Characterization of Anthracnose Resistance in Common Bean

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The common bean (*Phaseolus vulgaris* L.) is an important food crop in the world. Anthracnose of common bean, caused by the fungus *Colletotricum lindemuthianum*, is a major and worldwide destructive disease. A population of 126 F$_{5:6}$ recombinant inbred lines (RILs) of a cross between the resistant navy bean Bolt and the susceptible H4784A-29844, was phenotyped in the field and growth room trials and genotyped with genome-wide single nucleotide polymorphic markers. The resistance appeared to be controlled by a major QTL on Pv01, likely associated with the anthracnose resistance gene Co-1. *In-Silico* search in the *Phaseolus* reference genome resulted in identification of four candidate genes in the QTL region. Sequence comparison of the amplified coding regions of these genes revealed structural differences in resistant and susceptible genotypes.
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List of Abbreviations

ANOVA = Analysis of Variance
AUDPC = Area Under the Disease Progress Curve
AVR = Avirulence
°C = Degree Celsius
cv= Cultivar
DNA = Deoxyribonucleic Acid
rDNA = recombinant Deoxyribonucleic Acid
endo-PG = endo-Polygalacturonase
LOD= Log of odds
ha = Hectare
hg = Hectogram
HPRGs = Hydroxyproline-rich glycoproteins
HR= Hypersensitive response
hr = hour
INA = Isonicotinic Acid
m = Meter
MAS=Marker assisted selection
mm = Millimeter
μl = Micro liter
PAL = Phenylalanine Ammonia-Lyase
PDA = Potato Dextrose Agar
PCR = Polymerase Chain Reaction
PGIPs = Polygalacturonase Inhibiting Proteins

pv = pathovar

QTL = Quantitative trait loci

RIL = Recombinant inbred line

R = Correlation coefficient

R² = Coefficient of multiple determination

SAS = Statistical Application System

SNP = Single nucleotide polymorphism

SSR = Simple sequence repeat
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3.0. Abstract
Chapter 1: Introduction and Literature review

Introduction

The common bean (*Phaseolus vulgaris* L.) is grown widely around the globe, providing an important staple food crop for subsistence farmers in the developing countries and a high-value commodity crop in the developed world (Schwartz 2005).

Anthracnose caused by the fungus *Colletotrichum lindemuthianum*, is a major and destructive disease of common bean worldwide (Pastor-Corrales et al. 1987; Barcelos et al. 2011). The fungus *C. lindemuthianum* is primarily a seed-borne pathogen (Barrus 1921), which can attack all aerial plant parts and may cause yield losses as high as 100% (Zaumeyer and Thomas 1957; Schwartz et al. 2005). Infected seed is the main means of spreading from year to year and from location to location for the pathogen. The fungus is highly variable with more than 100 pathogenic variants and races reported in the scientific literature (Balardin and Kelly 1998).

Genetic resistance is the most environmentally-safe, and socially-acceptable disease management strategy for bean anthracnose (Mahuku and Riascos 2004; Kelly and Vallejo 2004). The genetics of anthracnose resistance has been studied for a long time, and is known to follow qualitative inheritance with involvement of single major genes in a gene-for-gene host-pathogen interaction (Flor 1955; Ferreira et al. 2013).
Literature Review

1.1: Phaseolus vulgaris L.

1.1.1: Taxonomy and morphological characteristics

The common bean (*Phaseolus vulgaris* L.) belongs to the legume family of *Fabaceae*, genus *Phaseolus*, subtribe Phaseolionae, tribe Phaseoleae, subfamily Papilionideae in the family Fabaceae (McClean et al., 2004; Singh, 2001; Debouck, 1991) classified as dicotyledonous. The term ‘common bean’ is the general term that refers to varieties of the species *P. vulgaris* L., while ‘dry bean’ is the term used to describe common beans that are grown to produce dry edible seeds commercially. Common bean include dry beans, green beans, shelling beans and popping beans. Previously, the genus *Phaseolus* included more than 180 species (Ditmer et al., 1937), but in more recent taxonomic classifications it includes only 50 species (Delgado-Salinas et al., 2006). All these species are native of Meso- or SouthAmerica. Only five species of *Phaseolus* have been cultivated. These include common bean (*P. vulgaris* L.), runner bean (*P. coccineus* L.), year bean (*P. dumosus* L.), tepary bean (*P. acutifolius* L.) and lima bean (*P. lunatus* Greenman) (Aragao et al., 2011; Gepts et al., 2008). Dry bean is the superior commercial class in *P. vulgaris*, not only in human consumption, but also due to the extensive extended scientific research that has been conducted on this species. All cultivated *Phaseolus* species and their wild relatives are diploid (2n = 2x = 22) with 11 chromosome pairs (Mercado-Ruaro & Delgado-Salinas, 1998). Common bean accounts for more than 85% of total world production of all cultivated *Phaseolus* species (Singh, 2001).

This species is diverse and, primary, secondary, tertiary and quaternary gene pools have been defined for *P. vulgaris*. The primary gene pool includes wild and cultivated forms of *P. vulgaris* (Singh et al., 1999). The secondary gene pool is comprised of *P. coccineus*, *P.

Common bean is an annual, self-pollinated, short-day plant, mostly grown in subtropical areas of the world. There are four growth habits for common bean: determinate bush, indeterminate bush, indeterminate prostrate and indeterminate climbers. Common bean cultivars are classified into two classes based on plant height: dwarf bean (20-60 cm) and climbing bean (≥2 m) (White & Laing, 1989). The biological development of common beans includes vegetative and reproductive phases. Each phase has 10 developmental stages.

1.1.2: The origin of common bean

Based on the discovery of wild common beans in Argentina (Burkart and Brücher 1953) and Guatemala (McBryde 1947) and the archaeological evidence found in the Americas (Kaplan and Kaplan 1988; Kaplan and Lynch 1999) the centre of origin of the common beans is believed to be the Americas (Van Schoonhoven and Voysest 1991; Mamidi et al. 2011). Moreover, morphological and historical evidence suggest that common beans were domesticated at least 7000 years ago in Mesoamerica and the Andes (Kaplan, 1981; Kaplan et al., 1973). Bitocchi et al. (2012) investigated the nucleotide diversity at five genetic loci of a large sample that represented the entire geographical distribution of the wild forms of P. vulgaris and provided evidence for a Mesoamerican origin of the species and the occurrence of a genetic bottleneck during the formation of the Andean gene pool that predates domestication. Their study suggested that both of the gene pools originated through different migration events from the Mesoamerican populations.
1.1.3: *Phaseolus vulgaris* Gene pools

Variation in morphology, seed protein, isozymes, mitochondrial DNA variation, amplified fragment length polymorphism (AFLP), short sequence repeats (SSR), and other molecular evidence indicate the existence of two major gene-pools of *P. vulgaris*, Andean and Middle-American (Beebe et al. 2001; Mamidi et al. 2011). Within the Andean gene pool, the evolutionary races Chile, Nueva Granada and Peru have been characterized. Genotypes of the Andean gene pool are generally large-seeded. Within the Middle American (MA) gene pool three evolutionary races, Durango, Jalisco (medium-seeded climber), and Mesoamerica (small-seeded), have been identified (Singh et al. 2000). Based on the discovery of common bean, the Middle American gene pool extends from Mexico through Central America and into Venezuela, while the Andean gene pool is found in Peru, Chile, Bolivia and Argentina (Mamidi et al. 2011).

Widely grown and consumed market classes in North America include: Great Northern, pink, pinto and small red beans of Durango descent, cranberry beans, yellow, light and dark red kidney beans of Nueva Granada descent, white pea beans (navy beans) and black beans of Mesoamerican descent and cranberry beans of Chile descent (Singh 1999; Voysest and Dessert 1991; Kelly et al. 2010) (Figure 1.1). In Ontario the navy, coloured, and Japanese (Otebo) classes are commonly grown. The more common coloured classes grown in Ontario are cranberry, black and kidney.

1.1.4: Production of common bean

Beans are considered profitable crops and with over 23 million metric tonnes (MT) produced annually, one of the most important legumes in the world (Broughton 2003). In 2013, a total of 22.8 million MT of dry beans and 21.37 million MT of green beans were grown
worldwide with an average yield of 0.8 Mg ha\(^{-1}\) and 13.8 Mg ha\(^{-1}\), respectively (FAOSTAT 2014). Dry and green beans were grown on 30.75 million ha of land in 2011 (FAOSTAT 2014). The commercial production of beans occurs throughout the world, Asia and Americas, approximately 45\% and 31\%, respectively of bean production in 2013 and include the top five countries for seed production (FAOSTAT, 2014) (Figure 1.2).

The developing countries in Asia, Africa, and Latin America have been increasing production of common bean, since Europe production has decreased throughout the last decade. India, Myanmar and Brazil have continued expanding common bean production in the last decade (Figure 1.3). Although Brazil and India are the largest producers of dry beans, but China produced the largest quantity of green snap beans in the last decade (FAOSTAT 2014).

Canada produced approximately 205,900 tonnes contributes 1.4 \% of the total bean production worldwide of dry beans in 2013, ranked among the top five exporters of this crop. On average, Canada exports almost all the beans produced to the USA and Europe (mostly to the United Kingdom and Italy), followed by Japan, Angola, Dominican Republic and Greece (Agriculture and Agri-Food Canada, 2014; FAOSTAT, 2014). Ontario, Manitoba, and Alberta are the major producers of dry beans in Canada with around 130,000 ha of harvested area, while Quebec and Saskatchewan produce smaller quantities of dry beans (Stats Canada 2012; FAOSTAT 2014; Ontario Bean Growers 2014). Ontario is the largest producer with approximately 42\% of bean production in the past ten years.

Other major exporting countries are Myanmar (1.09 Million MT), China (0.9 Million MT), USA (0.35 Million MT), and Argentina (0.24 Million MT) (FAOSTAT, 2012). In the last ten years the majority of export from these countries have been to India and the European Union, which have annually imported over 0.5 and 1.8 Million MT, respectively (FAOSTAT, 2012).
1.1.5: Agronomic and Nutritional values

Beans are grown in Eastern Africa, North, South and Central Americas, Eastern Asia, Western and south-eastern Europe (Adams et al. 1985; Sherf and Macnab 1986). Common beans are grown under very diverse climatic conditions at 50 to 3000 m above sea level in most continents, especially at 1200 – 2400 m above sea level. In developing countries beans are grown primarily by small-scale farmers who have limited resources and usually produce the crop under adverse conditions such as low input use, marginal lands, and intercropping with competitive crops like sorghum, maize and some other perennial tree crops like coffee (Aleligne, 1990). Dry beans grow best in temperatures ranging from 14 to 26°C, an annual precipitation between 400 and 1600 mm year\(^{-1}\), and a slightly acidic soil pH between 5 to 6. They show a wide range for time to maturity ranging from 70 to as high as 330 days and grain yield potential of 400 to 5000 kg ha\(^{-1}\) (Wortmann et al., 1998; Debouck, 1994) providing an important staple food to millions of people worldwide (Aragao et al., 2011).

Dry beans are generally high in fiber, protein, vitamin, and mineral content and low in fat. They are also low in sodium and are gluten free (Agriculture and Agri-food Canada 2009). Bean seed contains 20-25% protein (Ma and Bliss 1978), which contain amino acids such as methionine that is a common seed protein in the legume family and lysine that is deficient in cereal seed (Bressani 1983). Beans contain a large amount of free biotin, an essential cofactor for carboxylases and decarboxylases in metabolic organisms (Knowles 1989). Beans are also important sources of iron, phosphorus, magnesium, manganese, as well as zinc, copper, and calcium in a lesser degree (Broughton 2003).
1.1.6: Common Bean Diseases

A wide range of diseases attacks common beans. The important disease of beans are anthracnose (caused by *Colletotrichum lindemuthianum*), white mold (caused by *Sclerotinia sclerotiorum*), root rot (caused by *Fusarium solani*), angular leaf spot (caused by *Phaeoisariopsis griseola*), rust (caused by *Uromyces appendiculatus*), common bacterial blight (caused by *Xanthomonas* sp.), ascochyta blight (caused by *Ascochyta phaseolorum*), bean common mosaic virus, and golden mosaic virus. Among all, anthracnose is known to be the most predominant and highly destructive disease in all bean-growing countries around the world (Pastor-Corrales and Tu 1989; Tu 1992; Kelly et al. 1994).

1.2: *Colletotrichum lindemuthianum*

1.2.1: Taxonomy

Anthracnose of common bean, is caused by the fungus *Colletotrichum lindemuthianum* (telomorph: *Glomerella lindemuthianum* = *G. cingulate* f.sp. *Phaseoli*) (Sutton 1992) is a major and destructive disease, worldwide (Pastor-Corrales et al. 1987; Barcelos et al. 2011). The fungus is classified in the *Phyllachoraceae* family, *Phyllachorales* order, and *Ascomycota* group. *C. lindemuthianum* (Sacc. & Magnus) Scribner belongs to the fungi kingdom and is one of the asexual forms of *Glomerella cingulate* (Stoneman) Splaulding & Schrenk (Hawksworth et al. 1995). The fungus was first recognized as *Gloeosporium lindemuthianum* Sacc. & Magnus. In 1988, Scribner classified *Colletotrichum* for the fungus (Stevensen 1956) and named to *C. lindemuthianum* (Sacc. & Magnus) Scribner or *C. lindemuthianum* (Sacc. & Magnus) Briosi & Cavara. The species of *C. lindemuthianum* is distinguished from *Glomerella cingulate* by the slow growth of conidia and darker pigment in culture media (Morude 1971). The first scientific
A report of anthracnose was in 1875 in Italy and Germany (Sherf and Macnab 1986). In North America, anthracnose was first reported in the United States in 1885 (Sherf and Macnab 1986).

Anthracnose of bean is primarily a seed borne disease (Barrus 1921), which may cause yield losses as high as up to 100% (Zaumeyer and Thomas 1957; Schwartz et al. 2005). Bean yield reductions due to anthracnose infection are a result of poor seed germination, poor seedling vigour, seed abortion, pod abortion and loss of photosynthetic area (Dillard and Hunter 1988). Moreover, seed quality can be significantly reduced by anthracnose infection (Begum et al. 2008). Seed-borne infection plays an important role in the dissemination and initiation of the disease (Pastor-Corrales and Tu 1989) and significantly influences germplasm exchange and international seed trade (Neergaard 1977; Agarwal and Sinclair 1997).

Common bean is the major host of *C. lindemuthianum*. However, other legumes including climbing bean (*P. coccineus*), tepary bean (*P. acutifolius*), lima bean (*P. lunatus*), cowpea (*Vigna unguiculata*), and broad bean (*Vicia faba*) are also considered to be slightly susceptible (Pastor-Corrales and Tu 1989; Tu 1992a).

**1.2.2: Disease symptoms and signs**

Anthracnose can infect all plant parts including stems, leaves, pods and seeds (Agrios 2005). Early anthracnose symptoms are dark brown to black sunken lesions on the seeds, cotyledons and stems. The growth of the plants is stunted due to infection. Diseased areas may girdle the stem and eventually kill the seedlings. Small pink spores are produced in the lesions in moist conditions and may spread from stems to the leaves. Symptoms on leaves occur as linear, dark brick-red to black lesions on the lower leaf surface along the veins at the primary and trifoliate leaf stage. The discoloration appears on the upper leaf surface, as the disease progresses. Leaf symptoms are often not obvious and plants may appear normal until the disease
is advanced. This is because infection is initially restricted to the lower leaf surface (Hall 1994). Dark brown eyespots also develop on the stem and on young seedlings. Infection may cause the stem to rot and die. The most obvious symptoms of anthracnose are on the pods. Small reddish-brown to black-blemishes and distinct circulated dish-brown lesions are typical symptoms on the pods. Mature lesions are surrounded by a circular reddish-brown to black border with a grayish-black interior (Dillard and Hunter 1988; Del Rio and Brandley 2002). Symptoms on the seeds are yellowish to brown sunken lesions (Agrios 2005).

1:2:3: Disease identification

A rapid and easy way to identify C. lindemuthianum with minimal laboratory equipment (CTA 1999) is by inducing the germination of conidia to produce appressoria on the host surface on many natural media. C. lindemuthianum can be identified based on the absence of septa in germinating conidia (Bailey et al. 1996). Similarly, anthracnose infected seeds on moist blotters is used for a blotter test (Prasana 1985).

1.2.4: Disease development

Depending on environmental conditions, the fungus survives on plant debries in the seed, air, and water (Schwartz et al 2005) or in the plant residue. Research conducted in Canada has shown that the fungus can survive up to 5 years stored at 4° C in infected bean pods and seeds (Dillard and Hunter, 1988). Seed-borne infection plays an important role in the development of the disease. Alternate dry-wet cycles affect the survival of the pathogen in the soil (Tu 1983).

Infection by C. lindemuthianum in P. vulgaris as a hemi-biotroph grows intra-cellularly in plant tissue. The pathogen exhibits both biotrophic infection, where it grows in living host plant cells, and necrotrophic infection, growing on dead host plant cells, (Perfect et al. 2000).
The necrotrophic phase is linked to the increased expression of enzymes such as endo-polyglacturonases (endo-PG) and pectin lyase of plant cell wall (Wijesundra et al. 1989). The infection process includes attachment, host recognition, penetration, proliferation, and nutrition (Kahman and Basse 2001). Spore and appresorium adhere to the surface of the plant during the infection (Young and Kauss 1984). Adhesion of *C. lindemuthianum* may be caused by splashing water during heavy rain in the field (Wheeler 1969). Conidia in *C. lindemuthianum* are sticky and whenever they reach the host, adhere to their surface. 20 to 50% of conidia adhere to the bean hypocotyls in one hour in the water (Young and Kauss 1984).

Conidia that reach the plant surface germinate in 6 to 9 hours forming 1 to 4 germ tubes and appressoria that attach to the host cuticle by a gelatinous layer (Pastor-Corrales and Tu 1989). A compatible interaction between a susceptible cultivar of bean and a virulent race of *C. lindemuthianum* begins with the adhesion of conidia. Infective hyphae grow from the appressorium and penetrate the cuticle and grow between the cell walls and protoplast for 2 to 4 days without apparent symptoms on the infected tissue. Several days later, infection damages the host cuticle and cell walls are degraded enzymatically resulting in the appearance of water soaked lesions that darkens because of the presence of tannins (Schwartz et al. 2005). Some other species of *Colletotrichum* could not penetrate the membranes since they are melanin-deficient strains (Katoh et al. 1988) and they need cutinase to penetrate the host cuticle (Dikman and Patil 1986). As infection progress, the mycelium grows and aggregate within lesion resulting in formation of acervulii (forms of mycelium) that ruptures the host cuticle. Conidia are then formed within the water-soluble gelatinous matrix and serve as secondary inoculum (Dillard 1988).
Cool to moderate temperature (13 to 21°C with an optimum of 17°C) during a period of high humidity is necessary for development of anthracnose. Moisture is required for development, spread, and germination of the anthracnose spores and for infection of the plant (Brown-Rytlewski and Kirk 2006; Dillard 1988). Anthracnose is generally not a problem in dry conditions (Agrios 2005). The disease is spread by rainfall and wind (Brown-Rytlewski and Kirk 2006). The time from infection to visible symptoms ranges between 4 to 9 days, depending on the temperature, bean variety, and age of the tissues. Movement of people, machinery and wind in wet weather can carry the spores to healthy plants (Dillard and Hunter 1988).

1:2:5: Disease inoculation under controlled conditions

Different inoculation techniques are implemented in the growth room and under field conditions in order to characterize anthracnose races or to screen plant materials for resistance, based on the research objectives and available facilities. These methods include inoculation with a spore suspension to inoculate the seed (Bigirimana and Hofte 2001), sprayer and (or) syringe methods for stem inoculation (Schwartz et al. 1982), and brushing spores on to leaves or leaf inoculation (Tu 1986).

1.2.6: Disease control

Prevention of anthracnose epidemics in the field by minimizing pathogen inoculum may involve reducing the percentage of infected seed through producing seed in disease-free environments, cultural and chemical control measures, and host genetic resistance (Del Rio and Bradley 2002). The most desired control measure is the use of genetically-resistant varieties. Moreover integrated use of cultural and chemical control practices can significantly reduce the spread of the pathogen and the severity of the disease in a cropping system.
1.2.6.1: Cultural control

Bean anthracnose is caused by a seed-borne pathogen, which can survive in the alternate dry and wet soil conditions (Tu 1983). Among cultural practices, incorporation of the plant debris in the soil would hasten the breakdown of the debris, which would reduce fungal inoculum, which in turn will reduce the amount of initial inoculum source (Hall 1994), crop rotations that include crops other than legumes, especially in the areas where anthracnose has been identified and avoiding seeding earlier than the recommended dates have been recommended (Bush 2009; Schwartz et al. 2005).

1.2.6.2: Chemical control

Chemical control of anthracnose may include seed treatment with chemicals such as thiram, ceresam and ferbam (Tu 1992b) in different part of the world, pyraclostrobin for foliar treatment, and azoxystrobin, metalaxyl and fludioxonil are the major seed treatments used in North America (Gillard et al. 2012). Several chemicals for foliar application on bean crops have also been reported to provide some protection against anthracnose. These include Amistar, Armicarb, Bravo Ultrex, Bravo Weatherstik, Captafol, Carbendazim, Chlorothalonil, Echo Zn, Echomate Arnicarb, Equus, Headline, Maneb, Quadris Opti, Thiophanate methyl (Schwartz et al. 2005; Brown-Rytlewski and Kirk 2006). In Canada, registered products include azoxystrobin, pyraclostrobin and thiophanate-methyl with high efficacy, and mancozeb and chlorothalonil with lower efficacy (Del Rio et al. 2002). Two fungicides, pyraclostrobin, and azoxystrobin from Syngenta are effective against C. lindemuthianum in decreasing the severity of the disease (Gillard et al. 2012).

Nevertheless, seed fungicide is often not very effective when the pathogen is located in the embryo. A wide range of fungicides such as benomyl, carbendazim, chlorothalonil, and
thiophanate methyl are not sensitive against some isolates of *C. lindemuthianum* (Tu 1983; Maringini and Barros 2002).

Fungicide application is recommended as an effective measure at the early bloom, or at the late vegetative stages, but later on in the growth cycle, near the end of flowering or pod stage, may not provide enough protection, especially under heavy inoculum pressure (Gillard et al. 2012). In spite of that, 10-14 days after the first application, a second application may be considered because of the continued spread of disease infection (Gillard et al. 2012). However, fungicide application may have negative effects on the environment, farmers, and consumers.

Moreover, a hot-water treatment of bean seeds has also been reported to result in reduced fungus activity without reducing seed germination (Bush 2009). In this method, seeds are soaked at 18 to 22°C for 15 hours and then at 47°C for 25 minutes. This hot water treatment, however, has not become a routine practice among growers due to its impracticality on a large scale. Studies by Friesen et al. (2014) revealed microwave radiation of seed infected with *C. lindemuthianum decreased* disease incidence.

### 1.2.6.3: Biological control

Systemic Acquired Resistance (SAR) and Induced Systemic Resistance (ISR) through the application of the product of CGA 41396 (2,6-dichloroisonicotinic acid-INA) and CGA 245704 can play a role in control of *C. lindemuthianum* (CTA 1999). In a study by Bigirimana et al. (2000), *Trichoderma harzianum* were used in a liquid media on susceptible bean leaves against *C. lindemuthianum*. The study reported the observation of strong protection against *C. lindemuthianum* on beans when susceptible bean leaves were treated with *Trichoderma harzianum* in a liquid medium.
1.2.6.4: Genetic resistance

Genetic resistance against bean anthracnose is the most effective and environmentally-safe disease management strategy (Mahuku and Riascos 2004; Kelly and Vallejo 2004), least expensive and also easiest for farmers to adopt (Pastor-Corrales et al. 1995; Schwartz et al. 1982). However, high variability in the pathogen population and the possibility of resistance break down are the major constrains. There are no resistance genes that provide resistance against all known anthracnose races. Pyramiding of race-specific anthracnose resistance genes into a common background is expected to result in a more durable resistance in dry beans against various races of the pathogen (Mahuku et al. 2002).

1.3: Host-pathogen interaction in common bean-anthracnose pathosystem

Host-pathogen interaction in general is the outcome of different biological pathways both in the pathogen and the host, which involve several proteins that play important roles in controlling pathogenicity and virulence on the pathogen side and in recognition, and resistance response signaling in the host (Herbert et al. 2004).

In general, two groups of resistance mechanisms have been defined in plants. The first group of mechanisms is the pre-existing structural or chemical defense mechanisms that exist in plants independent of the pathogen attack. The second group of mechanisms is structural or chemical defense mechanisms that are induced and therefor activated following the attack and invasion by the pathogen (Agrios 1997). In *P. vulgaris* there are some barriers such as cuticle in the epidermal cell walls as well as chemical substances including hydroxyproline-rich glycoproteins (HPRGs) and polygalacturonase inhibiting proteins (PGIPs) in the cell walls, which have been reported in low amounts in non-infected bean plants and large amounts
following infection (Esquerre-Tugaye et al. 1992; De Lorenzo et al. 1990). Common bean defense against *C. lindemuthianum* is known to be elicited by compounds such as polygalacturonases (Devoto et al. 1997) and pectin fragments (Hargreaves and Bailey 1978), produced by the fungus and by the invaded bean cells, respectively, as well as some hydroxyproline-rich glycoproteins (HPRGs) that are stored in infected beans with resistance mechanisms (Mazau and Esquerre-Tugaye 1986). The resistance of common bean against *C. lindemuthianum* has been reported to be the outcome of hypersensitive response (Esquerre-Tugaye et al. 1992), in which cells around the infection sites collapse and diminish the growth of the pathogen. Different mechanisms such as increased phytoalexin activity have been reported to play a role in this hypersensitive response (Skipp and Bailey 1976). Besides, pathogenesis-related proteins (PR protein), which are produced by common bean, may play an important role in bean-anthracnose interaction (Daugrois et al. 1990).

Several studies have revealed that the resistant and susceptible phenotype in the host, common bean, or virulence and avirulence phenotype in the pathogen, *C. lindemuthianum*, are controlled genetically (Beebe and Pastor-Corrales 1991; Neema et al. 1994). Host resistance, conditioned by these major resistance genes is believed to follow the gene-for-gene interaction (Flor 1947; Goswami et al 2011). While complete resistance in the bean-anthracnose pathosystem is the most-widely studied resistance mechanism, partial resistance (Van der Plank 1963), identified by a reduced disease severity in a susceptible interaction is likely to play a role in overall resistance.

The major difficulty in the bean-anthracnose pathosystem, especially when it comes to breeding for disease resistance is the existence of several races of *C. lindemuthianum* in different countries around the world, which in addition to a high probability of development of new races
in the pathogen populations may result in the break-down of resistance either due to migration of races to different geographical regions or through mutations that result in new virulence patterns. More than 130 different races of *C. lindemuthianum* have been reported from different bean growing regions all over the world. Up to 15 races have been identified from Europe, USA and Brazil (Tu 1992; Jorge et al. 1998).

1.4: Anthracnose resistance genes in common bean

The gene symbol *Co* was proposed by Kelly and Young (1996) for anthracnose resistance genes. There are 29 independent major genes reported in *C. lindemuthianum*-common bean interaction, *Co-1* to *Co-14* and *Co-u, Co-v, Co-w, Co-x, Co-y, and Co-z*. *Co-9* gene is not included as it was found to be an allelic form of the gene *Co-3*. Some of the 29 genes are different allelic forms of the same locus. The gene *Co-1, Co-12, Co-13, Co-14, Co-w, and Co-x* are primarily from the Andean gene pool, while other genes are from the Middle-American gene pool (Ferreira et al. 2013). The gene *Co-8* is the only recessive anthracnose resistance gene, while the other genes are dominant (Dongfang et al. 2008; Kelly and Young 1996; Balardin et al. 1997; Young et al. 1998; Geffroy et al. 1999; Melotto et al. 2000; Alzate-Marín et al. 2001; Ragagnin et al. 2003; Kelly and Vallejo 2004).

The gene *Co-1* (formerly designated *A*) gene from the Andean gene pool was the first anthracnose resistance gene identified (Bulkholder 1918). It was discovered by McRosite (1919) in the differential variety Michigan Dark Red Kidney (MDRK). In 1960, Mastenbroek identified the gene *Co-2* (formerly designated *Are*) due to appearance of new races of bean anthracnose in the Middle American differential variety Cornell 49242. This gene was defeated following the appearance of new races like 31, and 89 (Kruger et al. 1977; Fouilloux 1976; Hubbelingg 1977). The gene *Co-3* (formerly designated *Mexique* 1) was discovered by Bannerot (1965) in the
Middle American differential variety Mexico 222. In 1969, Co-4 (formerly Mexique 2) and Co-5 (formerly Mexique 3) genes were discovered in the Middle American differential varieties by Bannerot (1965). The gene Co-4 was found in the variety TO and the gene Co-5 in the varieties TU, G23333, and SEL 1360.

The gene Co-1, Co-x, and Co-w have been mapped on linkage group Pv01 (Barrus 1915; Freyre et al. 1998; Méndez de Vigo 2001; Kelly et al. 2003; Rodriguez-Suarez et al. 2007; Geffroy et al. 2008), Co-u on Pv02 (Geffroy et al. 2008), Co-J3 on Pv03 (Lacanallo et al. 2010), Co-3/Co-9 on Pv04 (Geffroy et al. 1999; Rodriguez-Suarez et al. 2007), and Co-10 on Pv04 (Alzate-Marín et al. 2003; Rodriguez-Suarez et al. 2007), Co-y, Co-z on Pv04 (Bannerot 1965; Méndez-Vigo et al. 2005), Co-5 on Pv07 (Fouilloux 1976; Campa et al. 2005), Co-6 on Pv07 (Young and Kelly 1996; Kelly et al. 2003), Co-v on Pv07 (Campa et al. 2009), Co-4 on Pv08 (Fouilloux 1976; Melotto et al. 2004; Méndez de Vigo 2001; Rodriguez-Suarez et al. 2007), Co-2 on Pv11 (Adam-Blondon et al. 1994; Freyre et al. 1998). All these resistance loci segregate independently from one another except Co-3 and Co-9, which were later identified as two allelic forms of the same gene (Méndez-Vigo et al. 2005).

1.5: Races of C. lindemuthianum

Races of C. lindemuthianum are identified using a set of 12 differential lines (Pastor-Corales et al. 1987), each with a binary code ranging from 1 to 2048. Each race is designated a number which is the sum of the binary codes of the differential lines for which the interaction is compatible (susceptible). In Canada, race 23 was first reported in 1976, race 55 was reported in 1979 and race 89, which was characterized from a seed source grown in Ontario, was reported in 1992 (Wallen 1979; Tu 1994; Dongfang et al. 2007). More recent reports in Canada have detected anthracnose races of 23, 31, 73, 81, and 105 have in different provinces (Wallen 1979;
Tu 1994; Dongfang et al. 2007). The *C. lindemuthianum* races 23, 31, and 73 in Ontario, 23, 31, 73, and 105 in Manitoba (Dongfang et al. 2007; Del Rio et al. 2003a) and races 7, 73, 89, 1153 and 1161 in North Dakota (Goswami et al. 2011; Del Rio et al. 2003b) have been reported. Some races, such as race 73, are widely distributed across different common bean growing countries, from Argentina to Canada, while other races are reported only in specific regions of the world (Balardin et al. 1997). Unpublished results indicate that the prevalent race in Ontario is race 73, which was first reported in 2001 in Manitoba and later in 2003 in Ontario, Michigan and North Dakota (Kelly et al. 1994; Tu 1994; del Rio et al. 2003). This race is the predominant race in dry bean growing areas in Canada (Dongfang et al. 2008).

1.6: Breeding for anthracnose resistance

Genetic resistance against plant pathogens, including *C. lindemuthianum* of common bean, is known as the most efficient, environmentally-safe and socially-acceptable disease control measure as it can offer a long-lasting control strategy, (Mahuku and Riascos 2004; Kelly and Vallejo 2004). However, the occurrence of the new pathovars of the pathogen, which may defeat the resistance genes (Tu 1992) has often been a major limitation, when it comes to the durability of resistance against highly variable pathogens such as *C. lindemuthianum*.

Even though sources of resistance to anthracnose have been reported in both Mesoamerican and Andean gene pools, search of 20,000 accessions held at CIAT gene bank on host plant resistance revealed that some Mesoamerican accessions were resistant to all known anthracnose races, while the Andean beans were mostly susceptible (Pastor-Corrales et al., 1995).
1.7: Genetic mapping

Genetic mapping studies have identified seven main chromosome regions on Pv01, Pv02, Pv03, Pv04, Pv07, Pv08, and Pv11, which contain clusters of resistance genes where each member of the cluster conditions resistance to a specific race. These resistance genes (R-gene) encode proteins that are often involved in the infection recognition process. These proteins are coded by the members of the resistance gene analog family, which are known to encode different family of proteins including NBS-LRR (Nucleotide-Binding Site-Leucine rich repeat), eLRR (extracytoplasmic LRR), or LRR-Kinase proteins. The NBS-LRR genes in plants are often located in clusters of genes at linked loci (Michelmore and Meyers 1998).

1.8: Molecular markers linked to anthracnose resistance genes

In most breeding programs, the type of markers that have been proven most useful are SCAR (Sequence Characterized Amplification Region) markers (Young et al. 1998). Markers are mostly used in marker assisted selection (MAS) or marker-assisted backcrossing (Garzón et al. 2008; Ferreira et al. 2012). There are several markers for MAS linked to the Co-genes at distances of less than 5 cM, but a few are more tightly linked at less than 1 cM (Kelly and Vallejo 2004). Differential lines used for Colletotrichum lindemuthianum race identification of the major Co genes are shown in Table 1.1. Major molecular markers linked to resistance to specific isolates or races of C. lindemuthianum are localized in different Co-gene cluster regions.

Major markers reported to be linked to resistance to anthracnose race 73 in different sources are OF10530 (Young and Kelly 1997; Rodríguez-Suárez et al. 2007; Campa et al. 2009, 2011), CV542014 (Gonçalves-Vidigal et al. 2011), and TGA1.1 (Gonçalves-Vidigal et al. 2011) on Pv01, localized in Co-1 cluster, SW12 (Miklas et al. 2000; Rodríguez-Suárez et al. 2007) on Pv11, localized in Co-11 cluster.

1.9: Common bean genome

The common bean genome was recently sequenced (McClean et al. 2010; Schmutz et al. 2014). The current version V1.0 of the reference genome sequence of the common bean is available at Phytozome V10 (www.phytozome.net). The genome assembly is approximately 521.1 Mbp. There are 27,197 loci containing 31,638 protein-coding transcripts and 4,441 alternatively spliced transcripts in total.

In plants, resistance genes (R genes) in a host interact with Avirulence genes (Avr) in the pathogen as an activator or detector against pathogen (Chisholm et al. 2006). The R gene family includes genes that encode NBS-LRR (Nucleotide-Binding Site-Leucine rich repeat), eLRR (extracytoplasmic LRR), or LRR-Kinase proteins. The NBS-LRR genes in plants are often located in clusters of genes at linked loci (Michelmore and Meyers 1998).

The genomic regions contain clusters of resistance genes where each member of the cluster conditions resistance to a specific race. These resistance genes (R-gene) encode proteins that are often involved in the infection recognition process. These proteins are coded by the members of the resistance gene family (Michelmore and Meyers 1998).
1.10: Objectives and Hypothesis

The objectives of this research were to:

1) Study the inheritance of resistance to race 73 of *C. lindemuthianum* in the navy bean line Bolt,
2) Identify the genomic region(s) controlling resistance to *C. lindemuthianum* race 73 in Bolt,
3) Study the gene effect of resistance gene(s) in Bolt,
4) Identify molecular marker(s) associated with resistance to *C. lindemuthianum* race 73, and
5) Characterize candidate resistance gene(s) associated with *C. lindemuthianum* race 73 resistances in Bolt

**Hypotheses:**

The progeny of the cross between Bolt and H4784A-29844 (race 73 susceptible) are similar in their response to *C. lindemuthianum* race 73 infection in the field and growth room evaluations. Segregation of anthracnose resistance alleles at the resistance locus in the RIL population follows a Mendelian 1:1 ratio. Furthermore, I hypothesize that there is at least one molecular marker linked to the gene(s) that cause resistance to race 73 of *C. lindemuthianum* in Bolt, the sequence of the candidate anthracnose resistance gene(s) is non-polymorphic between Bolt and H4784A-29844, and that the protein translated from the candidate anthracnose resistance gene(s) is similar in Bolt and H4784A-29844.
1.11: Figures and Tables

Table 1.1. Differential lines used for *Colletotrichum lindemuthianum* race identification (Dongfang *et al.*, 2008)

<table>
<thead>
<tr>
<th>Differential Line</th>
<th>Gene Pool</th>
<th>Binary No.</th>
<th>Resistance Gene</th>
<th>23</th>
<th>31</th>
<th>73</th>
</tr>
</thead>
<tbody>
<tr>
<td>Michelite</td>
<td>MA</td>
<td>1</td>
<td><em>Co-1</em></td>
<td>S</td>
<td>S</td>
<td>S</td>
</tr>
<tr>
<td>MDRK</td>
<td>AN</td>
<td>2</td>
<td><em>Co-1</em></td>
<td>S</td>
<td>S</td>
<td>R</td>
</tr>
<tr>
<td>Perry Marrow</td>
<td>AN</td>
<td>4</td>
<td><em>Co-1</em>^3*</td>
<td>S</td>
<td>S</td>
<td>R</td>
</tr>
<tr>
<td>Cornell 49242</td>
<td>MA</td>
<td>8</td>
<td><em>Co-2</em></td>
<td>R</td>
<td>S</td>
<td>S</td>
</tr>
<tr>
<td>Widusa</td>
<td>AN</td>
<td>16</td>
<td><em>Co-1</em>^5*, <em>Co-9</em>^3*</td>
<td>S</td>
<td>S</td>
<td>R</td>
</tr>
<tr>
<td>Kaboon</td>
<td>AN</td>
<td>32</td>
<td><em>Co-1</em>^2*</td>
<td>R</td>
<td>R</td>
<td>R</td>
</tr>
<tr>
<td>Mexico 222</td>
<td>MA</td>
<td>64</td>
<td><em>Co-3</em></td>
<td>R</td>
<td>R</td>
<td>S</td>
</tr>
<tr>
<td>PI 207262</td>
<td>MA</td>
<td>128</td>
<td><em>Co-4</em>^3*, <em>Co-9</em></td>
<td>R</td>
<td>R</td>
<td>R</td>
</tr>
<tr>
<td>TO</td>
<td>MA</td>
<td>256</td>
<td><em>Co-4</em></td>
<td>R</td>
<td>R</td>
<td>R</td>
</tr>
<tr>
<td>TU</td>
<td>MA</td>
<td>512</td>
<td><em>Co-5</em></td>
<td>R</td>
<td>R</td>
<td>R</td>
</tr>
<tr>
<td>AB136</td>
<td>MA</td>
<td>1024</td>
<td><em>Co-6</em>, <em>co-8</em></td>
<td>R</td>
<td>R</td>
<td>R</td>
</tr>
<tr>
<td>G2333</td>
<td>MA</td>
<td>2048</td>
<td><em>Co-4</em>^2*, <em>Co5</em>^2*, <em>Co7</em></td>
<td>R</td>
<td>R</td>
<td>R</td>
</tr>
</tbody>
</table>
Figure 1.1. Dry bean market classes in North America Photo courtesy of L. Copeland
Figure 1.2. Distribution of common bean production regions in the world in 2013.
Figure 1.3. Dry bean production in selected countries (FAOSTAT, 2014).
Chapter 2. Inheritance of resistance to race 73 of *Colletotrichum lindemuthianum* in the navy bean Bolt

2.0. Abstract

Anthracnose of common bean (*Phaseolus vulgaris* L.), caused by the fungus *Colletotrichum lindemuthianum*, is an important disease, worldwide. Resistance to race 73 of the fungus *C. lindemuthianum*, in a population of 126 F$_{5:6}$ recombinant inbred lines of a cross between the resistant navy bean cultivar Bolt and the susceptible line H4784A-29844 was phenotyped in growth room and two field locations over two years, 2012 and 2013, in Ontario, Canada and genotyped using SNP markers. Six QTL were identified for the Area Under Disease Progress Curve (AUDPC) and seven QTL were detected based on the Seed Infection Index (SII), while only the QTL on Pv01 was found to be repeatedly significant with major effect across all test environments. The identified QTL accounted for 10 to 79% and 11 to 64% of the phenotypic variation in AUDPC and SII, respectively. Two linked SNP markers PvSNP8p1922017 and PvSNP8p1574781, on Pv01 were associated with the major effect anthracnose resistance in cultivar Bolt. In the genomic region of the major effect QTL on Pv01, where the *Co-1* cluster is located, 27 putative candidate genes from the resistance gene family were identified.
2.1. Introduction

2.1.1: Anthracnose

Anthracnose of common bean (*Phaseolus vulgaris* L.), caused by the fungus *Colletotrichum lindemuthianum*, (Sacc. & Magnus) Lams.-Scrib, is a destructive disease worldwide (Pastor-Corrales and Schoonhoven 1987; Barcelos et al. 2011). The disease is most problematic in areas where beans are grown in relatively cooler and more humid environments in temperate regions of the world or at higher altitudes in the tropics. The ideal environment for conidial growth and tissue infection is a temperature of 13 to 26 °C with an optimum of 17 °C and with higher than 92 % humidity (Schwartz et al. 2005). *C. lindemuthianum* is primarily a seed-borne pathogen (Barrus 1921), which can attack all aerial plant parts and may cause yield losses as high as 100% (Schwartz et al. 2005). Infected seed is therefore the main means of pathogen dispersal from year to year, while frequent rain, especially when accompanied by wind or splashing rain, in addition to movement of machinery, animals, and humans through the field are important for local dispersion of the conidia (Schwartz et al. 2005; Tu 1994).

2.1.2: Pathogenicity and resistance against anthracnose

The fungus *C. lindemuthianum* is highly variable, with more than 100 pathogenic variants and races reported in the scientific literature (Balardin and Kelly 1998). These variants are identified based on their pathogenic response on a set of 12 differential cultivars using a standardized method (Pastor-Corrales 1991), in which *C. lindemuthianum* races are designated a binary code, which can range from 1 to 2048. Using this standardized set of differentials, it has been demonstrated that some races, such as race 73, are widely distributed across different growing regions and in many countries, from Argentina to Canada, while other races are reported only in specific regions of the world (Balardin et al. 1997). In Canada, races 23 was first
reported in 1976, race 55 was reported in 1979 and race 89, which was characterized from a seed source grown in Ontario in 1992 had historically been reported, while more recent reports have also reported (Wallen 1979; Tu 1994; Dongfang et al. 2007). Recent unpublished results indicate that the current prevalent race in Ontario is race 73, which was first reported in 2003 in Manitoba and earlier in Ontario, Michigan and North Dakota (Kelly et al. 1994; Tu 1994; del Rio et al. 2003). Races of *C. lindemuthianum* are known to have *co*-evolved in the two centers of domestication of common bean, Andean and Middle American (Gepts 1998) and therefore races of the pathogen tend to be associated with one gene pool or the other, however races with virulence on both Andean and Mesoamerican gene pools exist (Balardin et al. 1997).

### 2.1.3: Breeding and genetics of anthracnose resistance

Genetic resistance is the most environmentally-safe and socially-acceptable disease management strategy for many plant diseases, including bean anthracnose (Mahuku and Riascos 2004; Kelly and Vallejo 2004), and can offer a long-lasting control measure. However, the appearance of new races of *C. lindemuthianum*, which may overcome the host resistance, can cause a major challenge for bean breeders. Pyramiding of race-specific anthracnose resistance genes is, therefore, expected to result in durable resistance in dry beans against various races of the pathogen.

Up to 29 anthracnose resistance genes (designated *Co*- followed by a number) have so far been described in common bean; *Co-1* to *Co-14* and well as *Co-u, Co-v, Co-w, Co-x, Co-y*, and *Co-z*. The list does not include *Co-9*, as it was found to be an allelic form of the gene *Co-3*. Some of these genes are different allelic forms of the same locus. The genes *Co-1, Co-1², Co-1³, Co-12, Co-13, Co-14, Co-w, and Co-x* have been reported in the genotypes from the Andean gene pool, while the other genes are reported in genotypes from the Middle American gene pool.
(Ferreira et al. 2013). All anthracnose resistance genes except $co-8$ are reported to have a complete dominance gene effect.

### 2.1.4: Molecular mapping

Genetic mapping studies have identified seven main chromosome regions on linkage groups Pv01, Pv02, Pv03, Pv04, Pv07, Pv08, and Pv11, as the genomic positions of the anthracnose resistance genes. These genomic regions are thought to contain clusters of resistance genes where each member of the cluster conditions resistance to a specific race. These resistance genes (R-gene) encode proteins that are often involved in the infection recognition process. These proteins are coded by the members of the resistance gene analog family, which are known to encode a different family of proteins including NBS-LRR (Nucleotide-Binding Site-Leucine rich repeat), eLRR (extracytoplasmic LRR), or LRR-Kinase proteins. The NBS-LRR genes in plants are often located in clusters of genes at linked loci (Michelmore and Meyers 1998).

Genetic studies of *C. lindemuthianum* resistance have often reported a qualitative mode of inheritance with major genes that follow a gene-for-gene interaction model (Flor 1955). These studies are often based on the phenotypic classification of members of a segregating population(s) into resistant and susceptible, based on visual disease evaluations conducted in a controlled environment. In other host-obligate pathogen systems, however, quantitative forms of resistance, such as partial resistance (Parlevliet 1978) known to be conditioned by few to several genes with additive effects have also been extensively investigated *e.g.*, cereal-rust pathosystems, in which partial resistance has been defined as a compatible host-pathogen interaction where the final disease severity is low. The quantitative mechanisms of resistance are expected to condition more durable resistance (Johnson 1984). However, the study by Geffroy et
al, (1999), where a relationship between genes controlling partial and specific resistance was reported, is the only study to examine partial resistance against anthracnose in common bean.

2.1.5: Objectives

The objectives of this research were to 1) study the inheritance of resistance to race 73 of anthracnose in the navy bean variety ‘Bolt’ as a qualitative trait in both growth room and field disease nursery trials and 2) identify the genomic regions that contribute to race 73 anthracnose resistance.
2.2. Materials and Methods

2.2.1: Plant materials:

A population of 126 F\textsubscript{5/6} recombinant inbred lines (RIL) was developed from a cross between the race 73 resistant navy bean cultivar Bolt and the susceptible line H4784A-29844 using the single seed descent approach in a greenhouse at the Greenhouse and Processing Crops Research Centre-Agriculture and Agri-Food Canada (GPCRC-AAFC) in Harrow, Ontario, Canada. Bolt with the pedigree OAC\_Rex/A98083//AC\_Compass/B98143/3/RESW2138/B981045//B98213/Kippen is a navy bean variety resistant to race 73 of \textit{C. lindemuthianum}. H4784-29844 is an anthracnose susceptible navy bean line derived from the cross OAC Rex//Kippen/Vista.

2.2.2: Field trial

2.2.2.1: Experimental design

A total of 144 entries, including 126 RILs, the 2 parental lines, the 12 anthracnose differential lines (Pastor-Corrales, 1991), and 4 common bean checks were evaluated for their response to \textit{C. lindemuthianum} race 73 in the artificially inoculated field disease nurseries at the University of Guelph Elora Research Station (ERS) and Huron Research Station (HRS) in Ontario, Canada in 2012 and 2013. The experimental design in each site-year was a 12 × 12 unbalanced square lattice design (Cochran and Cox 1957) with two replications. The experimental unit in all field locations consisted of a hill plot with seven seeds, planted with a row spacing of 60 cm, hill spacing of 60 cm, and planting depth of 3.5 cm.
2.2.2.2: Disease inoculation

Anthracnose was artificially initiated in the field disease nurseries by growing spreader rows, using C. *lindemuthianum* race 73 infected seed, as the border rows around the experiment and as a solid row, planted every third row within the experiment, so that each hill plot was bordered on one side by an anthracnose spreader row. Artificial inoculation at a rate of 10 ml m\(^{-2}\) using fresh, sporulating conidial cultures of anthracnose race 73, with a spore concentration of 1 x 10\(^5\) spores per mL with Hemacytometer and diluted by distilled water, was carried out at Huron in 2012 and in both locations in 2013.

2.2.2.3: Disease rating

Anthracnose severity was rated when pods were in early stages of seed filling using a 0-10 visual score, corresponding to a percentage of pods showing infection (0=no visible symptoms; 1=1-10%; 2=11-20%; 3=21-30%; 4=31-40%; 5=41-50%; 6=51-60%; 7=61-70%; 8=71-80%; 9=81-90%; 10=91-100%). Disease rating was repeated four times during the growing season, starting when the susceptible check was 60% infected and repeated every seven days thereafter until maturity. The area under the disease progress curve (AUDPC), as a measure of disease severity over time, was then estimated for each plot as:

\[
AUDPC = \sum_{i=1}^{n} \frac{y_i + y_{i+1}}{2} \times (t_{i+1} - t_i)
\]

Where, \(y_i\) is the \(i^{th}\) severity rating and \(t_i\) is number of days after inoculation.

After maturity, harvested seed from each experimental unit was visually inspected and sorted into five seed infection groups (0 to 4) based on the severity of seed infection, in which seed samples with no visible symptoms were placed in seed infection group 0, 1-25% in group 2, 26-50% group 3 and plots with more than 75% of the seed surface showing severe anthracnose
infection were placed in seed infection group 4. A seed infection index (SII) was then computed for each experimental unit as:

\[ SII = \sum (n_i \cdot S_i) \]

where, \( n_i \) is the number of seeds in each infection group and \( S_i \) is the seed infection severity score of the group (0-4).

2.2.3: Growth room trial

2.2.3.1: Experimental design

A sub-set of 73 RILs from the population and the parental lines were evaluated for their response to race 73 of *C. lindemuthianum* in a growth room at the University of Guelph. Ten single plants of each RIL were grown in 10 cm fiber pots, which were filled with sterilized soil mixture (Irvin, California) in a growth room, maintained at 23 °C with 8 hours dark and 16 hours light, at the University of Guelph. Pots were irrigated once a week and fertilized with liquid fertilizer (20-20-20 NPK) as required.

2.2.3.2: Disease inoculation

Young seedlings were inoculated 10 days after planting. The inoculum was produced from fresh sporulating *C. lindemuthianum* cultures grown on Mathur’s medium at 25°C following the protocol described by Pastor-Corrales et al (1995), with modifications. A conidial suspension with concentration of \( 1.2 \times 10^6 \) conidia per L in sterile water was prepared and sprayed on the fully expanded unifoliate leaves of plants until run-off was evident. A hemacytometer was used to measure the spore concentration. Inoculated plants were kept in a humid chamber with > 95% humidity at 22-25°C for 48 h. After this incubation period, the growth room temperature was set to 28°C with a 16 hour photoperiod.
2.2.3.3: Disease rating

Disease reactions were evaluated at 7, 14, and 21 days after inoculation using a 1 to 9 scale (Pastor-Corrales et al. 1995). Plants with the scores 1 to 3 were considered resistant, and with scores 4 to 9 were considered susceptible. Genotype of the F5 single plant, from which each F5:6 RIL was derived, was imputed by grouping the RILs into three genotypic groups: parental type resistant (PTR), when all single plants within the RIL were resistant, parental type susceptible (PTS), when all single plants within the RIL were susceptible, and segregating (Seg), when single plants within the RIL had different responses. The conformity of the observed segregation ratios against the expected 0.46875 PTR: 0.625 Seg: 0.46875 PTS segregation ratios for one dominant gene in F5 generation were tested using a chi-square ($\chi^2$) test (Yates 1934).

2.2.4: Genotyping

2.2.4.1: DNA extraction

Leaf samples were taken from the first trifoliate leaves of the parental lines and the 126 F5:6 RIL and lyophilized. Genomic DNA was extracted from 1 to 2 g of lyophilized leaf tissue according to the method explained of Yu et al. (1999) using a DNeasy plant Mini DNA Kit (QIAGEN Inc, Irvin, California) following the manufacturer protocol, with minor modifications. Grinding was performed using a FastPrep grinder (BIO-101, Irvin, California) using grinding beads in screw-cap tubes. DNA concentration was measured using DNA NanoDrop spectrophotometer (Thermo Fisher Scientific, DE, USA) and was diluted to 50 ng/μL and stored at -20°C.
2.2.4.2: Marker assays

SNP Genotyping. DNA samples of a subset of 92 F$_{5:6}$ RILs and two sets of the two parental lines were genotyped at the University of Saskatchewan, using the Pv768 common bean Illumina GoldenGate array (Illumina Inc., San Diego, CA) developed at the University of Saskatchewan, Canada (Bett, unpublished). Genotyping was carried out according to the standard assay protocol (http://www.illumina.com/technology/goldengate_genotyping_assay.ilmn). Products generated by this assay were read with an Illumina HiScan (Illumina Inc., San Diego, CA) and the resulting data were clustered for allele calling using Genome Studio software version 2010.3 (Illumina Inc., San Diego, CA). Allele calls were visually inspected for errors in automatic allele calling and corrected where deemed necessary. Any calls that were not clearly one allele or the other were reported as missing data to avoid errors.

2.2.4.3: linkage mapping

A linkage map was constructed using JoinMap 4 software (Van Ooijen JW 2006) with genotypic data of 86 polymorphic SNP markers. Linkage groups were established with the LOD threshold of 7 using Kosambi mapping function (Kosambi 1944).

2.2.4.4: Physical mapping

The physical locations of the SNP markers in the bean reference genome were determined by alignment of the flanking sequence information with Phaseolus vulgaris reference
genome (Pv1.0) available at Phytozome (http://www.phytozome.net/). MapChart 2.2 (Voorrips 2002) was then used to draw the physical and linkage maps and to compare the alignment of markers on the two maps.

2.2.5: **Statistical analyses**

Statistical analyses of the quantitative data (AUDPC and SII) were performed using SAS 9.3 (SAS Institute, Cary, NC). Each location-year of data was first analyzed separately using the PROC MIXED procedure, in which block and sub-block nested within block were considered random effects and genotype a fixed effect. A combined analysis of variance was then performed using the PROC MIXED procedure, where environment (combination of locations and years), block within environment, sub-block within environment were considered random effect and genotype was considered a fixed effect. Least square means (lsmeans) and confidence limits (P < 0.05) were calculated for AUDPC and SII for each location-year separately and for the combined analysis by using lsmeans statement in the PROC MIXED procedure.

2.2.5.1: **QTL analysis**

Single marker QTL analysis was conducted using the PROC GLM procedure to examine the linear model, in which the phenotypic value is tested as a function of the SNP genotype at each locus. The coefficient of determination of the model ($R^2_p$) was computed as a measure of the proportion of phenotypic variance accounted for by each marker locus. The additive effect at each marker locus was estimated as half the difference of the Lsmean estimates of the two homozygous genotypic groups. Multi-locus models were computed by using PROC REG procedure using stepwise selection to determine the QTL with highest contribution in AUDPC and SII.
In order to identify putative candidate genes associated with the significant QTL, G-Browse on Phytozome v 10 (http://www.phytozome.net) was used to search the available common bean genome sequence and identify the most likely genes from the resistance gene family in the QTL regions.
2.3. Results

2.3.1: Field-testing

A wide continuous variation in disease reaction scores was observed for both quantitative measures of resistance, AUDPC and SII, across the RIL population in all four environments tested and when data was combined across location years (Fig. 2.1). The ERS location in 2012 had a lower average AUDPC (3.11) compared to the other environments, due to an unusually dry season and the use of passive inoculation from spreader rows only. In every other environment tested, ERS 2013, HRS 2012, and HRS 2013, the anthracnose severity of susceptible lines reached the highest disease rating. The parental lines of the population were highly separated along the disease distribution in terms of both AUDPC and SII in all four environments. The resistant parental genotype, Bolt, showed resistant responses in all environments with AUDPC scores of 0, 7.5, 0, 37, while H4784-29844 had AUDPC values 97.5, 175, 165.5, 228 at ERS 2012, ERS 2013, HRS 2012, and HRS 2013, respectively. Statistical analysis of each environment separately showed highly significant genotypic effects for both traits measured ($P < 0.05$). The statistical analyses presented (Table 2.1) are, however, for all four environments combined due to the high level of repeatability across environments. Bolt had an overall average AUDPC score of 22 while H4784-29844 scored 165.07; SII average values were 0.001 and 52.13 for Bolt and H4784-29844, respectively. The AUDPC and SII mean for the RIL population was 37.18 (5% confidence limit of the mean (CL 5%) = 7.07) and 4.92 (CL 5% = 1.54), respectively (Fig 2.1).

The genotypic effects were highly significant ($P \leq 0.001$) for both traits, AUDPC and SII. For the four environments combined (Table 2.1). The effect of environment was significant for both AUDPC and SII, although the magnitude of the effect was much larger for AUDPC than SII.
(F-values of 328.1 and 52.4, respectively). The genotype by environment interaction was also significant for both AUDPC and SII, although there were very few major rank changes where genotypes classified as susceptible in one environment were resistant in another environment, and vice-versa.

2.3.2: Growth room testing

Segregation of the three disease reaction groups in the growth room test in a sub-set of 73 RILs from the mapping population conformed to the expected segregation ratios of 0.46875 PTR: 0.6250 Seg: 0.46875 PTS ($\chi^2=1.12; p < 0.05$) for one dominant gene in F5 generation. These phenotypic groups co-segregated with the SNP markers PvSNP8p1922017 and PvSNP8p1574781 with minimal discrepancies: only two RILs phenotyped as parental-type resistant had heterozygote SNP marker genotypes (Table 2.2).

2.3.3: Linkage map and QTL analysis

A final genetic map for this population was constructed using 86 polymorphic SNP markers, resulting in total map coverage of 624.81 cM across 11 linkage groups with an average distance between two markers of 6.77 cM (Table 2.3). The 11 linkage groups represent 10 chromosomes, with no representation of Pv04 and with two unjoined linkage groups representing segments of Pv06. The linkage map aligned well with the physical map (Fig 2.2). Among the 86 markers used, 24 markers showed significant segregation distortion (nine markers at p < 0.05; six at p < 0.01; and nine at p < 0.001). Thirteen of these significantly distorted markers were on Pv06 (Table 2.3).

Six single loci QTL for AUDPC were found on linkage groups Pv01, Pv02, Pv03, Pv08, Pv10 and Pv11 and seven QTL were found for SII on linkage groups Pv01, Pv02, Pv03, Pv05, Pv06, Pv08, and Pv11 at individual environments. A QTL on Pv01 with a very large effect was
found consistently effective across location years. This QTL, significantly associated with six SNP markers in a 0.1 Mbp range, corresponding with 1.5 cM on the linkage map, was positioned at 90.6 cM corresponding with 50.17 Mbp in the physical map. These six SNPs co-segregated with the three phenotypic groups in the growth room test, each conforming to the segregation of a single Mendelian locus in the \( \chi^2 \) test (P > 0.05). When data of the three phenotypic groups of the growth room test were incorporated into the mapping data, the most likely position of the resistance gene was identified between the SNP markers PvSNP8p1922017 and PvSNP8p1574781 at 0.0 cM.

In addition to the major effect QTL on Pv01 (\( R^2_p = 0.79 \)), in a combined analysis across environments, two other QTL with smaller effects were detected for AUDPC on Pv03 and Pv08, accounting for 17 and 10 % of the variation, respectively. The same analysis for SII, detected the major effect QTL on Pv01 (\( R^2_p = 0.64 \)) in addition to seven other QTL with smaller effects on Pv02, Pv03, Pv05, Pv06, Pv08, Pv09, and Pv11 (Table 2.4), each accounting for 11 to 18 % of the variation. In terms of AUDPC, in Elora 2012, 2013 and Huron 2012, SNP marker PvSNP8p1922017 on PV01, in Huron 2012, PvSNP8p1574781 on Pv01, in Huron 2012, PvSNP02p2514658 on Pv02, in all four locations, PvSNP15p37366 on Pv03, and in Huron 2013, PvSNP126p592970, PvSNP908p88850, and PvSNP10p525994 on linkage groups Pv08, Pv10, and Pv11 were identified as significant QTL, respectively. Due to the relatively low infection rate, SII data was not collected for Elora 2012. In terms of SII, in Huron 2012 and 2013, PvSNP8p1922017 on Pv01, in Huron 2012, PvSNP02p2514658 on Pv02, in Huron 2012 and 2013, PvSNP15p37366 on Pv03, in Elora 2013, PvSNP68p856579 on Pv05, in Huron 2013, PvSNP11p2016349 on Pv06, in Huron 2013, PvSNP12p711403 on Pv08, and in Huron 2013, PvSNP10p525994 on Pv11 were identified as being significant QTL (Table 2.4).
The coefficient of determination \( (R^2_p) \) of the significant QTL ranged from 0.10 to 0.79 for AUDPC and 0.11 to 0.64 for SII (Table 2.4). Major QTLs, \( \text{PvSNP8p1922017} \) and \( \text{PvSNP8p1574781} \) for AUDPC and \( \text{PvSNP8p1922017} \) for SII were located in the same region on \( \text{Pv01} \). QTL in this region explained up to 0.79 of total variation in AUDPC and 0.64 for SII and had an additive effect of up to 0.87 for AUDPC and 0.85 for SII (Table 2.4).

The results of combined environments analyses based on multi-locus ANOVAs in a stepwise regression analyses revealed large QTL effect on linkage group \( \text{Pv01} \) for both traits, AUDPC and SII. In the multi-locus model, \( \text{PvSNP8p1922017} \) accounted for 79% and 72% of the variation of AUDPC and SII, respectively (Table 2.5). In addition to major effect QTL, three minor QTL on \( \text{Pv06} \), \( \text{Pv08} \) and \( \text{Pv11} \) fit in the multi-locus model for SII, however, no minor QTL added additional resolution to the multi-locus model for AUDPC.

Since the presence of a major QTL can reduce the likelihood of detecting a smaller QTL, a second analysis of single factor QTL was performed on the sub-set of 92 RILs that did not carry the resistant allele of the two highly significant markers associated with the major effect QTL on \( \text{Pv01} \). The highest coefficients of determination were found for \( \text{PvSNP10p427656} \) \( (R^2_p = 0.43) \), \( \text{PvSNP10p474186} \) \( (R^2_p = 0.43) \), and \( \text{PvSNP10p525994} \) \( (R^2_p = 0.43) \) on \( \text{Pv11} \) for AUDPC in Elora 2013, and \( \text{PvSNP12p711403} \) \( (R^2_p = 0.43) \) on \( \text{Pv08} \) for SII in Huron 2013.

The JBrowse search in the common bean genome sequence, performed in a 1.1 Mbp region around the significant genomic region (50.17 Mbp on \( \text{Pv01} \)), identified 27 putative candidate genes, of which one included Leucine Rich Repeat (LRR) domain, five were protein kinase genes, and 13 had ATP or GTP binding site domains. In addition, four kinase genes with ATP binding domain and four kinase genes with both LRR and ATP binding site domains were found in the significant region of the genome (Table 2.6).
2.4. Discussion

Studying an F$_{5:6}$ population from the cross Bolt × H4784A-29844, inoculated with anthracnose race 73, identified a genomic region at 0.0 cM corresponding with 50.17 Mbp on the physical map of Pv01 with a major effect on anthracnose disease progression. Two tightly linked SNP markers (PvSNP8p1922017 and PvSNP8p1574781) on Pv01 were associated with anthracnose resistance in Bolt, consistently across location-years. Of the previously identified anthracnose resistance genes, the Co-1 cluster has been reported on Pv01 (Mendez de Vigo 2001; Kelly et al. 2003; Kelly and Vallejo 2004). The cultivar Seafarer, a navy bean of Middle American origin, was included in the parentage of Bolt and has previously been reported as carrying anthracnose resistance alleles at the Co-1 locus (Melotto and Kelly 2000; Beaver et al. 2003; Kelly and Vallejo 2004). The phenotypic ratios of the 73 F$_{5:6}$ RILs inoculated with race 73 of C. lindemuthianum in growth room studies revealed a segregation pattern that fits the expected ratios for the presence of one single dominant allele. All six SNPs with significant association with phenotype co-segregated with a resistant response and were also segregating in the expected 0.46875 PTR: 0.625 Seg: 0.46875 PTS Mendelian ratios in the F$_{5:6}$ population. It was therefore concluded that anthracnose resistance in the navy bean Bolt is conditioned mainly by one of the alleles at the Co-1 locus.

Multiple genes have been reported at the Co-1 cluster including Co-1, Co-1$^2$, Co-1$^3$, Co-1$^4$, and Co-1$^5$ (Kelly and Vallejo 2004; Goncalves-Vidigal et al. 2012). The resistant parent of this population, Bolt, had a susceptible response against race 23 (virulent on Co-1, Co1$^3$, and Co-1$^5$) and race 105 (virulent on Co-1$^2$) and a resistance response against race 2 (virulent on Co-1) (Dr. R. L. Conner, AAFC, Morden, MB, Canada, personal communication). This suggests that Co-1
is the most likely resistant allele of Co-1 in Bolt. This speculation may need further verification using allelic studies, which is beyond the scope of this research.

In previous studies, Kelly and Vallejo (2004), Vallejo and Kelly (2008) identified an STS marker in an Andean mapping population, which was linked to the Co-1 locus. Kelly and Vallejo (2004) also reported that Co-1 is located near two genes, the determinacy gene fin which controls determinant growth habit and the photoperiod response gene Ppd, on Pv01 in both Andean and Mesoamerican gene pools. However, the determinant phenotype occurs more in the Andean gene pool. The SNP marker PvSNP8p1922017 on Pv01 in the current study had the closest association with anthracnose severity, accounted for the highest amount of phenotypic variation in both quantitative traits, AUDPC and SII, and co-segregated with the resistance phenotype in growth room studies and therefore is a suitable candidate to be used to develop breeder-friendly markers for selection in plant breeding programs.

This study also attempted to identify QTLs of minor effects which may interact with the major anthracnose resistance loci in the navy bean Bolt. For this reason, the phenotypic variation for anthracnose response was studied in the field trials in 2012 and 2013 for disease severity ratings using two quantitative measure of anthracnose resistance, AUDPC and SII. The AUDPC was computed as a measure of disease severity over time and SII was estimated as a quantitative measure of the effect of anthracnose on the final plant product, seed, which is important considering the seed-borne nature of anthracnose disease in common bean (Tu 1983; Pastor Corrales and Tu 1989). In both cases, continuous variation of phenotypic values coupled with the presence of transgressive segregation was evident in the frequency distributions, which suggest the involvement of additional minor genes contributing to the overall anthracnose resistance response in this population. These additional QTL seem to have additive effects. However, even
though the identified minor effect QTLs were similar in some cases, they were not repeatedly
detected as being significant for AUDPC and (or) SII across years and locations. Additionally,
when tested in the absence of the major QTL on Pv01, there were not significant repeatable QTL
for both AUDPC and SII in all locations.

Similarly, QTL analysis of the combined data, in addition to the major effect QTL on
Pv01 corresponding with the Co-1 cluster, identified additional QTL with smaller effects for
both AUDPC and SII. While three QTL were identified for AUDPC, a total of 8 QTL were
identified to contribute to SII in single marker analysis, from which four QTL on Pv01, Pv06,
Pv08, and Pv11 were selected in the multiple QTL analysis using step-wise selection model.
While the additional QTL may be related to other non-specific types of resistance mechanisms,
the number of identified QTL may suggest a higher level of complexity of anthracnose response
in SII compared with AUDPC. Such non-specific QTL may suggest the possibility of breeding
for partial resistance against anthracnose in common bean. Partial resistant was originally
defined by Parlevliet (1975) in leaf rust barley host-pathogen interaction as a resistance
phenotype, in which a relatively small disease severity is accompanied by high (susceptible)
infection type. This type of resistance, which is often controlled by few to several genes with
additive effects, is known to condition more durable resistance against obligate parasites in plant-
pathogen interactions (Singh and Rajaram, 2002). The study by Geffroy et al. (2000) reported the
co-localization of QTL for quantitative partial resistance against anthracnose with resistance
major genes and reported possible relationship between genes for specific and partial resistance
against anthracnose. Among the chromosomes carrying QTL for quantitative anthracnose
resistance in this population, only Pv06 has no major anthracnose resistance genes reported
(Ferreira et al. 2013).
Resistance gene (R gene) products in the host act as an activator of defense responses or detectors of pathogens (Chisholm et al. 2006). The types of genes typically involved in resistance responses include: serine-threonine protein kinases, lucine-rich repeats (LRRs), receptor-like kinases, and nucleotide binding site (NBS)- LRRs (Lopez et al. 2003). The largest groups of R genes are those that carry the NBS-LRR domains. These are classified into two groups, based on the N-terminal domain. The resistance genes are often located in tightly linked clusters (Ferreira 2013). A recent study by Richard et al. 2014, in a study of the Co-x gene on Pv01, identified eight genes, of which four were kinase genes, and suggested that Co-x is not a classical NBS-LRR gene. The authors concluded that Co-x is not a member of the Co-1 cluster and they are separated by 190Kb, based on the position of markers close to Co-1<sup>4</sup> and Phg-1 but this may need further evidence. Geffroy et al. (1999) identified a resistance gene cluster on Pv04 with high levels of complexity. The region was reported to correspond with Co-y and Co-z in the Andean gene pool and the Co-3 cluster in the Middle American gene pool. Melotto et al (2004) characterized the expression of a gene at the Co-4 locus and reported the presence of putative resistance related genes in that region. Geffroy et al (1998) in the sequence of PvH2O gene identified an open reading frame including six LRR genes linked to Co-2 anthracnose resistance gene. Lopez et al (2003) also isolated 15 classes of RGAs, where eight of them were NBS-LRR and seven were in the TIR domain. The Gbrowse search around the significant SNPs in this study identified 27 putative candidate genes with potential roles in disease resistance, which six of them are more involved.

In summary, this research identified a genomic region at the terminal end of Pv01, the genomic position of the Co-1 cluster, associated with anthracnose race 73 resistance in Bolt. In addition to the major effect on Pv01, a number of QTL with minor effects were found to be
involved in overall field resistance. Moreover, Gbrowse search in the bean genome sequence identified 27 putative genes from the resistance gene family which may be involved in anthracnose resistance. Future studies including expression analysis are required to characterize these genes.
### 2.5: Figures and Tables

**Table 2.1.** Analysis of variance for AUDPC (Area Under Disease Progress Curve) and SII (Seed Infection Index) traits for the F$_{5:6}$ population of Bolt × H4784A-29844 combined across Elora and Huron Research Stations in 2012 and 2013.

<table>
<thead>
<tr>
<th>Fixed factor</th>
<th>AUDPC</th>
<th>SII</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>F-value</td>
<td>P-value</td>
</tr>
<tr>
<td>Environment</td>
<td>328.1</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>Genotype</td>
<td>10.49</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>Genotype × Environment</td>
<td>1.88</td>
<td>&lt;.0001</td>
</tr>
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</table>

<table>
<thead>
<tr>
<th>Random factors</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Variance component</td>
<td>P-value</td>
</tr>
<tr>
<td>Block(Environment)</td>
<td>0.007</td>
<td>0.24</td>
</tr>
<tr>
<td>iBlock(Block × Environment)</td>
<td>0.01</td>
<td>0.31</td>
</tr>
<tr>
<td>Residual</td>
<td>0.79</td>
<td>&lt;.0001</td>
</tr>
</tbody>
</table>
Table 2.2. Two-way segregation frequencies representing co-segregation of SNP marker genotypes with disease response groups in the growth room tests of F₅₆ RILs derived from Bolt x H4784A-29844 cross.

<table>
<thead>
<tr>
<th>Marker</th>
<th>Marker Genotype</th>
<th>Growth room segregation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>PTR</td>
</tr>
<tr>
<td>PvSNP8p1574781</td>
<td>PTR</td>
<td>57</td>
</tr>
<tr>
<td></td>
<td>Seg</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>PTS</td>
<td>0</td>
</tr>
<tr>
<td>PvSNP8p1922017</td>
<td>PTR</td>
<td>57</td>
</tr>
<tr>
<td></td>
<td>Seg</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>PTS</td>
<td>0</td>
</tr>
</tbody>
</table>

* PTR: parental type resistant (RILs with resistant parent SNP genotype or all single plants showing resistance response; Seg: segregating (RILs heterozygote SNP genotype or different single plant responses; PTS: Parental type susceptible (RILs with susceptible parent SNP genotype or all single plants showing susceptible response).
### Table 2.3: Distribution of SNP markers on linkage map and the frequency of segregation distortion on each linkage group

<table>
<thead>
<tr>
<th>Linkage groups</th>
<th>Map length (cM)</th>
<th>No. of markers</th>
<th>Marker density (cM/marker)</th>
<th>Number of distorted markers</th>
<th>% distortion</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>p &lt; 0.05</td>
<td>p &lt; 0.01</td>
</tr>
<tr>
<td>PV1</td>
<td>133.42</td>
<td>13</td>
<td>10.26</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>PV2</td>
<td>87.19</td>
<td>8</td>
<td>10.90</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>PV3</td>
<td>62.99</td>
<td>13</td>
<td>4.85</td>
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<td>0</td>
</tr>
<tr>
<td>PV5</td>
<td>2.73</td>
<td>6</td>
<td>0.45</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>PV6</td>
<td>97.87</td>
<td>13</td>
<td>7.53</td>
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<td>5</td>
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<tr>
<td>PV7</td>
<td>80.83</td>
<td>12</td>
<td>6.74</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>PV8</td>
<td>69.95</td>
<td>9</td>
<td>7.77</td>
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<td>0</td>
</tr>
<tr>
<td>PV9</td>
<td>4.16</td>
<td>2</td>
<td>2.08</td>
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<td>0</td>
</tr>
<tr>
<td>PV10</td>
<td>49.83</td>
<td>5</td>
<td>9.97</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>PV11</td>
<td>35.83</td>
<td>5</td>
<td>7.17</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>624.81</strong></td>
<td><strong>86</strong></td>
<td><strong>6.77</strong></td>
<td><strong>9</strong></td>
<td><strong>6</strong></td>
</tr>
</tbody>
</table>
Table 2.4. Quantitative Trait Loci (QTL) for AUDPC (Area Under Disease Progress Curve) and SII (Seed Infection Index) traits identified by single marker analysis of F$_{5.6}$ RILs from Bolt (Resistant) x H4784A-29844 (Susceptible).

<table>
<thead>
<tr>
<th></th>
<th>Map Position</th>
<th>SNP</th>
<th>Mbp</th>
<th>cM</th>
<th>LOD</th>
<th>$R^2_p$</th>
<th>Additive effect</th>
<th>Map Position</th>
<th>SNP</th>
<th>Mbp</th>
<th>cM</th>
<th>LOD</th>
<th>$R^2_p$</th>
<th>Additive effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td></td>
<td>PvSNP8p1922017</td>
<td>50.17</td>
<td>96.18</td>
<td>52.23</td>
<td>0.79</td>
<td>0.87</td>
<td></td>
<td>PvSNP8p1922017</td>
<td>50.17</td>
<td>96.18</td>
<td>27.91</td>
<td>0.64</td>
<td>0.85</td>
</tr>
<tr>
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Table 2.5. Multi-locus model (stepwise selection) of multiple QTL analysis for AUDPC (Area Under Disease Progress Curve) and SII (Seed Infection Index) traits identified by single marker analysis of F\textsubscript{5:6} populations in Bolt (Resistant)x H4784A-29844 (Susceptible).

<table>
<thead>
<tr>
<th>Pv</th>
<th>Model Components</th>
<th>Mbp</th>
<th>cM</th>
<th>Estimate</th>
<th>St Error</th>
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<tr>
<td>Map Position</td>
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<th>Mbp</th>
<th>cM</th>
<th>Estimate</th>
<th>St Error</th>
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Table 2.6. Putative candidate resistance genes identified around the genomic region of the markers with close association with the QTL for anthracnose resistance on Pv01 in the navy bean Bolt.

<table>
<thead>
<tr>
<th>Gene ID</th>
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<th>Description/Activity</th>
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<tbody>
<tr>
<td>Phvul.001G240400</td>
<td>50026833 – 50031313</td>
<td>F-BOX/LEUCINE RICH REPEAT PROTEIN</td>
</tr>
<tr>
<td>Phvul.001G242500</td>
<td>50212324 – 50218347</td>
<td>Predicted unusual protein kinase, aarF domain-containing kinase</td>
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<tr>
<td>Phvul.001G243500</td>
<td>50289347 – 50291144</td>
<td>Protein tyrosine kinase, ATP binding</td>
</tr>
<tr>
<td>Phvul.001G243600</td>
<td>50293967 – 50295727</td>
<td>Protein kinase domain, Serine/threonine protein kinase</td>
</tr>
<tr>
<td>Phvul.001G243700</td>
<td>50296080 – 50297315</td>
<td>Protein kinase domain, Serine/threonine protein kinase</td>
</tr>
<tr>
<td>Phvul.001G243800</td>
<td>50300459 – 50303474</td>
<td>Protein kinase domain, Serine/threonine protein kinase</td>
</tr>
<tr>
<td>Phvul.001G243900</td>
<td>50331097 – 50336473</td>
<td>AAA domain (Cdc48 subfamily), ATP binding</td>
</tr>
<tr>
<td>Phvul.001G245300</td>
<td>50447480 – 50452182</td>
<td>Leucine rich repeat N-terminal domain, Serine/threonine protein kinase, ATP binding</td>
</tr>
<tr>
<td>Phvul.001G246000</td>
<td>50512380 – 50517494</td>
<td>DEAD/DEAH box helicase, nucleic acid binding, ATP binding</td>
</tr>
<tr>
<td>Phvul.001G246400</td>
<td>50545702 – 50550616</td>
<td>SNF2 family N-terminal domain, DNA binding, ATP binding</td>
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<td>Phvul.001G252800</td>
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<td>Phvul.001G253300</td>
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<td>Ras family, GTP binding</td>
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<td>IBR domain, ATP dependent helicase activity, ATP binding</td>
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<td>Phvul.001G256200</td>
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<td>ABC transporter transmembrane region, ATP binding</td>
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<td>Phvul.001G256700</td>
<td>51302885 – 51307354</td>
<td>TCP-1/cpn60 chaperon family, ATP binding</td>
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<td>U-box domain, Kinesin-associated protein (KAP)</td>
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<td>Protein of unknown function, DUF258, GTP binding</td>
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<tr>
<td>Phvul.001G264100</td>
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<td>DEAD/DEAH box helicase, ATP-DEPENDENT RNA HElICASE, ATP binding</td>
</tr>
<tr>
<td>Phvul.001G264500</td>
<td>51808537 – 51815269</td>
<td>Kinesin motor domain, Kinesin-like protein, ATP binding</td>
</tr>
<tr>
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<td>Leucine Rich Repeat, Protein kinase domain, Serine/threonine protein kinase, ATP binding</td>
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<td>51930350 – 51940813</td>
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<td>51990109 – 51995766</td>
<td>TBC domain, GTPase-activating protein</td>
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<td>52017663 – 52023449</td>
<td>Helicase conserved C-terminal domain, ATP binding</td>
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</tbody>
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Figure 2.1. Average distribution of the area under the disease progress curve (AUDPC) on left and seed infected index (SII) on right estimated for 126 RILs of navy bean in the F_{5:6} Bolt x H4784A-29844 population. Arrows show parental lines. The AUDPC mean for the RIL population was 37.18 (CL 5% = 7.07) and SII mean was 4.92 (CL 5% = 1.54).
Figure 2.2. Genetic linkage maps aligned with physical location of navy bean in the F_{5:6} Bolt x H4784A-29844 are shown. 86 polymorphic SNP markers used to describe resistance to anthracnose race 7. The 11 linkage groups represent 10 chromosomes, with no representation of chromosome Pv04 and with two un-joined linkage group on chromosome Pv03. The maps were constructed with MapChart 2.2 (Voorrips 2002). Map distances on centimorgans are listed on left and Mega base pair are on right site of each chromosome with an LOD minimum 2.0 and Kosambi function on JoinMap 4 (Van ooijen JW, 2006).
Chapter 3. Characterization of Candidate Genes in the distal end of chromosome 1 associated with resistance to race 73 of Colletotrichum lindemuthianum

3.0. Abstract:

The navy bean (Pheseolus vulgaris L.) variety Bolt is resistant to Colletotrichum lindemuthianum (Sacc. & Magnus). In order to characterize the resistance genes involved in the host-pathogen interaction, 4 candidate resistance genes were chosen, based on their proximity to a major QTL on Pv01 in Bolt. All four genes belong to the serine-threonine protein kinase subfamily, and may relate to Nucleotide binding site (NBS)-LRR family of genes in a 50.3 Mbp region. Sequencing of the predicted coding regions, amplified using the genomic DNA of resistant and susceptible genotypes, were compared in nucleotide and amino acid levels. Significant sequence similarity was revealed among the four candidate genes. In addition, one insertion–deletion (indel) in the coding region of the gene Phvul.001G243500, resulted in premature stop codon and therefore shortened a protein in the susceptible genotype. Within qRT-PCR analyses, genes Phvul.001G243500, Phvul.001G243600, and Phvul.001G243700 were significantly expressed. Furthermore, the relative expression of genes, quantified at 0, 2, and 4 days post infection, revealed regulation of transcripts upon pathogen infection. While the results point to possible roles for these genes in the resistance response against race 73 of C. lindemuthianum, there is a need for further investigation of the resistance mechanism pathway.
3.1. Introduction

Anthracnose of common bean (*Phaseolus vulgaris* L.), caused by the fungus *Colletotrichum lindemuthianum*, (Sacc. & Magnus) Lams.-Scrib, is a major destructive disease worldwide (Pastor-Corrales et al., 1987; Barcelos et al., 2011). Cool temperatures of 13 to 26 °C with an optimum of 17 °C with higher than 92 % humidity is required for infection (Brown-Rytlewski and Kirk 2006; Dillard 1988). Anthracnose can infect all plant parts including stems, leaves, pods and seeds (Agrios, 2005). *C. lindemuthianum* is primarily a seed-borne pathogen (Barrus 1921), which may cause yield losses as high as 100% (Schwartz et al. 2005). *Colletotrichum* species is biotrophic and follow two main infection strategies: intracellular colonization or sub-cuticular intramural colonization. *Colletotrichum lindemuthianum* on bean exhibit the intracellular colonization infection type (Bailey et al., 1992). The anthracnose-common bean pathosystem has been studied for many years, providing knowledge on the molecular and cellular bases of fungal pathogenicity, concerning fungal differentiation and fungal–plant interactions.

Genetic resistance against anthracnose of common bean is known as the most effective and environmentally-safe disease management strategy (Mahuku and Riascos 2004; Kelly and Vallejo 2004). Genetic studies of *C. lindemuthianum* resistance have often reported a qualitative mode of inheritance with major resistance genes that follow a gene-for-gene interaction model (Flor 1955). So far, up to 29 anthracnose resistance genes (*Co-1* to *Co-14* and *Co-u, Co-v, Co-w, Co-x, Co-y, and Co-z*) have been described in common bean (Ferreira et al. 2013). Genetic mapping studies have identified seven main chromosome regions on linkage groups Pv01, Pv02, Pv03, Pv04, Pv07, Pv08, and Pv11, as the genomic positions of the anthracnose resistance genes (Michelmore and Meyers 1998).
The identification of resistance gene analogs (RGAs) is a great development in resistant cultivars. Resistance genes (R genes) present in plants interact with Avirulence genes (Avr) in the pathogen as a detector and an activator of a cascade of biochemical events that result in a resistance response against the pathogen (Chisholm et al. 2006). These resistance genes (R-gene) encode proteins that are often involved in the infection recognition process. The major R-gene groups are serine-threonine protein kinase, Lucien-rich repeat (LRR), receptor-like kinase, and Nucleotide binding site (NBS)-LRR (Lopez et al. 2003). The largest group of the R-genes is the NBS-LRR group are often classified into two groups based on the N-terminal domain and they are often located at the tightly linked clusters in plant genomes (Ferreira 2013). Interestingly, protein kinase involved in R-gene may require for resistance by NBS-LRR (Warren et al. 1999; Swiderski and Innes 2001).

The navy bean cultivar, ‘Bolt’ with the pedigree OAC_Rex/A98083//AC_Compass/B98143/3/RESW2138/B981045//B98213/Kippen is resistant to race 73 of *C. lindemuthianum*. Results presented in previous studies pointed to the *Co-1* complex located at the distal end of Pv01 (Mendez de Vigo 2001; Kelly et al. 2003; Kelly and Vallejo 2004) as being responsible for resistance against race 73 of *C. lindemuthianum*. Based on the findings presented in Chapter 2, two tightly linked SNP markers (PvSNP8p1922017 and PvSNP8p1574781) on Pv01 were associated with anthracnose resistance in Bolt.

In addition, JBrowse search in the significant genomic region, in the DNA sequence level, identified 27 putative genes, which may be involved in anthracnose resistance. The closest genes to the SNP markers PvSNP8p1922017 and PvSNP8p1574781 were Phvul.001G242500, Phvul.001G242600, Phvul.001G242700, and Phvul.001G242800.
3.1.1. Objectives

The main objectives of this chapter were to 1) Characterize four candidate genes, based on their proximity to a major QTL for resistance against race 73 of *C. lindemuthianum* in cultivar Bolt and 2) investigate the expression of the four candidate genes upon infection with the pathogen.
3.2. Materials and Methods

3.2.1: Plant materials:

Parental lines of the recombinant inbred line (RIL) population studied in Chapter 2, the anthracnose resistant navy bean variety Bolt and the susceptible line H4784A-29844, were used in this study. Genomic DNA was extracted from 1-2 g of freeze-dried leaf tissue according to the method explained by Yu et al. (1999) using a DNeasy plant Mini DNA Kit (QIAGEN Inc, Toronto).

3.2.2: In-silico sequence analysis:

From the *In silico* search in the Phaseolus reference genome (http://www.phytozome.net), in the Pv01 genomic region cultivar Bolt associated with race 73 resistance in the RIL population studied in Chapter 2 (Table 3.3), four putative genes were chosen as candidate genes for anthracnose resistance based on their proximity with the genomic region associated with anthracnose resistance in cultivar Bolt. The designations of these genes are Phvul.001G243500, Phvul.001G243600, Phvul.001G243700, and Phvul.001G243800. BLAST search was performed in Phytozome v9.1; http://www.phytozome.net and the National Center for Biotechnology Information (NCBI) public databases (http://www.ncbi.nlm.nih.gov). DNA and amino acid sequences for the resistant and susceptible genotypes were aligned using ClustalW2 software (http://www.ebi.ac.uk/Tools/msa/clustalw2).
3.2.3: Gene specific primer design:

Ten primer pairs (Table 3.4) were designed based on the sequence of the four candidate genes, using the software Primer3Plus (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi). Each primer pair was designed to amplify less than 1000 bp of the coding regions of the candidate genes. Primers were synthesized at the University of Guelph, Guelph Molecular Oligo Super center, Guelph, ON, Canada. In general, annealing temperature was 60°C and primer optimum was 30 GC% for all four candidate genes. Also, in the advanced settings icon in the software, the maximum 3’ self-complementarity and stability were set at 3 base pairs as the initial preference.

3.2.4: Polymerase chain Reaction and Agarose Gel Electrophoresis:

DNA amplifications were conducted in a 25 μL PCR mix containing 2.5 μL of 10X PCR buffer (500 mM potassium chloride, 15 mM magnesium chloride, 10 mMTris-HCl pH 9.0, 1% Triton X-100), 3 μL MgCl2 (25 mM) , 1 μL of dNTP (Invitrogen® 10 mM dNTP), 1 μL of Taq DNA Polymerase (Sigma®),1 μL of the forward and reverse primers (10μM) and 50ng genomic DNA. This mix was overlaid with light mineral oil before loading into the PCR machine. PCR amplification was performed in a programmable thermocycler (Bio-Rad® iCycler™) using numbers of reaction cycles depending on the target (Table 3.1). The PCR cycle consisted of a 5 min of initial denaturation step at 94°C, followed by 35 cycles of 1 min annealing step at variable annealing temperatures (50 to 70 ºC depending on the primer pair) and 1 min elongation step at 72°C. An initial 5 min denaturation step at 95°C and a final 5 min elongation step at 72°C also were included (Table 3.1).
PCR products were size-fractionated by gel electrophoresis. The 1% agarose gel was made by agarose suspended in 1X TBE running buffer (Tris base, boric acid and EDTA). The agarose solution was heated in a flask in a microwave for 1-1.5 min until all particles were dissolved. When it was cooled down to approximately 45 ºC, 60µl/ml of 10mg/ml ethidium bromide was added to the solution and mixed well to load into the wells for gel to solidify for around ½ hour. 15µL of PCR product were loaded into the gel with 2 µL loading buffer. All gels contained a 1kb DNA ladder (Invitrogen) as a size reference. Gels were run at 100V for 40min- 1h and visualized under UV light using a Gel Doc 1000 (BioRad, Hercules, CA, USA).

3.2.4.1: PCR Product Purification:

The PCR product was isolated from a 1% agarose gel using the PureLink® Quick Gel Extraction Kit (Invitrogen, Carlsbad, CA). The gel slice containing the desired DNA fragment was excised using a clean sharp razor blade under UV light and transferred into 1.5 ml microcentrifuge tube. The gel slice was weighed using a scale sensitive to 0.001 g. To dissolve the gel, the tube(s) containing the gel slice and Gel Solubilization Buffer was placed into a 50°C water bath for at least 10 minutes. The tube was inverted by hand every 3 minutes to mix and additional 5 minutes incubation was suggested after the gel slice was dissolved. The dissolved gel piece containing the target DNA fragment was placed onto the center of a Quick Gel Extraction Column inside a Wash Tube to centrifuge for 1 minute at >12,000 × g in order to bind the DNA. 500 µL Wash Buffer (W1) containing ethanol was added to the Quick Gel Extraction Column and centrifuged at >12,000 × g for 1 minute. To remove any residual Wash Buffer and ethanol, the column was centrifuged again at maximum speed for 1–2 minutes. 50 µL Elution Buffer (E5) was added to the center of the Quick Gel Extraction Column on a
Recovery Tube. After 1 minute incubation at room temperature, the column was centrifuged at >12,000 × g for 1 minute. The purified DNA collected in the recovery tube was stored at 4°C for immediate use or stored at –20°C for long-term storage.

3.2.4.2: PCR and PCR product cleanup:

DNA fragments extracted from the gels were further amplified using PCR. The PCR reactions were conducted using respective gene specific primer sets using the JumpStart Taq DNA Polymerase kit (Sigma-Aldrich, Co, St. Louise, MO, USA). PCR reactions were performed in 25μL volume containing 15.2μL of molecular biology grade H₂O, 2.5μL of 10X PCR buffer (25mM), 3.0μL of MgCl₂, 1.0μL of dNTP (10mM), 1.0μL, 25μL of 10μL of forward and reverse primers, 0.4μL of Taq DNA Polymerase of 2.5U μL⁻¹, 0.5μL of DNA. The PCR cycles included an initial denaturation step at 94°C for two minutes, followed by 34 cycles of 30 seconds 94°C, annealing temperatures at 60°C, and 45 seconds at 72°C, and hold at 4°C. The PCR product (25μL) was mixed with an equal volume of gel-loading buffer by electrophoresis in 5% a polyacrylamide gel containing 5% glycerol.

Cycle sequencing cleanup was then used to purify PCR reactions in 14μL volume containing 9.0μL of molecular biology grade H₂O, 2.0μL of 5X sequencing buffer, 1.0μL of BigDye terminator, 1.0μL of 10μm forward or reverse primers, 1.0 to 2.0μL of template DNA depending on the DNA concentration using Quick PCR Purification Kit, PureLink ™ (Invitrogen, Carlsbad, CA).

The PCR cycle was programed as 96°C for two minutes, followed by 30 cycles 95°C for 30 seconds denaturation, annealing temperatures at 55°C due to the determination of each gene specific primers designed temperature for 1.5 minute, and 60°C for 1.3 minutes extension, and then after finishing 30 cycles, 72°C for four minutes for final extension, and hold at 6°C.
3.2.4.3: Gene sequencing:

For each gene, cleaned up PCR solutions derived from both forward and reverse primer amplifications were sent to the University of Guelph Genomics Facility (Science Complex, University of Guelph, Guelph, Ontario, Canada) for sequencing using the Sanger method (Sanger et al. 1997) which uses an Applied Biosystem BioDye Terminator version 3.1 Cycle sequencing Kit and Applied Biosystems 3730 sequencer.

3.2.4.4: Sequence alignment

The DNA and the predicted translated amino acid sequence of the four candidate genes were aligned individually using ClustalW2 software (http://www.ebi.ac.uk/Tools/msa/clustalw2) using the amplified coding regions in the resistant and susceptible genotypes. Sequence alignment was also performed with the sequence of a homologous gene (XM_003537442.1) in *Glycine max*, extracted from NCBI (http://www.ncbi.nlm.nih.gov).

3.2.5: Expression analysis

3.2.5.1: RNA Extraction

RNA was isolated from leaf samples of Bolt and H4784A-29844 following inoculation with *C. lindemuthianum* race 73. Leaf tissue was harvested at three different times (0, 2, and 4 days after inoculation. Plants were grown in 10 cm fiber pots, filled with sterilized soil mixture in a growth room condition at 23 °C with 8 hours dark and 16 hours light, at the University of Guelph. One-gram samples taken from fully-expanded leaves were ground in liquid nitrogen. RNA extraction was done as described by Gasic et al. (2004). RNA integrity and quality was verified by formaldehyde gel electrophoresis and then the RNA was treated with DNase I using
the TURBO DNA-free kit (Applied Biosystems, Austin, TX) according to the manufacturer’s instructions. One microgram total RNA was used for first-strand cDNA synthesis with Oligo(dT)\textsubscript{20} and Superscript III (Invitrogen, Carlsbad, CA) at 50°C for 30 min followed by 55°C for another 30 min.

### 3.2.5.2: Gene specific primer design

Primers used for quantitative real-time PCR (qRTPCR) were designed using Integrated DNA Technology online software (http://www.idtdna.com/Primerquest/Home/Index) and unique sequences for each gene. Primers used for RT-PCR are listed in Supplementary Table 3.6. The reference gene (Actin) was identified from the Bean Genome Database (F: GAAGTICTCTTCCAACCATCC, R: TTTCTTGTCTCTTCGTCCG).

### 3.2.5.3 Real-time PCR (qPCR):

#### 3.2.5.3.1: Gradient PCR

In order to optimize the annealing temperature for each one of the four genes, gradient PCR reaction was conducted in a LightCycler 480 II (Roche Diagnostics GmbH, Mannheim, DE). The temperature gradient was programmed with different annealing temperatures between 55°C and 58°C. The reaction mix contained 10 ng of cDNA, 0.25 μM of each primer and 1× Taq PCR Master Mix (Qiagen, Gaithersburg, MD, USA) under condition of 2 minutes at 95°C, 40 cycles of 30 seconds at 94 °C, 30 seconds of (56-60) °C, 10 seconds at 72 °C followed by 5 minutes at 72 °C and hold at 4 °C.
3.2.5.3.2: Relative Standard Curve

Relative expression and data analysis were determined using the $2^{-\Delta Ct}$ method (Livak et al. 2001). Two technical replicates were conducted for each biological replicate, and the average ±SE of three biological replicates was determined for each time point. Actin and Skip16 (SKP1/ACK-interacting protein 16) were used as the reference genes due to their high expression in all tissues in common bean (Aline Borges et al. 2012). Relative standard curves for target genes (Phvul.001G243500, Phvul.001G243600, Phvul.001G243700, and Phvul.001G243800), and a reference gene, Actin, were generated. Each sample was four-fold serially diluted six times and the dilutions were used through the experiment to calculate primers efficiency test based on $E=10^{-1/slope}$.

3.2.5.3.3: Efficiency of Primers

Serial dilution of cDNA template was conducted to ensure primer efficiency of 90-105%. The PCR conditions included 95°C for 2 min, then 40 cycles of 95°C for 10 s, 55°C for 30 s and 72°C for 15 s to amplify 80-100 bp fragments. The reaction was detected using quantitative real-time PCR performed on diluted cDNA (1:10 in DEPC treated water) using SYBR green dye in a LightCycler 480 II (Roche Diagnostics GmbH, Mannheim, DE).

3.2.5.3.4: Data Analysis for Quantification of Real-Time PCR (qPCR)

Real time PCR results were expressed as Ct (cycle threshold) values. Ct values were transformed to relative quantities at which the fluorescence of the SYBR Green dye reached above the threshold or background fluorescence value. In quantifying expression level of the target genes (Phvul.001G243500, Phvul.001G243600, Phvul.001G243700, and Phvul.001G243800) in different samples, the relative expression level was obtained by the cycle differences of the target genes and the reference gene (Actin).
Quantitative real-time PCR was performed on diluted cDNA (1:10 in DEPC treated water) using SYBR green dye in a LightCycler 480 II (Roche Diagnostics GmbH, Mannheim, DE) in a volume of 20μL reaction containing 3 μl Nuclease-free water, 1 μl of each forward and reverse primers (300 nM), 10 μL iQ™ SYBR Green PCR supermix, and 5 μl cDNA dilution template (100ng). The reaction replicates were performed in the 96 cell plates using an initial denaturing cycle (2 min at 95°C), followed by pre-incubation for 10 min at 94°C, followed by 45 cycles of 95°C for 10s, 55–58.8 °C (optimized for each gene) for 5s and polymerization for 10s at 72°C. Melting curve analysis was set at the end of each PCR before cooling down in three steps: 95°C for 10s, cooling to 65°C for 1 min and heating to 97°C as described in Table 3.2.

3.2.5.3.5: Relative Expression

The equivalency of the PCR efficiencies of each gene between the target and reference gene was used to calculate the relative quantitation for each template dilution by running standard curves. Regression analysis was performed on log (10) transformed data. The slope of the regression line was used as a validation experiment.

3.2.5.3.6: Fold Change:

Fold change in RNA expression was estimated by the ΔΔCT method using threshold cycles.

3.2.6: Statistical analyses:

Statistical analyses of the relative expression data were performed using SAS 9.3 (SAS Institute, Cary, NC). Relative expression of each sample was log transformed prior to analysis. Transformed data were subjected to analysis of variance using the PROC GLM procedure, in
which sample, time and sample by time interaction were included in the model statement. Least square means (lsmeans) and confidence limits (P < 0.05) were calculated for each candidate gene’s expression for each genotype in each time interval separately.

Fold changes were calculated using the Relative Expression Software Tool (REST) version 2009 (Qiagen, Valencia, CA) to compare control and treatment based on the PCR efficiencies and the mean crossing point deviations between them.
3.3. Results

3.3.1: Gene structure comparison

Analysis of the genomic sequences of Phvul.001G240500, Phvul.001G240600, Phvul.001G240700, and Phvul.001G240800, using the sequence of the amplified regions with site-specific primers between Bolt (resistant parent) and H4784A-29844 (susceptible parent) revealed numerous polymorphic regions for all for candidate genes (Table 3.5).

3.3.2: Sequence comparison of the gene Phvul.001G240500

From the coding region of the gene Phvul.001G240500, a total of 1797 bp was amplified and sequenced using two sets of primers, K500-F1 and K500-R1, K500-F2 and K500-R2 (Table 3.4). Sequence analysis identified 99.17% sequence similarity between the resistant and susceptible parents and 99.67% similarity with the published sequence of the gene in the reference genome sequence (Table 3.5, Figure 3.1). Comparative sequence analysis of the sequence of Phvul.001G243500 between the two genotypes identified five SNPs and a frame shift mutation caused by a 10 bp insertion in the susceptible genotype that leads to premature stop codon and a shortened protein (Figure 3.1; 3.5).

3.3.3: Sequence comparison of the gene Phvul.001G240600

From the coding region of the gene Phvul.001G243600, a total of 1760 bp was amplified and sequenced using two sets of primers, K600-F1 and K600-R1, K600-F2 and K600-R2 (Table 3.4; 3.5). A total of 11 SNPs and one indel were identified (Table 3.5) in sequence comparison between the two genotypes. The published sequence of the gene
Phvul.001G243600 in the reference genome sequence shared 99.55% and 99.20% similarity with the resistant and susceptible genotypes, respectively (Figure 3.2).

3.3.4: Sequence comparison of the gene Phvul.001G240700

From the coding region of the gene Phvul.001G243700, a total of 1235 bp was amplified and sequenced using two sets of primers, K700-F1 and K700-R1, K700-F2 and K700-R2 (Table 3.4; 3.5). A total of 11 SNPs and one indel were identified (Table 3.5) in sequence comparison between the two genotypes. The published sequence of the gene Phvul.001G243600 in the reference genome sequence shared 99.13% and 98.27% similarity with the resistant and susceptible genotypes, respectively (Figure 3).

3.3.5: Sequence comparison of the gene Phvul.001G240800

From the intronic region of the gene Phvul.001G243800, a total of 3015 bp was amplified and sequenced using four sets of primers, K800-F1 and K800-R1, K800-F2 and K800-R2, K800-F3 and K800-R3, K800-F4 and K800-R4 (Table 3.4, 3.5). A total of 34 SNPs and two indels were identified (Table 3.5) in sequence comparison between the two genotypes. The published sequence of the gene Phvul.001G243600 in the reference genome sequence shared 97.14% and 98.95% similarity with the resistant and susceptible genotypes, respectively (Figure 3.4).
3.3.6: Comparison of candidate gene sequences with a *Glycine Max* gene

In sequence comparison of the four candidate genes with a paralogous gene (XM_003537442.1) in *G. max*, of the gene Phvul.001G243800 had the highest nucleotide sequence similarity, resulting in 86.58% amino acid sequence similarity (*Figure 3.8*). The amino acid sequence similarities with the orthologous gene XM_003537442.1 of *Glycine max* (L.) Merr. for the genes Phvul.001G243500 (*Figure 3.5*), Phvul.001G243600 (*Figure 3.6*), and Phvul.001G243700 (*Figure 3.7*) were 35.7 %, 52.2 %, and 47.2 % respectively.

3.3.7. Gene expression analysis

The transcript level of each of the four candidate genes was normalized with the actin gene as a reference gene. The analysis of variance of the qRT-PCR data indicated that the effect of genotype for all genes was significant (*p < 0.05*). However, the effect of time and the interaction of time and genotype were not significant (*Table 3.7*). For all candidate genes the susceptible genotype had higher expression than the resistant genotype at the time of infection and the level of expression was reduced over time after infection. Only minor changes in the levels of gene expression were observed over time for all genes, except the gene Phvul.001G243800 which was over-expressed 96 hr after infection (*Figure 3.10*).

The fold changes between the two genotypes from 48 to 96 hrs after infection were compared with the non-infected check. There was a significant decrease between 48 to 96 hrs after infection, comparing the susceptible cultivar with the non-infected check for the gene Phvul.001G243500. However, there were no significant differences between 48 to 96 hrs after infection (*Figure 3.11.a*). A similar pattern of expression was found for gene Phvul.001G243700, which showed similar responses as for the gene Phvul.001G243500 in
both genotypes (Figure 3.11.c). The result of gene Phvul.001G243800 showed that while there was a significant decrease from 48 to 96 hrs after infection, there was a significant increase from 48 to 96 hrs after infection compared with the non-infected leaves for the resistant genotype. An exception was the gene Phvul.001G243600, in which both susceptible and resistant genotypes had decreased fold change from 48 to 96 hrs after infection compared with the non-infected leaves (Figure 3.11).

Furthermore, the transcript level of the gene Phvul.001G243500 was increased by 1.8- and 2.4-fold at 48 and 96 hrs after infection relative to time of infection in the resistant genotype and decreased 2.5- and 10.9-fold in the susceptible genotype. Similarly, the expression in the resistant genotype was by 3.8- and 4.6-fold at 48 and 96 hrs after infection and 1.8- and 7-fold at 48 and 96 hrs after infection for the gene Phvul.001G243700 and Phvul.001G243800, respectively. In general, comparison of the expression levels in the non-infected and anthracnose-infected leaves, revealed that all four candidate genes in the resistant genotype were significantly up-regulated, while they were all down-regulated in the susceptible genotype except for the gene Phvul.001G243600 (Figure 3.11).
3.4. Discussion

This chapter reports the characterization of four full-length bean genes Phvul.001G243500, Phvul.001G243600, Phvul.001G243700, and Phvul.001G243800 that may encode R-like proteins. All four genes belong to the serine-threonine protein kinase, and may relate to Nucleotide binding site (NBS)-LRR family of genes. These genes were chosen based on identified proximity to the significant effect SNP markers, as reported in Chapter 2. These resistance genes are likely to play a role in the cascade of events leading to resistance response against anthracnose.

A recent study by Richard et al. (2014) revealed that candidate genes Phvul.001G243500, Phvul.001G243600, Phvul.001G243700, and Phvul.001G243800 were expressed in resistance response for different common bean cultivars. However, there weren’t any specified candidate genes. Moreover, protein sequence in Phvul.001G243500, Phvul.001G243600 and Phvul.001G243700 encoded only shorten C-terminal part of the kinase, whereas, Phvul.001G243800 encoded a lengthen CrInKIY4 related 3 protein.

The nucleotide sequences and amino acid obtained in this study were searched using the BLAST algorithm, using the sequence of the amplified regions of the four genes, using site-specific primers that were designed to amplify the coding region of the genes Nucleotide sequences of the amplified regions were also translated into predicted amino acid sequences, in order to perform comparison of the amplified regions in the resistant and susceptible genotypes both in nucleotide and in amino acid sequence levels. Comparison at the amino acid level is believed to be more reliable than nucleotide-nucleotide sequence comparison, due to higher homology with NBS-LRR region of many RGAs than nucleotide and also greater chance of
amino-acid sequence to be conserved around the structural motifs (Totad et al. 2005; Thirumalaiandi et al. 2008).

All four genes sequences were found to be polymorphic between the resistant and susceptible genotypes in the sequence level. However, the difference between the sequences of the resistant and susceptible genotypes for the gene Phvul.001G243500, in which a 10 bp insertion resulted in pre-mature stop codon and therefore a shortened protein in the susceptible genotype, pointed to the gene Phvul.001G243500 as the strongest candidate among for with a possible role in resistance response to anthracnose. Richard et al. (2014) also reported potential role for the gene Phvul.001G243500 in resistance against anthracnose race 73 in the region of 58 kb that encode truncated kinases.

The changes in the transcript levels of the resistant and susceptible genotypes in response to anthracnose were exactly opposite. All four genes had increased in both resistant and susceptible cultivar compared to the non-infected leaf tissue, except the gene Phvul.001G243600, which had increased expression in resistant and decreased in susceptible one. The transcript fold increase in the resistant genotype and decrease in the susceptible one indicated that the pathogen invasion in plant tissues occurred at the end of infection. These findings are similar to previous reports in the chitinase gene in the bean genotype Ef-1α (Mahe et al. 1992). However, the time intervals of this study for induction were later (8 hours) compared to our study.

Several studies provide further evidence for a role of protein kinases in pathogenicity of fungi. In a study by Lee and Dean (1993), cAMP was a regulatory element of appressorium differentiation in M. grisea. A similar study in rice against fungus M. grisea revealed the identification of the cpkA gene encoding a cAMP protein kinase (Sweigard et al. 1996;
Mitchell and Dean 1995). Dufresne et al. (1998), which demonstrated the involvement of a kinase gene in the early steps of the infection process, revealed the gene from the serine/threonine protein kinase family by cDNA sequences called clk1 (*Colletotrichum lindemuthianum* kinase 1), although, it was weakly expressed and undetectable during the infection. In a similar study, the tomato gene Pti1 encodes a serine/threonine kinase that is phosphorylated by Pto and is involved in the hypersensitive response (Zhou et al. 1995).

Interestingly, Borges et al. (2012) identified the relative expression (RE) levels of 12 defense-related transcripts during incompatible interaction between the resistant common bean genotype SEL1308 and anthracnose pathogen race 73 which were dependent on the tissue and time post inoculation. The expression of the gene COK-4 that encodes for a serine threonine kinase protein mapped to the *Co-4* region was assessed by RT-PCR in a study by Melloto et al. (2004). In a second study, study by Oblessuc et al. 2015, gene COK-4 was suggested as a defense-response activator, and gene FER-ike as an inhibitor which are predicted to code for a receptor-like kinase and regulated by immune response in common bean against *C. lindemuthianum* on Pv08 that suggested COK-4 is part of the *Co-4* locus conditioning resistance to *C. lindemuthianum* in bean.

Additionally, Genetic analyses at iTAK-Plant Transcription factor and Protein Kinase Identifier and Classifier (http://bioinfo.bti.cornell.edu/cgi-bin/itak/db_align.cgi?trans_ID=Phvul.001G243600.1) showed that the gene Phvul.001G243600 is classified as a receptor like cytoplasmic kinase VI and protein tyrosine kinase. It should be noted that a large number of effector proteins into host cells assist proliferation in plants and capable of signaling pathway to suppress plant immunity (Block et al. 2008; Fu et al. 2007; Gimenez-Ibanez et al. 2009; Kim et al. 2005; Nomura et al. 2006;
Xiang et al., 2008). AvrPto in *P. syringae* acts as a kinase inhibitor to block immune signaling from FLS2 and EFR (Xiang et al. 2008).

In summary, the characterization of the four candidate genes in this study may contribute to a better understanding of the defense mechanism of common bean against *C. lindemuthianum*. Moreover, revealing the transcriptional profile of genes highlights the importance of defense processes that common bean may have to contain the growth of a pathogen. Phvul.001G243500, Phvul.001G243600, Phvul.001G243700 and Phvul.001G243800 are more likely part of a mechanism for fugal pathogen recognition rather than defence. Future development of this study are required to better understand the signaling pathway involved in resistance against *C. lindemuthianum* infection process. It is expected that the regulation of others genes may contribute to the resistance of cultivar Bolt of common bean to the race 73 of *C. lindemuthianum*. 
### Table 3.1. Genomic position, transcript name, and the activity of four candidate resistance genes in a 50.3 Mbp region on Pv01 with association with anthracnose resistance in the navy bean Bolt.

<table>
<thead>
<tr>
<th>Candidate Gene locus name</th>
<th>Physical position in the reference genome</th>
<th>Transcript name</th>
<th>Description/Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phvul.001G243500</td>
<td>50289347--50291144</td>
<td>Phvul.001G243500.1</td>
<td>Protein tyrosine kinase protein kinase activity protein amino acid phosphorylation ATP binding</td>
</tr>
<tr>
<td>Phvul.001G243600</td>
<td>50293967--50295727</td>
<td>Phvul.001G243600.1</td>
<td>Protein tyrosine kinase Serine/threonine protein kinase protein kinase activity protein amino acid phosphorylation</td>
</tr>
<tr>
<td>Phvul.001G243700</td>
<td>50296080--50297315</td>
<td>Phvul.001G243700.1</td>
<td>Protein kinase domain Serine/threonine protein kinase protein kinase activity protein amino acid phosphorylation</td>
</tr>
<tr>
<td>Phvul.001G243800</td>
<td>50300459--50303474</td>
<td>Phvul.001G243800.1</td>
<td>Protein kinase domain Serine/threonine protein kinase protein kinase activity protein amino acid phosphorylation</td>
</tr>
</tbody>
</table>

* Reference genome sequence extracted from Pv 1.0 available at [http://www.phytozome.net/](http://www.phytozome.net/)
Table 3.2. Nucleotide sequence, expected amplification and target fragment size for specific primers designed to amplify coding regions of four candidate genes for anthracnose resistance in the Navy bean Bolt. All primers are listed in the 5’ to 3’ direction from left to right.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer name</th>
<th>Primer sequence</th>
<th>Amplification fragment size</th>
<th>Target fragment size</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phvul.001G243500</td>
<td>K500-F1</td>
<td>TGATCCAATAGTCAAGGGAAGC</td>
<td>987</td>
<td>856 bp</td>
</tr>
<tr>
<td></td>
<td>K500-R1</td>
<td>GACGACCCAAGAAGGTCACT</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>K500-F2</td>
<td>ACGAAACCAGTCCCAAGATG</td>
<td>990</td>
<td>940 bp</td>
</tr>
<tr>
<td></td>
<td>K500-R2</td>
<td>CCAGCATCCCCAAAATTTGAC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phvul.001G243600</td>
<td>K600-F1</td>
<td>CCGAGGACAATTCAAAGGT</td>
<td>980</td>
<td>889 bp</td>
</tr>
<tr>
<td></td>
<td>K600-R1</td>
<td>GATGTGTACGGGGCTTTGAGT</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>K600-F2</td>
<td>TCCAAACTTTAACGTTGTCTT</td>
<td>1000</td>
<td>852 bp</td>
</tr>
<tr>
<td></td>
<td>K600-R2</td>
<td>TCAATCTGCTGATGCAGAGTAGGAA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phvul.001G243700</td>
<td>K700-F1</td>
<td>TCCATCATTTATTCATTCACAA</td>
<td>840</td>
<td>661 bp</td>
</tr>
<tr>
<td></td>
<td>K700-R1</td>
<td>ACGTCCCAAGAATTGTTG</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>K700-F2</td>
<td>TCCATGACCTCATACACCAA</td>
<td>896</td>
<td>717 bp</td>
</tr>
<tr>
<td></td>
<td>K700-R2</td>
<td>GCAAGTGGGAAAACAAAAA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phvul.001G243800</td>
<td>K800-F1</td>
<td>TCGTCTAATGTTAGGAAACAGTGG</td>
<td>960</td>
<td>859 bp</td>
</tr>
<tr>
<td></td>
<td>K800-R1</td>
<td>TGTGTATGGGCTTGAGTTG</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>K800-F2</td>
<td>CAAAATACGAGGCACCTGCAA</td>
<td>900</td>
<td>869 bp</td>
</tr>
<tr>
<td></td>
<td>K800-R2</td>
<td>ATGGGGAGGTGCTCTATAT</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>K800-F3</td>
<td>TCTGGCGCCTCATATCTAT</td>
<td>999</td>
<td>817 bp</td>
</tr>
<tr>
<td></td>
<td>K800-R3</td>
<td>GTCTGCTTCCGGGAATTTT</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>K800-F4</td>
<td>GCGGCAAGACTCAACATGGA</td>
<td>936</td>
<td>822 bp</td>
</tr>
<tr>
<td></td>
<td>K800-R4</td>
<td>AAGCACCACAGGACTGAA</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 3.3. Number of single nucleotide polymeric regions and indels observed in the sequence comparisons of the four candidate genes amplified in resistant (Bolt) and susceptible (H4784A-29844) bean varieties.

<table>
<thead>
<tr>
<th>Candidate gene</th>
<th>Sequence analyzed (bp)</th>
<th>SNPs</th>
<th>Indels</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phvul.001G243500</td>
<td>1797</td>
<td>5</td>
<td>1</td>
</tr>
<tr>
<td>Phvul.001G243600</td>
<td>1760</td>
<td>11</td>
<td>1</td>
</tr>
<tr>
<td>Phvul.001G243700</td>
<td>1235</td>
<td>11</td>
<td>1</td>
</tr>
<tr>
<td>Phvul.001G243800</td>
<td>3015</td>
<td>34</td>
<td>2</td>
</tr>
</tbody>
</table>
Table 3.4. Primers designed for quantitative RT-PCR of four candidate genes for anthracnose resistance in the Navy bean Bolt. All primers are listed in the 5’ to 3’ direction from left to right.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer name</th>
<th>Primer sequence</th>
<th>Amplification fragment size</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phvul.001G243500</td>
<td>Rt500-F</td>
<td>CTTGTTGATGGTAGGGAGGTAG</td>
<td>104 bp</td>
</tr>
<tr>
<td></td>
<td>Rt500-R</td>
<td>CGACCCAAGAAAGGTCACTAAA</td>
<td></td>
</tr>
<tr>
<td>Phvul.001G243600</td>
<td>Rt600-F</td>
<td>TCGCAGGGCGTGTTATTT</td>
<td>113bp</td>
</tr>
<tr>
<td></td>
<td>Rt600-R</td>
<td>CGCTGTATGGG</td>
<td></td>
</tr>
<tr>
<td>Phvul.001G243700</td>
<td>Rt700-F</td>
<td>GTGGAGGAGAACGTCTAGTTTG</td>
<td>110bp</td>
</tr>
<tr>
<td></td>
<td>Rt700-R</td>
<td>CCTCATACACAAACACCCCTTT</td>
<td></td>
</tr>
<tr>
<td>Phvul.001G243800</td>
<td>Rt800-F</td>
<td>GGAAGGGAAAGATAGACCAACC</td>
<td>100bp</td>
</tr>
<tr>
<td></td>
<td>Rt800-R</td>
<td>AGAGCCACTGGAATGCTATC</td>
<td></td>
</tr>
</tbody>
</table>
### Table 3.5. Analysis of Variance for gene expression of cultivar Bolt and H4784A-29844

<table>
<thead>
<tr>
<th>SV</th>
<th>df</th>
<th>Phvul.001G243500</th>
<th>Phvul.001G243600</th>
<th>Phvul.001G243700</th>
<th>Phvul.001G243800</th>
</tr>
</thead>
<tbody>
<tr>
<td>Genotype</td>
<td>1</td>
<td>2.645**</td>
<td>2.512**</td>
<td>0.991*</td>
<td>0.244</td>
</tr>
<tr>
<td>Time</td>
<td>2</td>
<td>0.096</td>
<td>0.238</td>
<td>0.018</td>
<td>0.009</td>
</tr>
<tr>
<td>Genotype × Time</td>
<td>2</td>
<td>0.465</td>
<td>0.412</td>
<td>0.487</td>
<td>0.415</td>
</tr>
<tr>
<td>Error</td>
<td>11</td>
<td>0.168</td>
<td>0.139</td>
<td>0.167</td>
<td>0.135</td>
</tr>
</tbody>
</table>

* and ** are significant at P < 0.05 and P < 0.01, respectively
Fig 3.1: A and B nucleotide sequence comparison for the gene Phvul.001G243500 in the reference genome (Pv1.0), available at Phytozome (http://www.phytozome.net/), compared with the amplified sequences in the resistant (-R) and susceptible (-S) parental lines. Identified single nucleotide polymorphism is shown shaded in light gray, while dashes and the dark gray shaded region identify a 10 bp indel in the intron region. Length of the Amplified sequence and percent similarity are presented below the figure.

<table>
<thead>
<tr>
<th>SeqA</th>
<th>Name</th>
<th>Length (bp)</th>
<th>SeqB</th>
<th>Name</th>
<th>Length (bp)</th>
<th>Similarity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Phvul.001G243500</td>
<td>1998</td>
<td>2</td>
<td>Phvul.001G243500-R</td>
<td>1817</td>
<td>96.97</td>
</tr>
<tr>
<td>1</td>
<td>Phvul.001G243500</td>
<td>1998</td>
<td>3</td>
<td>Phvul.001G243500-S</td>
<td>1829</td>
<td>98.41</td>
</tr>
<tr>
<td>2</td>
<td>Phvul.001G243500-R</td>
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**Fig 3.2:** A and B nucleotide sequence comparison for the gene Phvul.001G243600 in the reference genome (Pv1.0), available at Phytozone (http://www.phytozone.net/), compared with the amplified sequences in the resistant (-R) and susceptible (-S) parental lines. Identified single nucleotide polymorphism is shown in shaded light gray, while dashes and the dark gray shaded regions identify 77 bp, 17 bp and 1 bp indel in the intron region. Length of the Amplified sequence and percent similarity are presented below the figure.
**Fig 3.3:** A and B nucleotide sequence comparison for the gene Phvul.001G243700 in the reference genome (Pv1.0), available at Phytozome ([http://www.phytozome.net/](http://www.phytozome.net/)), compared with the amplified sequences in the resistant (-R) and susceptible (-S) parental lines. Identified single nucleotide polymorphism is shown in shaded light gray, while dashes and the dark gray shaded regions identify 64 bp and 6 bp indel in the intron region. Length of the Amplified sequence and percent similarity are presented below the figure.

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**Fig 3.3:** A and B nucleotide sequence comparison for the gene Phvul.001G243700 in the reference genome (Pv1.0), available at Phytozome ([http://www.phytozome.net/](http://www.phytozome.net/)), compared with the amplified sequences in the resistant (-R) and susceptible (-S) parental lines. Identified single nucleotide polymorphism is shown in shaded light gray, while dashes and the dark gray shaded regions identify 64 bp and 6 bp indel in the intron region. Length of the Amplified sequence and percent similarity are presented below the figure.
Fig 3.4: A and B nucleotide sequence comparison for the gene Phvul.001G243800 in the reference genome (Pv1.0), available at Phytozome (http://www.phytozome.net/), compared with the amplified sequences in the resistant (-R) and susceptible (-S) parental lines. Identified single nucleotide polymorphism is shown in shaded light gray, while dashes and the dark gray shaded regions identify a 9 bp indel in the intron region. Length of the Amplified sequence and percent similarity are presented below the figure.

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**Phvul.001G243800**

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- **R**
  - ACAACCGAACATCTGAATCTCTCTTTATAGCACAACCATGTTCAGCTCCAAGCGCCAACCCA
  
**Phvul.001G243800**

- **S**
  - AACATGGACATGTT
  
- **R**
  - AACATGGACATGTTGGACATGTTCCCAAATACACTCTCAATGCGTTCCGTTAAATCCGAA
  
**Phvul.001G243800**

- **S**
  - ACGCTGGTGTCTCCCCAGCACCGAACCCGATAACTATTCTTCAAAATTCCGCAAGAGAAG
  
- **R**
  - ACGCTGGTGTCTCCCCAGCACCGAACCCGATAACTATTCTTCAAAATTCCGCAAGAGAAG
  
**Phvul.001G243800**

- **R**
  - CCAGTCCCAGATGAAATCGAAGCGAATTTATCAGTCGCAGAAGGAAGCTGAAACGTGTCA
  
**Phvul.001G243800**

- **S**
  - TTCCTCCCACCCGAAATCGTTGAAAACTAGCGTTGGGAGTGATGGAGGCAATCTGCCCC
  
- **R**
  - TTCCTCCCACCCGAAATCGTTGAAAACTAGCGTTGGGAGTGATGGAGGCAATCTGCCCC

**Phvul.001G243800**

- **S**
  - TGACGGTAACACTCAATGCGGCGCGTGGACTCGCGTGCCACGACGGCGCACACGGTGGAG
  
- **R**
  - TGACGGTAACACTCAATGCGGCGCGTGGACTCGCGTGCCACGACGGCGCACACGGTGGAG

**Phvul.001G243800**

- **S**
  - GAGGCGTCAGAGATGGTAAGGGTGGCGCCGGAGCCGAGGCCATGGGAAGAGCGTGGTAGA
  
- **R**
  - GAGGCGTCAGAGATGGTAAGGGTGGCGCCGGAGCCGAGGCCATGGGAAGAGCGTGGTAGA

**Phvul.001G243800**

- **R**
  - TTGAACTTTGAGGGAGTAGTTGTGGACTCTTCAATCTCCCATTTTGCTAAGACATTTCTT
  
**Phytozome**: [http://www.phytozome.net/](http://www.phytozome.net/)
Phvul.001G243500.1
Phvul.001G243500-S
Phvul.001G243500-R
Glycine
---

Phvul.001G243500.1
Phvul.001G243500-S
Phvul.001G243500-R
Glycine
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**Fig 3.5.** A and B amino acid sequence comparison for the gene Phvul.001G243500 in the reference genome (Pv1.0), available at Phytozome (http://www.phytozome.net/) compared with the inferred sequences based on the amplified intron regions in the resistant (-R) and susceptible (-S) and the sequence of a homologous gene (XM_003537442.1) in *Glycine max*, extracted from NCBI (http://www.ncbi.nlm.nih.gov). Dashes indicate the missing amino acids in the comparison.
Glycine                 MKTSPSSVNLCAVAAVVLILSVPSSGSGGTTLALSDATVCAVASESTRRIE 60
Glycine                 CYRQGQVQFIAPFNNFSSISSGGRNYFCGIMSSNSLCCWNTNSSFEMRRLYHKSIVPLEN 120
Glycine                 LAVGDTHVCATAVGDGTVCWRTGDTFRPSGSDQFASISSGSGFSCGILNGSKVRGW 180
Glycine                 DTNVAQQIENSFGNMSMSLVSAGGSNICGLNSTGFLCSCQSTDFTQTNLCGLDTNKLKVVCWGPWS 240
Glycine                 HGCAIRGSNGWVVCWGGNGQFSVNNVTEGVSFEVIGSVSNVFVCGLTSNMLKMEVFGAS 300
Glycine                 NYSNSSRFELPLPRVLPGPCVSFSCAECGSYVDSQTLCSGSGNICKPMTCRPQTTAPPPL 360
Glycine                 LPTTPPSSQPSPPPSPPPSSPPSSKRTGLRRPPAPFUPAIIGSGAFAGICTIVHCLWSGVCF 420
Glycine                 GKKKVHNSVQPTITRGSSGSSGGGASNNSNSSISSMIMRQTSIIMRRQFSSTKHPDR 480
Glycine                 ARLFTLAELKAATNNFSIHNKIFCAGSISVVHRGKLFDGRQVYKRAIESSMKMYQER- 539
Glycine                 PDRDHRPMKAAGTVGYIDPEYYGLNVLTAKSDVYGLGVHLLELLTGKRAIFKYGEDGGTP 599
Glycine                 N-WRMRKIVALDASRGIELYHKKHVFPSIIHRDINSSISSLDDATWCFSGFSSCFMPS 658
Glycine                 AE--------------------HVYSDFRTVLAKSDVYGLGVHLLELLTGKKAIFTKFGINRET- 718
Glycine                 PKFALSLA--------MALSTQKLALL-TALAILS-------44
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Phvul.001G243600-R
- SMVKIAGRUILGWKMKVILDPRUGAPHVNNEEALEVAHTAVSCVNSKRKDRPTMTQV 227
Glycine
LVSVDFAVPALAGELVILKIDPRUGPPGVN=EAEAVELVAYTAICVNLGKDRPTMADI 777
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- ILLNFT----------------------------------------------- 50
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**Fig 3.6.** A and B amino acid sequence comparison for the gene Phvul.001G243600 in the reference genome (Pv1.0), available at Phytozome (http://www.phytozome.net/) compared with the inferred sequences based on the amplified intron regions in the resistant (-R) and susceptible (-S) and the sequence of a homologous gene (XM_003537442.1) in *Glycine max*, extracted from NCBI (http://www.ncbi.nlm.nih.gov). Dashes indicate the missing amino acids in the comparison.
Fig 3.7. A and B amino acid sequence comparison for the gene Phvul.001G243700 in the reference genome (Pv1.0), available at Phytozome (http://www.phytozome.net/) compared with the inferred sequences based on the amplified intron regions in the resistant (-R) and susceptible (-S) and the sequence of a homologous gene (XM_003537442.1) in Glycine max, extracted from NCBI (http://www.ncbi.nlm.nih.gov). Dashes indicate the missing amino acids in the comparison.
**Fig 3.8.** A and B amino acid sequence comparison for the gene Phvul.001G243800 in the reference genome (Pv1.0), available at Phytozome (http://www.phytozome.net/) compared with the inferred sequences based on the amplified intron regions in the resistant (-R) and susceptible (-S) and the sequence of a homologous gene (XM_003537442.1) in *Glycine max*, extracted from NCBI (http://www.ncbi.nlm.nih.gov). Dashes indicate the missing amino acids in the comparison.

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Fig 3.9. PCR amplification of intronic genomic regions of DNA using sequence-specific primers designed based on the sequence of four candidate genes for anthracnose resistance in the navy bean Bolt. Each set of four lanes from left to right consist of resistance parent (Bolt), susceptible parent (H4784A-29844), OAC Rex, and Sanilac. 1 = K500-1, 2 = K500-2, 3 = K600-1, 4 = K600-2, 5 = K700-1, 6 = K700-2, 7 = K800-1, 8 = K800-2, 9 = K800-3, 10 = K800-4 as identified in Table 2.
Figure 3. Relative expression of cDNA in the leaves of the resistant (Bolt) and susceptible (H4784A-29824) common bean genotypes infected with Colletotrichum lindemuthianum in Phvul.001G243500 (a), Phvul.001G243600 (b), Phvul.001G243700 (c), and Phvul.001G243800 genes (d). Data represent means of relative expression for three inoculation times. The means were generated from three different inoculation time during infection course of 0, 2 and 4 days after inoculation with race 73 of C. lindemuthianum and bars indicated the standard error of the mean.
Figure 3. Fold increase/decrease in transcript levels of four serine/threonine kinase genes. (a) Phvul.001G243500, (b) Phvul.001G243600, (c) Phvul.001G243700, (d) Phvul.001G243800 in the infected relative to un-infected leaves of the resistant (Bolt) and susceptible (H4784A-29844) common bean genotypes infected with Colletotrichum lindemuthianum at 2 and 4 days after infection.
Chapter 4. Summary and future work

4.1: Summary of the study (Importance of anthracnose, breeding and genetic resistance)

The objective of this thesis was studying the inheritance of resistance to race 73 of *C. lindemuthianum* in the navy bean cultivar Bolt and identify the molecular markers and candidate resistance genes associated with resistance to this disease to help breeders to improve the seed quality by disease management.

The contributions of this work include:

1) The inheritance study of resistance to race 73 of *C. lindemuthianum* in the navy bean cultivar Bolt revealed presence of a major QTL on Pv01, likely associated with the Co-1 cluster as well as QTLs of minor effects, which may interact with the major anthracnose resistance locus in the navy bean Bolt.

2) Application of linkage mapping and in comparison with the physical map of the genomic regions in cultivar Bolt identified the distal end of Pv01 as the major contributor to resistance against race 73 of *C. lindemuthianum*.

3) Characterization of candidate genes in the significant region detected structural differences in the DNA sequence of the genes and therefore resulting amino acid sequences.

Outcomes of this research will help the breeders to develop markers in breeding strategies to very quickly introduce the gene into different backgrounds and across bean market classes. Since anthracnose is a very destructive disease and one of the main reasons why Ontario Bean Growers need to produce seed in disease-free environments with additional cost to the industry, developing durable anthracnose resistance will have significant impact in reducing input cost for the Ontario farmers.
The methodology used may have some limitations. In the laboratory, the use of different DNA and RNA kits, PCR and rt-PCR may have allowed for greater results, rather than the ones were used. In the field, environmental conditions played a large role in the disease pressure between locations. The disease incidence and severity were only determined based on the visual evaluation of infection or two field seasons.

4.2: Future research direction

The most difficult task in the management of bean anthracnose is its genetic variability due to the appearance of new races of the pathogen. Identification of resistant genes and introgression of these genes into commercial bean varieties adapted to different bean growing regions is important, which requires basic research and development approach with active involvement of plant pathologists, molecular geneticists and plant breeders.

A better understanding of the anthracnose host-pathogen interaction can help breeders in developing anthracnose resistant varieties. Future work could benefit from the genome sequences of bean genotypes. Genome sequences are becoming available to the scientific community, which might help better characterize the genes involved in important traits including resistance to diseases. Pyramiding host plant resistance genes can accelerate breeding of cultivars with resistances to several races of anthracnose pathogens.
References

Adam-Blondon AF, Sevignac M, Dron M, Bannerot H (1994) A genetic map of common bean to localize specific resistance genes against anthracnose. Genome 37:915-924


Barrus MF (1915) An anthracnose-resistant red kidney bean. Phytopathology 5:303–311


Burkholder WH (1918) The production of an anthracnose-resistance white marrow bean. Phytopathology 8: 353-359


CTA (1999) Improved control of bean anthracnose disease in Latin America and Africa through increased understanding of pathogen diversity. Summary report of European Commission supported STD-3 project, pp. 98-103


Flor HH (1947) "Inheritance of reaction to rust in flax". *J. Agric. Res. 74*: 241–262


Kaplan L, Lynch TF, Smith CE (1973) Early cultivated beans (Phaseolus vulgaris) from an intermontane Peruvian valley. Science 179: 76-77


McRostie GP (1919) Inheritance of anthracnose resistance as indicated by a cross between a resistant and a susceptible bean. Phytopathology 9: 141-148


Young RA, Kelly JD (1997) RAPD markers linked to three major anthracnose resistance genes in common bean. Crop Sci. 37:940–946


