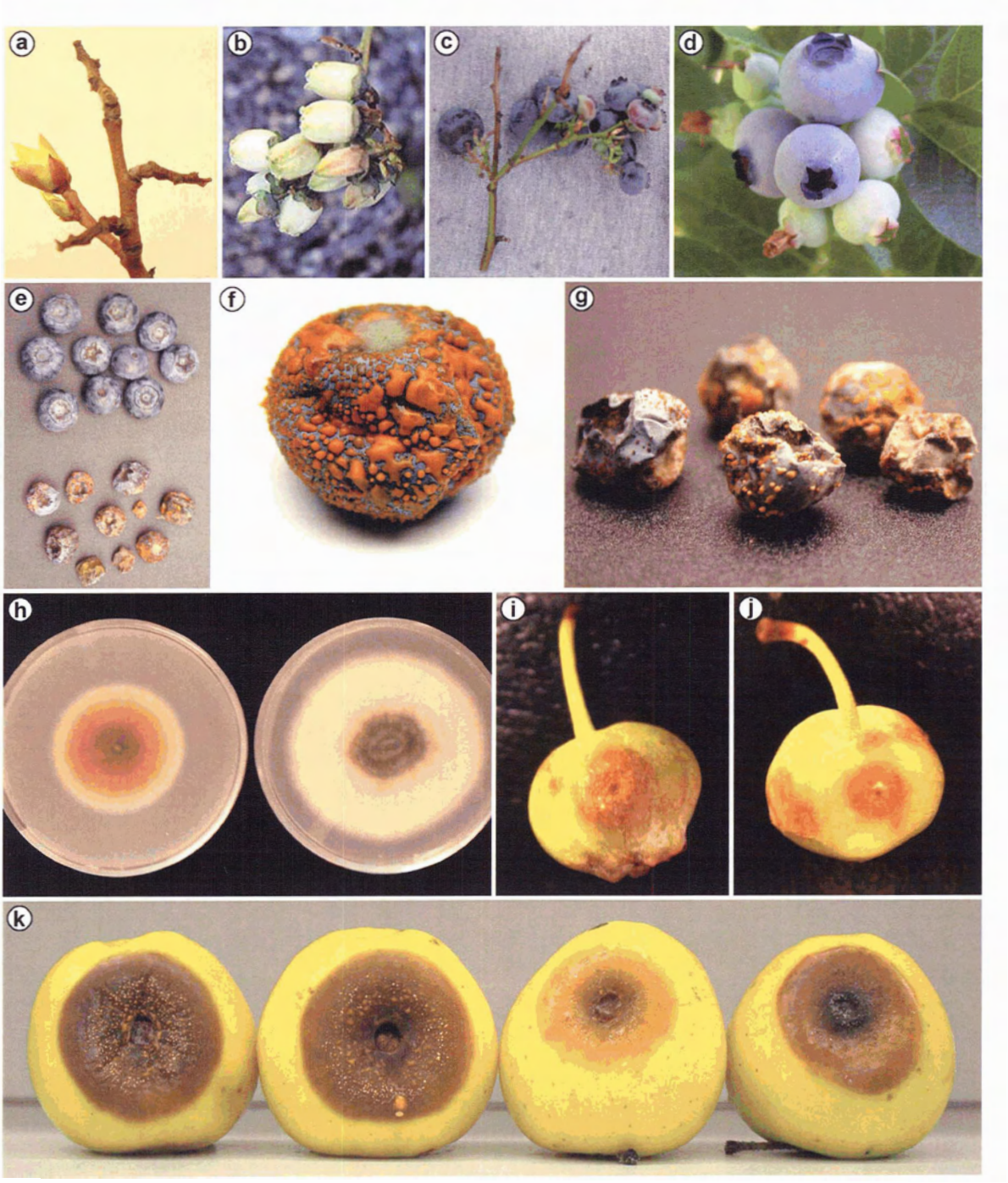

Figure 3.3 **Development of anthracnose and growth of *Colletotrichum acutatum*. a-d); Tissue samples collected from blueberry plants, a) fruit trusses and vegetative buds, b) blossoms, c) ripe berries, d) green berries. e-g); Visible spore masses of *C. acutatum* on diseased fruit. h); Comparison of *C. acutatum* and *C. gloeosporioides* colonies growing on PDA plates. i-j); Disease symptoms on immature green berries inoculated with a spore suspension *in vitro*. k); Apples inoculated with *Colletorichum* isolates displaying orange spore masses and decayed tissue.**

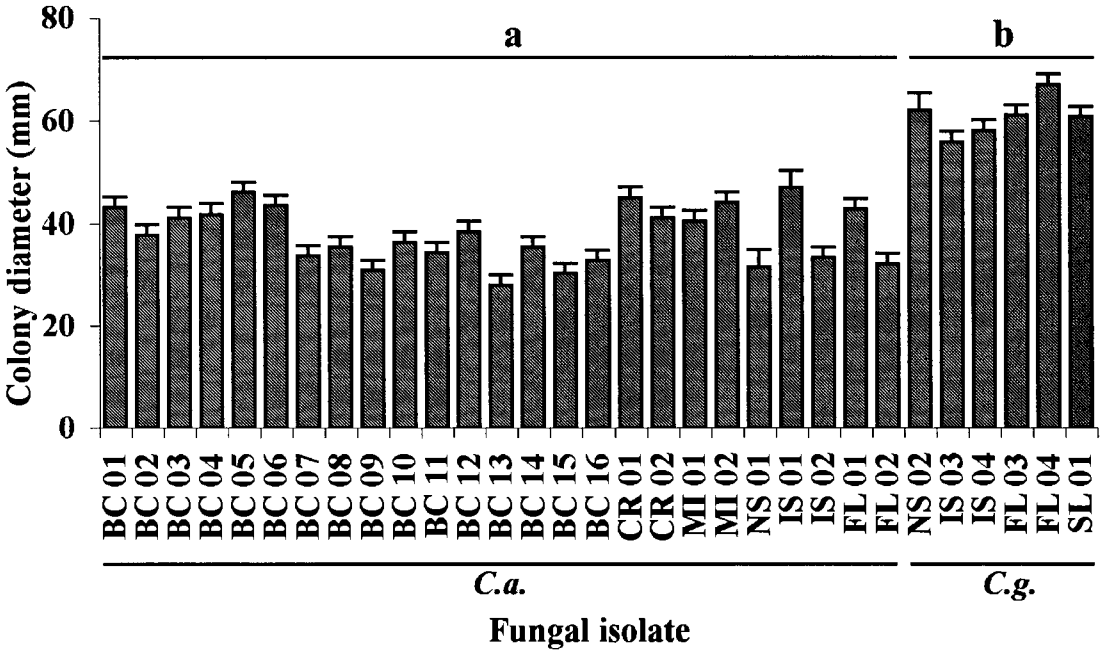

Figure 3.4. **Comparison of colony diameter of *Colletotrichum acutatum* (*C.a.*) and *C. gloeosporioides* (*C.g.*) after 14 days on PDA at 28°C. Isolate designations as in Table 2.1. Isolates designated BC were collected from blueberry fields in British Columbia during 2002-2004.**

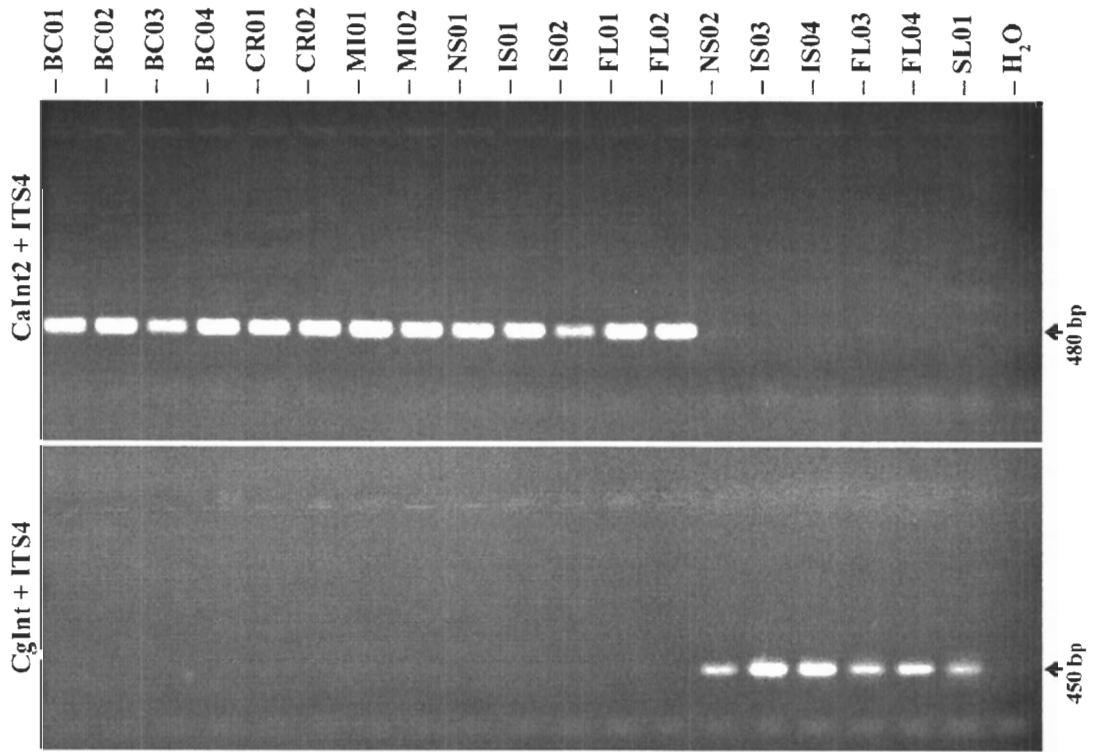

Figure 3.5. **Identification of *Colletotrichum acutatum* and *C. gloeosporioides* using species-specific primers. The CaInt2+ITS4 primer set identifies *C. acutatum*, and CgInt+ITS4 identifies *C. gloeosporioides*. Corresponding fragments are 480 and 450 bp in size, respectively. Positive controls are isolates CR01, CR02, MI01, MI02, NS01, IS01, IS02, FL01, FL02 (*C.a.*) and NS02, IS03, IS04, FL03, FL04, SL01 (*C.g.*). Water was included as a negative control (H_2O).**

2004; Xiao et al., 2004). The species-specific primer pairs used in this study effectively identified and distinguished *C. acutatum* from *C. gloeosporioides*.

3.4 Infection of blueberry and apple fruit

The optimal temperature for blueberry fruit infection was determined to be around 20°C ($p<0.0001$), although diseased berries were observed at all temperatures between 7-30°C (Figures 3.3 i-j; 3.6). This implies that *C. acutatum* has a wide physiological infection capability and can infect at temperatures as low as 7°C. Apples incubated at 20°C displayed the largest lesion diameter with evident sporulation as orange spore masses oozing out of decayed tissue (Figure 3.3 k). All 4 fungal isolates showed a similar trend of increased lesion diameter with increasing temperature (Figure 3.7).

3.5 Establishing period of susceptibility of blueberry plants

3.5.1 Trap plants and natural inoculum

The extent of berry infection on traps plants exposed to prevailing conditions in a field known to contain natural infection is shown in Figure 3.8. Plant growth phases were slightly different for equivalent weeks between years; however, peaks of infection (defined as >5% berry infection) occurred during comparable growth phases in each year, especially during full-blossom (FBL), early green berry (GBR) and ripe berry (BLB). There was one distinct period of infection early in the season (late-April to mid-May), which coincided with opening of flower and leaf buds. The next major infection period was observed mid-season (mid-June-July), coinciding with green berry development. A third infection peak in 2002 and 2003 coincided with mature green-ripe berry stage (late July-Aug). In 2001, sampling was discontinued in early July and no data were available

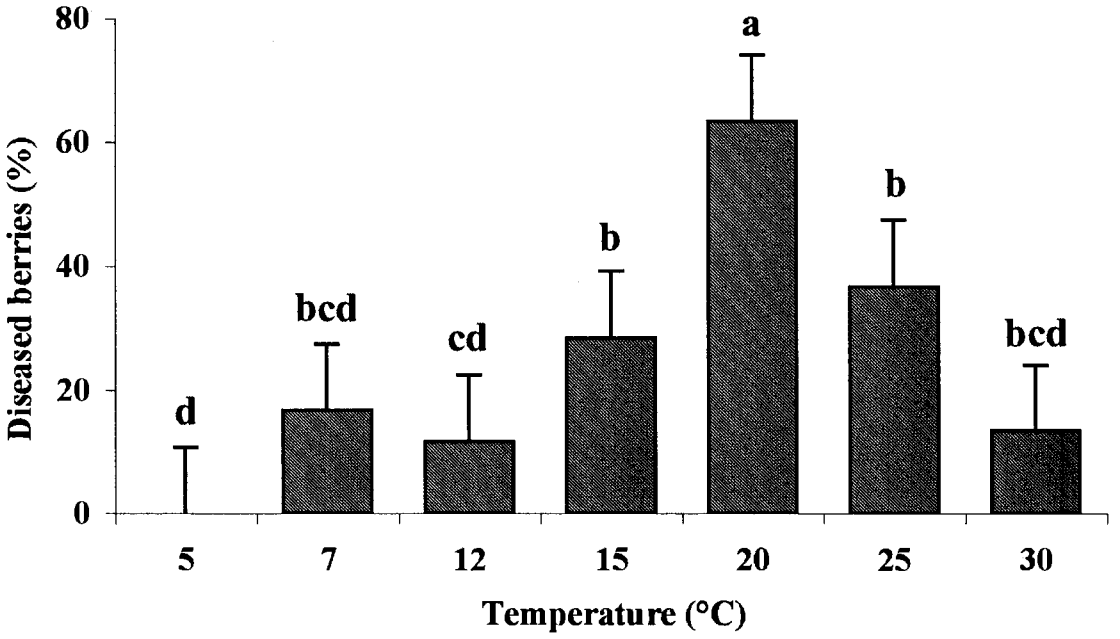

Figure 3.6. **Infection of blueberry fruit with *Colletotrichum acutatum* at different temperatures. Ripe fruit were inoculated with a spore suspension, incubated at specified temperatures for 14 days, and then rated for sporulation.**

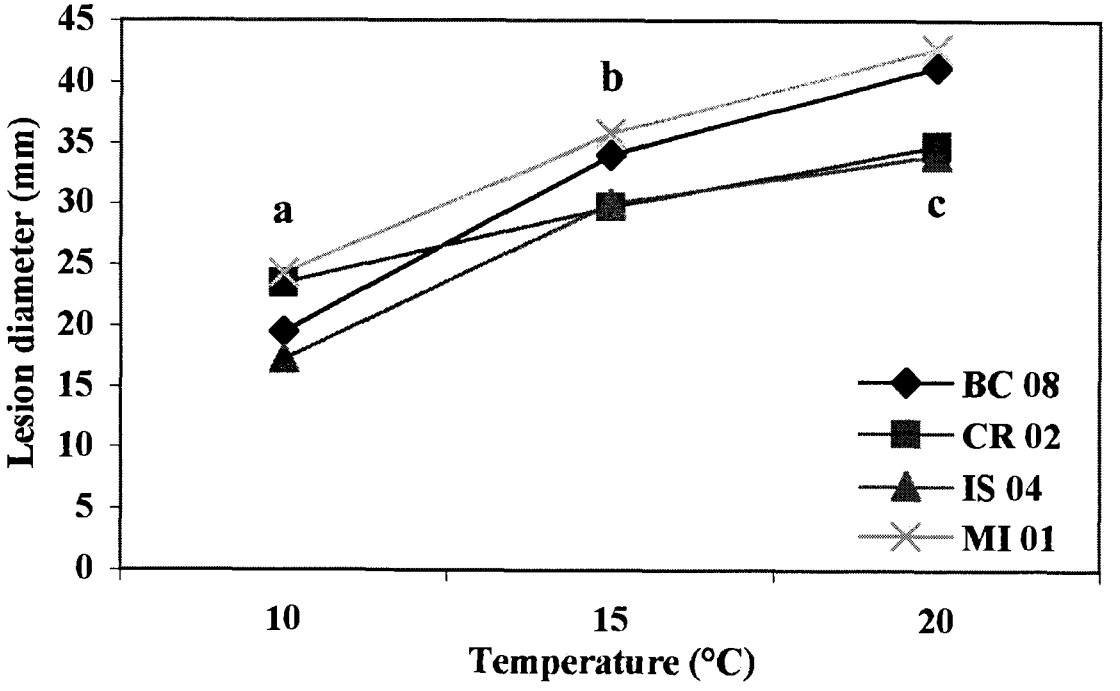

Figure 3.7. **Infection of apple fruit with three isolates of *Colletotrichum acuatum* (BC08, CR02, MI01) and one of *C. gloeosporioides* (IS04) at different temperatures. Apples were wounded with an 8mm cork-borer, inoculated with PDA plugs containing fungal mycelium, and incubated at specified temperatures for 7 days. Resulting radial growth from the inoculation site was measured.**

for the ripe berry stage. An infection period (IP), defined as a period of 10 hours of continuous leaf wetness (measured by the leaf-wetness sensor), and temperatures simultaneously at or above a specified value, was correlated with disease severity. These IP's occurred during the season, and were compared with infection levels to determine the prevailing weather conditions which would correlate with successful infection. Statistical analysis of these results indicated that the best correlative trend was obtained when these IP's occurred at 11°C or higher temperatures in 2001, 15°C or higher temperatures in 2002, and 14°C or higher temperatures in 2003, all with 10 hours or more of leaf wetness occurring simultaneously. Table 3.2 shows the prevailing temperature and leaf-wetness conditions during the weeks of the infection periods as evidenced by disease levels assessed from trap plants in all 3 years.

These field trap-plant experiments suggested that infection was occurring under cooler conditions than previously reported (Hartung, 1981). In studying avocado fruit rot in New Zealand, Everett (2003) reported that spores and appressoria of *C. acutatum* could survive at much cooler conditions than *C. gloeosporioides*, as low as 5.5°C. Results from this study demonstrated that *C. acutatum* can infect at temperatures as low as 11°C in the field, when provided with at least 10 hr of leaf wetness.

3.5.2 Artificial inoculation of plants

Results from artificial inoculation of potted plants in 2004 indicated that the plants were susceptible throughout the growing season. Figure 3.9 shows relatively high infection frequency in all weeks of the trial due to the conducive conditions provided by misting and irrigation. Using a similar statistical correlative analysis as in the field trap-plant experiments, IP's occurring at 12°C or higher temperatures with 10 hr of leaf

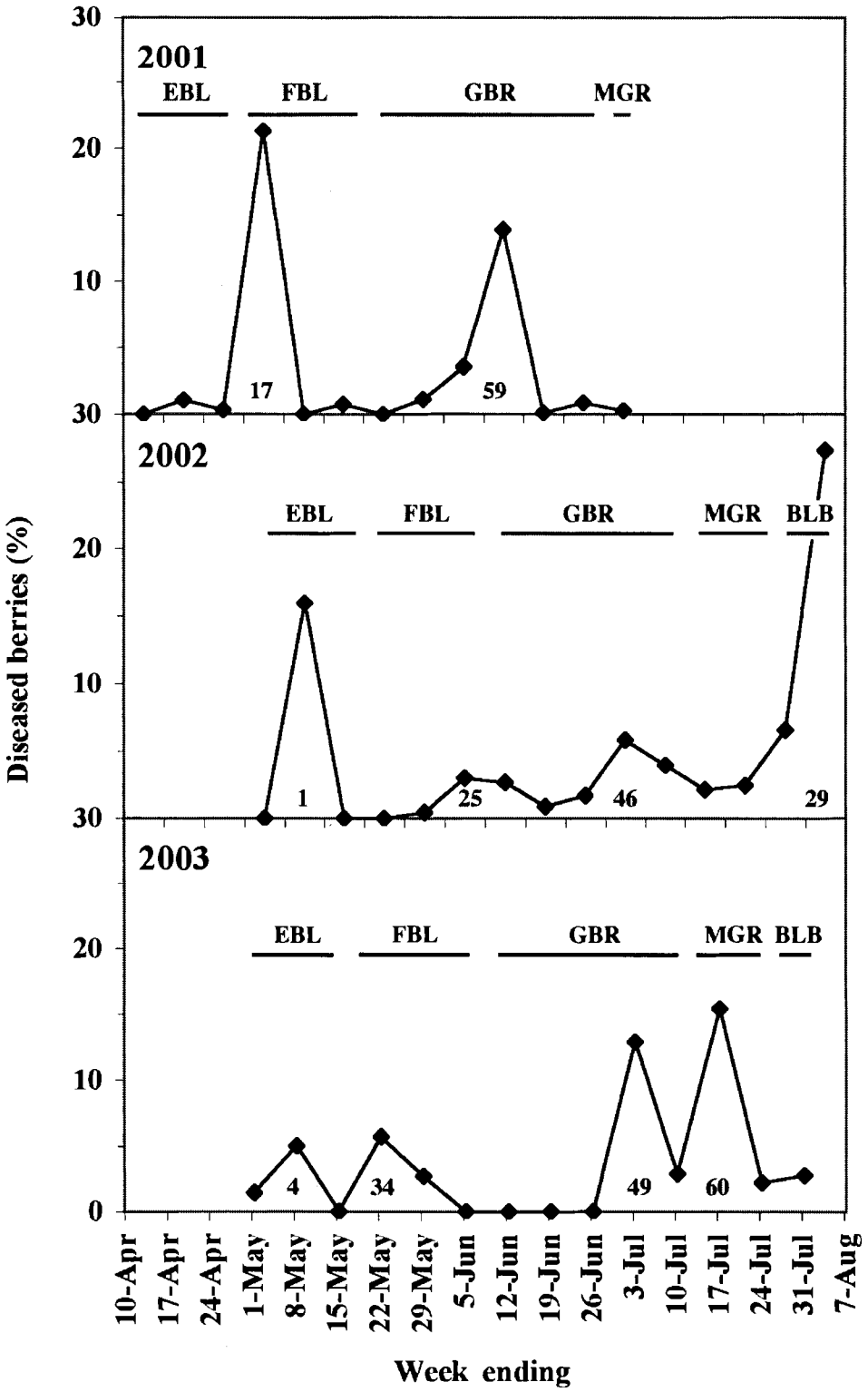

Figure 3.8. Incidence of fruit infection from natural inoculum sources of 2-year old blueberry trap plants in 2001, 2002, and 2003, corresponding to different growth stages; EBL-early blossom; FBL-full blossom; GBR-early green berry; MGR-mature green berry; BLB-ripe berry. Numbers within graphs represent number of infection periods (IP); 10 hours of leaf wetness at temperatures ≥10°C, for peak weeks.

Table 3.2. **Environmental monitoring data (temperature and leaf wetness duration) during field exposure of trap plants to natural conditions over 3 years. Corresponding growth stages are indicated for infection periods (IP), which are defined as 10 hours of leaf wetness at the indicated temperatures (10° or 12°C).**

Week ending	Growth phase[a]	Temp. Max. (°C)	Temp. Min. (°C)	Temp. Avg. (°C)	No. of wet hours	Infection Periods	
						IP ≥10°C	IP ≥12°C
2001							
1-May	EBL	25.1	-0.6	11.3	45	1.7	0.7
15-May	FBL	23.0	-0.3	12.2	49	3.3	0.6
29-May	GBR	32.8	6.6	17.1	41	1.4	0.5
12-Jun	GBR	24.7	8.0	13.3	80	5.9	2.6
26-Jun	MGR	30.6	7.3	17.5	32	2.5	0.4
2002							
1-May	EBL	25.7	-0.1	11.3	49	0.3	0.0
8-May	EBL	29.7	-1.9	8.3	25	0.1	0.0
15-May	EBL	29.2	-0.9	10.8	54	0.4	0.1
29-May	FBL	23.3	2.2	14.8	63	4.9	4.1
26-Jun	GBR	34.5	7.1	19.9	36	2.0	1.2
10-Jul	GBR	34.3	5.1	17.6	55	4.5	3.3
24-Jul	MGR	35.8	8.4	21.8	39	3.3	2.5
7-Aug	BLB	24.5	5.9	15.2	41	2.9	2.3
2003							
1-May	EBL	23.1	0.9	12.6	37	0.6	0.3
8-May	EBL	21.0	-0.2	9.8	45	0.4	0.1
15-May	EBL	22.3	1.2	12.3	47	1.3	0.5
22-May	FBL	21.2	0.6	10.7	49	3.4	1.9
29-May	FBL	26.8	3.9	16.0	49	4.9	4.3
26-Jun	GBR	28.9	7.4	14.6	76	4.8	1.7
3-July	GBR	35.3	7.1	17.2	62	4.9	3.3
10-July	GBR	35.9	8.7	19.0	60	4.3	2.9
17-July	MGR	30.4	7.3	18.7	66	6.0	5.5
24-July	BLB	33.2	8.3	21.2	57	4.5	3.7

[a] EBL - early blossom; FBL - full blossom; GBR - early green berry; MGR - mature green berry; BLB - ripe berry.

wetness best explained the disease levels observed (R^2 value = 0.53; p-value = 0.0022). These results are shown in Table 3.3, with corresponding plant growth phases.

Information on infection periods for a number of other diseases has enabled researchers to develop forecasting models to provide recommendations for disease management, as in botrytis blight of blueberry (Hildebrand et al., 2001), peanut leaf spot (Cu and Phipps, 1993), tomato anthracnose (Byrne et al., 1998), legume anthracnose (Chakraborty et al., 2004), and anthracnose of saskatoon berries (Holtslag et al., 2003). Results from this study suggest that similar predictions of disease occurrence based on IP could be developed for blueberry anthracnose.

3.5.3 Post-harvest disease

Postharvest disease and subsequent decay of blueberry fruit were reported to result from infection followed by an ensuing period of pathogen latency (Daykin and Milholland, 1984; Hartung et al., 1981). This latent behaviour has also been reported for *Colletotrichum* species on other hosts, such as citrus (Timmer et al., 1998), papaya (Dickman and Alvarez, 1983), peach (Zaitlin et al., 2000), cranberry (Olatinwo and Schilder, 2004), banana, avocado, and mango (Latunde-Dada, 2001; Wharton and Diéguez-Uribeondo, 2004). A review of pathogen quiescence in various postharvest diseases is presented in Prusky (1996). Results from this study indicated that while latent infections can result in postharvest rot in blueberry, plants remain susceptible to infection throughout the growing season, as shown by the artificial inoculation experiment conducted in 2004 (Figure 3.9). Under natural field conditions, however, given the distinct peaks of infection, host susceptibility may not be as crucial a factor as inoculum

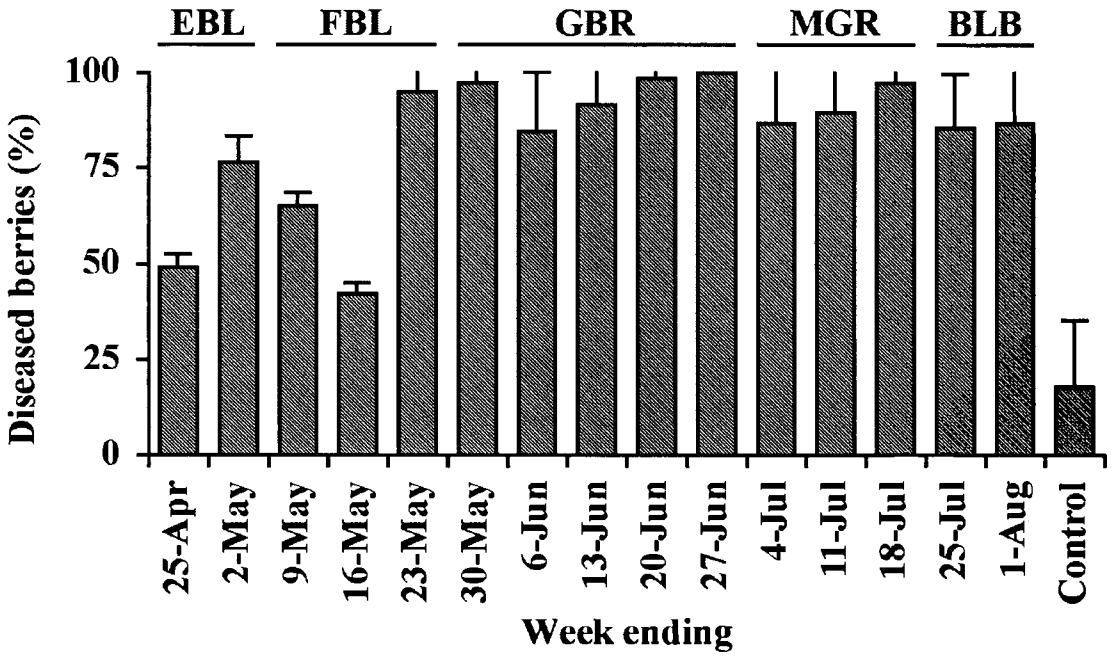

Figure 3.9. **Infection of blueberry fruit resulting from artificial inoculation of blueberry plants at weekly intervals during 2004. Corresponding growth stages are indicated; EBL-early blossom; FBL-full blossom; GBR-early green berry; MGR-mature green berry; BLB-ripe berry.**

Table 3.3. **Environmental monitoring data (temperature and leaf wetness duration) for artificially inoculated blueberry plants exposed to natural conditions in 2004. Corresponding growth stages are indicated for infection periods (IP), which were defined as 10 hours of leaf wetness with simultaneous temperatures of 12°C or higher.**

Week ending	Major growth phase*	Temp. Max. (°C)	Temp. Min. (°C)	Temp. Avg. (°C)	No. of wet hours	IP at 12°C
25-Apr	EBL	21.8	0.8	10.8	90	0.9
2-May	EBL	26.8	-0.1	13.6	88	2.6
9-May	FBL	20.8	0.7	12.8	108	4.6
16-May	FBL	22.3	2.1	12.7	97	1.8
23-May	FBL	24.8	5.6	15.1	89	5.2
30-May	FBL	24.3	4.2	13.6	105	5.7
6-Jun	GBR	28.3	5.6	15.6	86	4.4
13-Jun	GBR	27.2	5.3	14.6	91	5.7
20-Jun	GBR	33.0	4.2	18.4	79	2.9
27-Jun	GBR	32.2	9.1	19.1	82	6.5
4-July	GBR	28.1	7.3	18.3	86	5.6
11-July	MGR	26.7	7.3	16.8	87	6.6
18-July	MGR	31.6	7.9	20.4	75	5.2
25-July	BLB	36.0	9.3	22.0	65	5.4
1-Aug	BLB	29.5	7.8	19.7	47	3.3

[a] EBL - early blossom; FBL - full blossom; GBR - early green berry; MGR - mature green berry; BLB - ripe berry.

availability and conducive weather conditions for disease development.Waller (1992) suggests that the coincidence of susceptible crop stages with wet conditions usually precedes the development of *Colletotrichum* disease epidemics. When wet weather conditions were prevalent, damage due to disease was often severe, as observed in premature fruit drop of citrus (Denham and Waller, 1981), mango blossom blight (Fitzell and Peak, 1984), and coffee berry disease (Griffiths and Waller, 1971), all caused by *Colletotrichum* species.

3.6 Pathogen overwintering and perennial persistence

No *Colletotrichum* colonies were recovered from blueberry fruit left to overwinter in the commercial blueberry field from October 2003 to February 2004, and again from November 2004 to February 2005. Plating of tissues from artificially inoculated plants revealed that flower buds had the highest percentage of residual infection, followed by twigs and fruit trusses, and finally vegetative (leaf) buds (Figure 3.10). When placed onto water agar, *Colletotrichum* colonies were easily identified by their light pink colour when viewed from the underside. Colonies were then sub-cultured onto PDA to confirm identity. The period of inoculation in the previous year (2004) that resulted in highest inoculum persistence the following year (2005) was May 16 to July 18. In comparing tissues sampled from the same plants in December 2004 with February 2005, recovery of the pathogen was similar, perhaps a little higher in the latter. Levels of *Colletotrichum* recovery declined in the twigs and trusses when sampled in February 2005, as compared to December 2004, perhaps due to competition from other organisms, although this was not assessed.

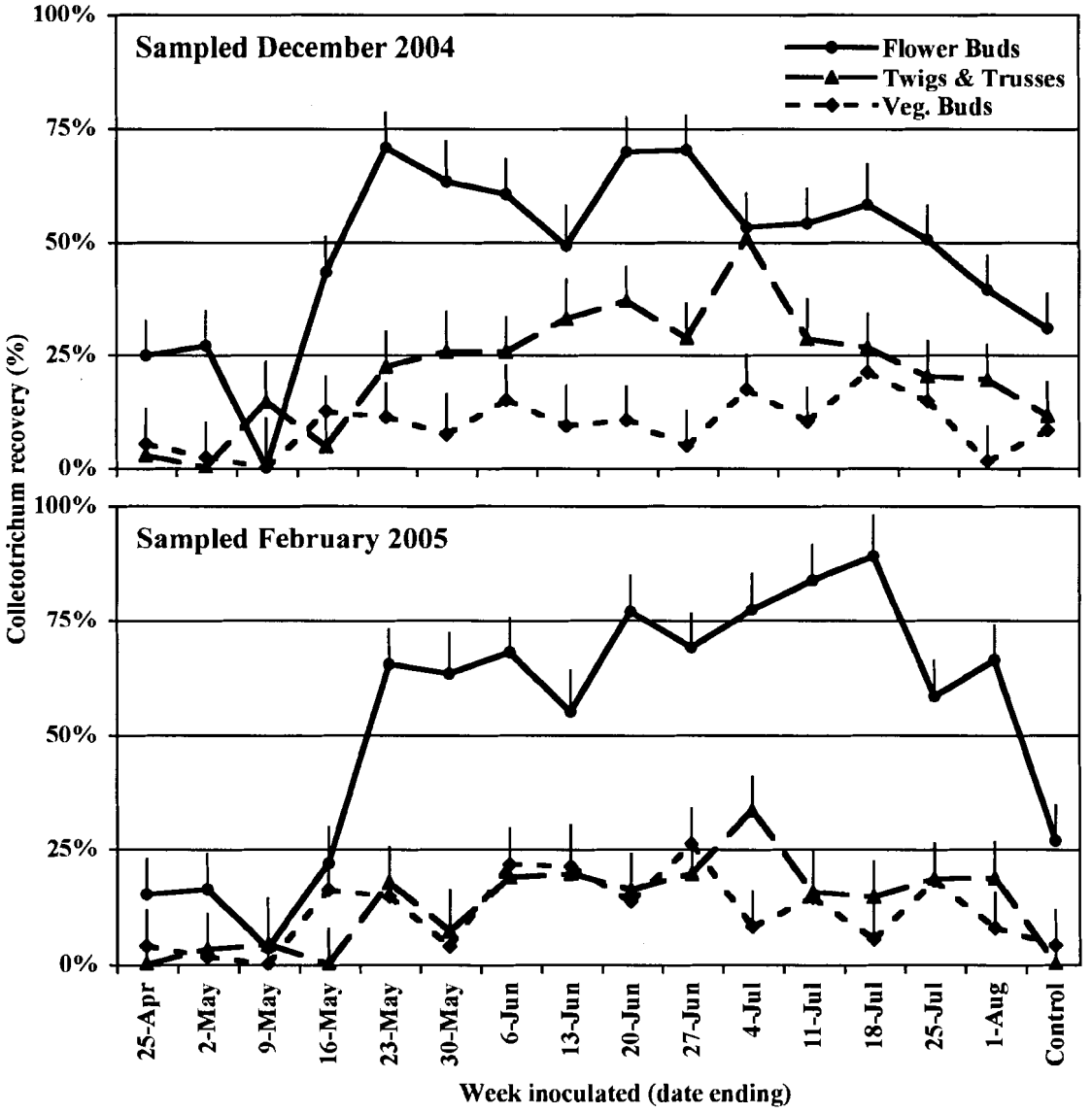

Figure 3.10. Recovery of *Colletotrichum* after overwintering, from blueberry plants artificially inoculated at weekly intervals during 2004. Plants were sampled twice; once in December 2004 and then again in February 2005.

A number of studies have been conducted on survival of *Colletotrichum* species under field conditions (DeMarsay and Oudemans, 2003; 2004; Eastburn and Gubler, 1990; Freeman and Shalev, 2002). Possible survival strategies for *Colletotrichum* species include overwintering in soil, vegetative tissues, and mummified fruit buried in the soil. Feil et al. (2003) demonstrated that colonization of buried strawberry plant tissue in soil ensured survival of *C. acutatum* over the winter months in California. Results from this study indicate that flower buds, twigs and spent fruit trusses are likely sources of inoculum for infection in the spring of the following year. Vegetative buds had the lowest reservoir of inoculum and mummified berries in soil are an unlikely source of inoculum. DeMarsay and Oudemans (2002; 2003; 2004) showed that pathogen overwintering and inoculum sources of *C. acutatum* in highbush blueberry fields in New Jersey were highest in flower buds (72%) of the cultivar 'Bluecrop'. Wharton and Diéguez-Uribeondo (2004) have described the infection cycle on flower buds, which is initiated by the fungus growing out of the buds at bud break, causing tissue necrosis, followed by pathogen sporulation on the dead tissue. Under field conditions, spore-trapping studies have shown that pathogen sporulation coincided with bud-break in the spring (Bristow and Windom, 2000; MacDonald et al., 2001; Wharton et al., 2002; Wharton and Diéguez-Uribeondo, 2004). Developing flower buds, therefore, are important targets for application of disease control methods.

3.7 Biological control experiments

The reduction in disease due to applications of *Gliocladium catenulatum* (Prestop) and *Trichoderma harzianum* (Plantshield) on potted plants in 2002, and to field plants in 2003 and 2004 is illustrated in Figure 3.11. In each year, applications of the biological

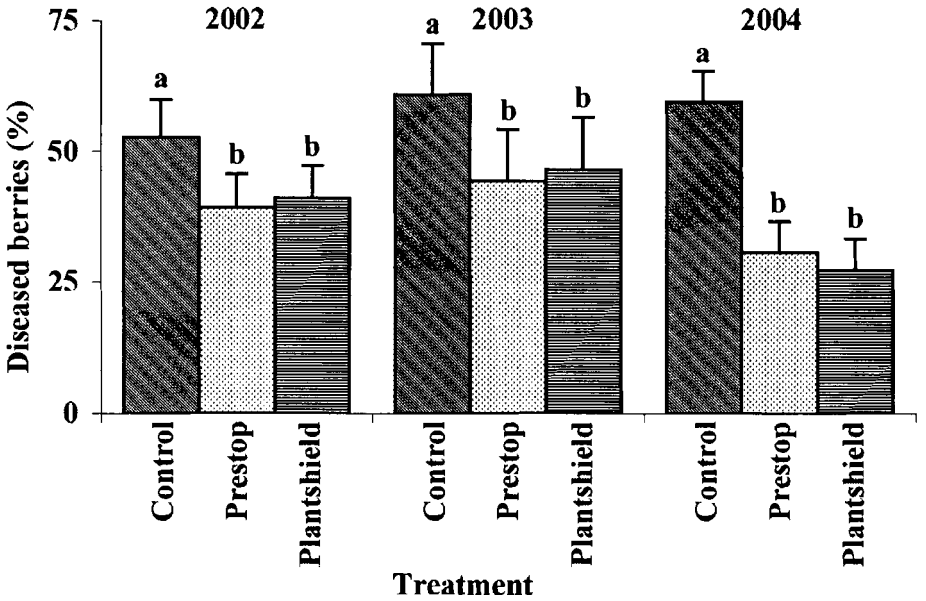

Figure 3.11. **Effect of *Gliocladium catenulatum* and *Trichoderma harzianum* on fruit infection at harvest over 3 years. In 2002, potted plants were used, while in 2003 and 2004, applications were made to plants in commercial blueberry fields. 3 applications of the biological control agents were made each year. Bars labeled with the same letter are not statistically different according to an ANOVA comparison of means.**

control agent reduced disease on fruit by 33%, 33%, and 45% respectively, when compared to plants sprayed with water alone. Both agents were equally effective in reducing disease.

The potential for biological control of *Colletotrichum* species using phyllosphere antagonists has been suggested previously (Lenné and Parbery, 1976). The use of microbial agents to mitigate post-harvest rots in fruit has been reviewed (Jeger and Jeffries, 1988; Korsten and Jeffries, 2000). The experiments in this study with *Gliocladium catenulatum* (Prestop) and *Trichoderma harzianum* (Plantshield) on potted and field plants showed they reduced disease incidence by up to 45% when three applications were made during the growing season. Biological control of *Colletotrichum* species has been demonstrated in other studies using *Trichoderma* species applied to strawberries with concomitant disease reduction (Freeman et al., 2003). *Trichoderma* species were also shown to reduce *C. acutatum* infection in detached 'Tahiti' lime flowers from citrus orchards in Belize (Moretto et al., 2001). It is believed that *Trichoderma* species are able to effectively compete for infection sites, thereby reducing pathogen infection success (Freeman et al., 2003; Jeffries and Koomen, 1992).

CHAPTER 4. SUMMARY AND FUTURE RESEARCH

4.1 Application of research results

Given the relative lack of information regarding blueberry anthracnose in BC compared to other blueberry-growing regions in North America, a basic understanding of its epidemiology in BC is essential towards effective management of disease. The results from this study indicate that environmental and pathogen monitoring, coupled with timely application of biological and chemical controls, can reduce disease development and subsequent pathogen persistence. Applications would need to coincide with predicted infection periods based on weather forecasting, to preclude infection under conditions optimal for pathogen development. Targeting control measures during periods of susceptible tissue development, for example at flower-bud development, could aid in reducing overwintering inoculum persistence.

The fungus responsible for blueberry anthracnose in BC was shown to be *Colletotrichum acutatum*, and has the ability to infect fruit at relatively low temperatures, as long as sufficient humidity (as measured by leaf wetness) is available. This suggests that growers should be aware of susceptible periods of infection based on prevailing weather conditions, and apply preventive controls appropriately. Additionally, peaks of *C. acutatum* presence in the field coincide with specific plant growth phases, especially blossoming and mature green-ripe berry stages, which also suggesting a specific period for applying preventive controls.

C. acutatum is present in all growing regions of BC, although disease severity varied in different fields, likely due to prior disease occurrence and irrigation practices. Due to the potential latent period involved in the pathogen's lifecycle, post-harvest management is essential in reducing disease levels. An integrated combination of cultural control tactics and chemical control options as currently recommended in the Berry Production Guide for Blueberry Growers (Anonymous, 2005b) should be practiced.

4.2 Directions for future research

As mentioned previously, the taxonomy of *Colletotrichum* is under constant re-evaluation as new diagnostic and identification techniques are applied to this field of study. Although this re-organization has its academic and pathological merit, one must be careful to make species distinctions only as necessary to avoid enlarging the number of species unnecessarily. Although traditional methods of classification based primarily on morphological criteria can be used in many cases, deviation from type-culture characteristics and the presence of intermediate forms can lead to mistaken identification in this genus (Freeman et al., 1998). Whether identifications of the pathogen in previous studies were incorrectly made due to prevailing knowledge at the time remains to be verified, as recent studies have demonstrated (Xiao et al., 2004).

Developing a disease forecasting model would require in-depth laboratory and field studies of inoculum levels and the effect of environmental factors on the various steps in successful pathogenesis. Temperature-controlled experiments in humidity chambers using entire plants through the entire growing season would provide valuable data for discerning specific infection requirements. These could then be tested in field studies through weather correlations and predictive models as suggested in this study.

The use of resistant cultivars could be an important part of an integrated approach to managing blueberry anthracnose, provided resistance to pathogen attack does not come at the expense of fruit quality. Since harvested fruit undergoes various physiological changes, the resistance mechanism(s) would have to survive these changes, without adversely affecting fruit taste and marketability. DeMarsay (2005) and Polashock et al. (2005) have already reported some varietal difference in anthracnose susceptibility among various blueberry cultivars. Studies on the biochemical composition of unripe green fruit as compared to ripe blue berries would provide information regarding infection requirements for the pathogen as well as possible host resistance mechanisms. Detailed information on the biology of the pathogen will further allow optimization of application and timing of available control tactics. For example, an improved understanding of the physiology and underlying biochemistry involved in the induction and maintenance of latency could lead to new disease control measures, targeted at susceptible phases of the pathogen's lifecycle.

It would also be interesting to characterize the various *C. acutatum* isolates collected to determine genetic relatedness of isolates from different fields. This data could be compared against reference fungal isolates from other parts of North America, including blueberry and other related hosts (strawberry, cranberry, wild *Vaccinium*, etc.). Any of the molecular techniques mentioned earlier could be employed for this purpose.

Although the preliminary biological control experiments in this study evaluated the efficacy of two microbial agents in reducing blueberry anthracnose incidence on potted as well as field plants, they did not study the effect these agents had on reducing the amount of overwintering inoculum for the next growing season. Since a different set

of plants were used in each year this study was conducted, this reduction in inoculum was not evaluated. A similar experiment using plants through succeeding growing seasons would provide insight into this aspect of the biological control agents' ability to reduce the pathogen's seasonal persistence. As shown in this study, tissues from treated plants could be assessed after an overwintering period to determine whether the biological control agents could effectively reduce inoculum survival, by how much, and in which specific tissues. Even more revealing would be studies on the detailed specifics of microbial ecology that underlie methods of disease suppression by these antagonists.

It is hoped that results from this study can aid the respective biological control companies in applying for registration of their products for blueberry anthracnose management. In addition to efficacy data, residue and toxicity analysis on crop and non-target organisms would be required for inclusion in the registration applications. Alternative control methods for post-harvest disease management, such as hot-water treatment, controlled atmospheres, and biological control applications should be further explored to determine if they could also aid in managing this disease. Pre-harvest management, possibly aided by microbial applications, should also have a profound effect on reducing post-harvest disease. Lastly, a synergistic integration of chemical controls with a biological component would be attractive to growers; however, the effect of pesticides on antagonistic microbes needs to be studied for this to be a viable choice.

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