# THE GENETICS OF ANTHRACNOSE RESISTANCE IN COMMON BEAN

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#### ABSTRACT

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*Colletotrichum lindemuthianum* is a widespread and highly variable pathogen that attacks common bean (Phaseolus vulgaris L.). Identifying new sources of anthracnose resistance in both Andean and Middle American bean types and developing efficient methods for incorporating resistance into elite bean lines is important to breeders worldwide for the development of more durable resistance. Accordingly, a highly diverse panel of 226 Andean beans was screened with eight races of anthracnose to identify and map new sources of resistance using a genome-wide association study (GWAS). Outputs from the GWAS indicated major QTL for resistance on three linkage groups: Pv01, Pv02, and Pv04 and minor QTL on Pv10 and Pv11. Candidate genes associated with the significant SNPs were detected on all five chromosomes. A QTL study with the black bean cultivar 'Jaguar,' known to possess resistance to anthracnose race 73, was conducted to determine the basis of the anthracnose resistance commonly used in the MSU breeding program. Resistance to anthracnose was investigated in an  $F_{4:6}$  recombinant inbred line (RIL) population developed from a cross between Jaguar and 'Puebla 152' (landrace cultivar known to be susceptible to race 73). Resistance in Jaguar was determined to be conditioned by the single dominant gene Co-1. Using the Illumina BARCBean6K\_3 BeadChip, the physical location of the Co-1 locus was mapped between 50.10 and 50.22Mb on chromosome Pv01. The genomic positions of the numerous resistance loci on Pv01, Pv02, Pv04 and Pv11 identified in the Andean panel and RIL population should prove useful for breeding programs interested in improving anthracnose resistance in cultivars using marker assisted selection.

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## **GENERAL INTRODUCTION**

#### **INTRODUCTION**

Anthracnose is a seed-borne disease of common bean (*Phaseolus vulgaris* L.) caused by the fungus *Colletotrichum lindemuthianum* (Sacc. & Magnus) Lams.-Scrib, and is cosmopolitan in distribution. It is one of the most economically important disease of common bean (Melotto et al., 2000), and can cause devastation to farmers' fields, resulting in yield losses as high as 95% in susceptible bean cultivars (Guzman et al., 1979).

*C. lindemuthianum* is a hemibiotrophic fungal pathogen (Ferreira et al., 2013) that relies on the common bean for nutrients prior to causing cell death in the plant. Although it is found worldwide, it is more prevalence in cooler, more humid environments such as the temperate regions and at higher elevation in the tropics (Pastor-Corrales and Tu, 1989). The optimal temperature for conidia development ranges from 18 to 22°C (Guzman et al., 1979), and at least 92% relative humidity and moisture present on the foliage for at least 12 hours after conidia deposition is essential for germination (Tu, 1982).

Typical symptoms are deep, shrunken lesions containing flesh-colored spores on bean pods that are the most distinctive symptoms of anthracnose. Lesions also commonly appear on stems, hypocotyls and leaf veins of seedling plants, with more advanced disease resulting in wilting and flagging of chlorotic leaves similar to that of other foliar pathogens (Ferreira et al., 2013). Further advancement of the disease leads to complete girdling and ultimately death of the plant. Infection of the bean pods results in rust-colored lesions that develop into sunken cankers with black ring boarders. Severely infected immature pods are often aborted, while pods that mature produce infected seed with dark cankers that make the seed unmarketable to consumers (Pastor-Corrales and Tu, 1989).

#### CONTROL MEASURES

Numerous methods exist for the control of anthracnose, with varying degrees of effectiveness. The greatest priority is given to genetically resistant varieties, but research has also focused on appropriate crop rotations, planting disease free seed, and pesticide application. Crop rotation is important because spores can survive in the soil on plant debris for as long as 22 months (Ferreira et al., 2013). Infected bean debris should be incorporated into the soil post-harvest, and beans should be in at least a three year rotation.

*C. lindemuthianum* is a seed borne pathogen and is thus particularly problematic for small scale farmers of many third world countries who save their own seed from year to year (Ferreira et al., 2013). Transmission of anthracnose through the seed is very efficient (Kelly and Vallejo, 2004), which makes clean seed an essential component of anthracnose prevention around the world. For growers in the temperate regions, it is important to plant seed from semi-arid regions where anthracnose is not a concern. Effective clean seed programs have resulted in diminished cases of anthracnose in America and Europe (Pastor-Corrales and Tu, 1989). However, clean seed is not a viable option for many farmers in Latin America and Africa as these programs require organized seed production in areas where the pathogen is not endemic (Ferreira et al., 2013). Thus, infected seeds of susceptible varieties still serve as a main source of disease inoculum and transmission in subsistence farming systems (Melotto et al., 2000).

Alternatively, seeds and plants can be treated with fungicides. Spraying for anthracnose can reduce the prevalence of anthracnose if applied early in the infection process; however, overall success has been limited (Pastor-Corrales and Tu, 1989). In addition, the costs associated with repeated chemical use, makes such an option out of the reach of small scale farmers in developing countries. Genetic resistance offers a more long-lasting and acceptable solution.

Utilizing genetic resistance to anthracnose is the area of research and development that contains the most promise for reducing the damaging effects of the pathogen in common bean. One specific aspect of genetic resistance is pyramiding multiple resistance genes to provide more durable resistance against anthracnose. This is especially important for a variable pathogen such as *C. lindemuthianum* as resistance quickly breaks down when single resistance genes are deployed in a given cultivar. Further, the *C. lindemuthianum* pathogen has been shown to become specialized on a single gene pool of the *P. vulgaris*, thus pyramiding resistance genes from the alternative gene pool may provide more durable resistance (Young and Kelly, 1997). Developing cultivars with multiple resistance genes for more durable resistance is a high priority among bean breeders.

#### PATHOGEN VARIABILITY

Variability in *C. lindemuthianum* was first described by Barrus (1911) when he noticed differences between virulence of two races of anthracnose against 139 bean cultivars. These first two races were identified as  $\alpha$  and  $\beta$ , and laid the foundation for the discovery of greater pathogenic variability. Since that time, new races of anthracnose have been documented worldwide. However, many researchers in various countries used local codes instead of the Greek letters to identify anthracnose (Melotto et al., 2000) and no standardized system was in place for screening the disease, which has limited knowledge of the global variability of *C. lindemuthianum*. In more recent years, a standardized system has been developed that determines isolate pathogenicity based on the use of 12 differential bean cultivars (Table 1) originating from both the Mesoamerican and Andean gene pools (Pastor-Corrales, 1991). Within the system, each differential bean variety is assigned a binary number which is then used to identify the particular

*C. lindemuthianum* pathogen. The sum of the cultivars with susceptible reactions gives the binary number of a specific race. For example, anthracnose race 73 is virulent on Mexico 222 [64], Cornell 49242 [8], and Michelite [1].To date, over 100 pathogenic races have been reported globally using the 12 differential cultivars and the binary naming system (Ferreira et al., 2013; Gonzáles et al., 2015).

#### HOST RESISTANCE

Although unproven, it is likely that resistance to anthracnose follows the gene-for-gene theory (Flor, 1947) in which a resistance gene in the plant corresponds with an avirulence gene in the pathogen to mediate an incompatible reaction. Many resistance genes (Co-genes) have been characterized in common bean (Ferreira et al., 2013), but avirulence genes have yet to be investigated in *C. lindemuthianum*. In such systems, resistance is triggered by a specific gene product of the plant in recognition of avirulent gene products produced by the pathogen. Specificity between *C. lindemuthianum* races and common bean cultivars is well known (Tu, 1992), with the incompatible reactions often being characterized by a hypersensitive reaction (HR).

In the case of HR, resistance is often characterized by localized cell death quickly after fungal penetration, preventing the formation of infection vesicles and ultimately preventing the spread of the fungus. Each anthracnose resistance gene confers resistance to numerous races of *C. lindemuthianum* and some genes have been identified as complex loci comprised of multiple alleles. As an alternative to major resistance genes conferring resistance to anthracnose, some authors have investigated the role played by quantitative trait loci (QTL) in partial resistance (Geffroy et al., 2000; Oblessuc et al., 2014; González et al., 2015). However, in many cases these

QTL have been shown to co-localize with major resistance genes suggested that partial resistance may vary due to the genetic background of the genotype in which it is expressed.

#### **RESISTANCE GENES**

To date, as many as 40 resistance genes (R genes) have been described that condition resistance to specific isolates of *C. lindemuthianum* in common bean (González et al., 2015). Each gene has been assigned a "Co" symbol as proposed by Kelly and Young (1996) in order to standardize the naming of resistance genes, and thus prevent unnecessary duplication in naming resistance genes. R genes confer resistance through their involvement in detecting specific effectors from the pathogen as well as playing a role in activating plant immune response to prevent further infection of the disease. Currently, over 70 R genes have been isolated from various plants (Liu et al., 2007). The majority of the R proteins contain conserved structural motifs such as leucine-rich repeat (LRR), nucleotide-binding site (NBS), protein kinase domain (PK), Toll-interleukin-1 receptor domain (TIR) and leucine zipper (LZ) structure or other coiled-coil (CC) structures. These domains are involved in R protein interaction with pathogen effectors and activating signal transduction pathways involved in plant immunity (Liu et al., 2007).

## CO-EVOLUTION OF P. VULGARIS AND C. LINDEMUTHIANUM

Common bean was domesticated in two separate regions, one domestication event occurring in Middle America and the other event occurred along the eastern edge of the Andes Mountains of South America (Gepts and Debouck, 1991). As a result, there are two unique gene pools within *Phaseolus vulgaris* that are partially reproductively isolated, and anthracnose resistance genes are categorized based on the gene pool of the susceptible host cultivar. In addition, *C. lindemuthianum* is also classified according to which gene pool of the host cultivar in which the anthracnose race was isolated (Balardin and Kelly, 1998). Coevolution could be the mechanism driving resistance and virulence diversity in common bean through a continuous series of reciprocal selection pressure applied by the plant and the pathogen (Geffroy et al., 1999). The Mesoamerican *C. lindemuthianum* pathotypes have a broad virulence range and attack mainly small-seeded Mesoamerican varieties, whereas the Andean pathotypes have a more narrow virulence range and predominately attack large-seeded Andean beans (Pastor-Corrales et al., 1995; Fabe et al., 1995).

#### BREEDING FOR ANTHRACNOSE RESISTANCE

The first information on the inheritance of anthracnose resistance in common bean was published by Burkholder (1918), starting the long process of breeding resistance to a pathogen with such high levels of variability. Breeding for resistance in gene specific disease recognition systems requires identification of resistant plants (Strange and Scott, 2005), which can then be crossed with elite varieties. Historically, the majority of resistance genes identified in breeding programs have been Middle American in origin. Kelly and Vallejo (2004) reported that of the known anthracnose resistance genes, only 10% were Andean in origin. In more recent years, additional Andean sources of resistance genes *Co-12*, *Co-13*, *Co-14*, and *Co-15* have been identified including additional alleles of the *Co-1* locus (Goncalves-Vidigal et al., 2008; 2009; 2011; 2012; Sousa et al., 2015). Identifying additional sources of resistance in both gene pools plays a crucial role in reducing the vulnerability of common bean to the constantly evolving *C*. *lindemuthianum* pathogen. Proper identification of resistance is necessary for efficiently

transferring resistance using marker assisted breeding for the pyramiding of resistance genes, resulting in a more durable resistant cultivar.

Breeders utilize both greenhouse and lab techniques in screening for anthracnose. In the greenhouse, the most prevalent method for inoculating the common bean with anthracnose involves spraying a suspension of  $10^6$  conidia ml<sup>-1</sup> of specific races *C. lindemuthianum* onto seedling plants. Plants are then maintained under high humidity (>80%) for a minimum of three days. Symptoms of anthracnose are observed 8-10 days after initial inoculation and are observed to be similar to symptoms in the field (Ferreira et al., 2013).

Results of inoculation in the greenhouse are often recorded in one of two ways. Some researchers use a scale, which can be useful in identifying genes that may confer partial levels of resistance conferred by a quantitative trait. Other scientists simply categorize reaction types into two broad categories of susceptible and resistant which implies an assumed qualitative resistance. The later method tends to be sufficient as most of the resistance of common bean to anthracnose tend to follow a qualitative mode, and two simple categories are useful when characterizing the different races of *C. lindemuthianum* (Ferreira et al., 2013).

In the lab, a number of molecular tools have been utilized to streamline the process of identifying anthracnose resistance in elite lines of common bean. One of the most useful tools for plant breeders is molecular markers. PCR-based markers such as sequence characterized amplified regions (SCARs) are currently the most commonly used markers in bean breeding (Ferreira et al., 2013). Molecular markers are useful because they enable researchers to detect DNA polymorphisms associated with specific phenotypes, allowing them to reduce the amount of field screening otherwise required. Currently, there are SCAR linked to nine of the 14 major Co-genes. Some of the earliest marker work identifying anthracnose resistance with molecular

markers included the identification of the OF10<sub>530</sub> RAPD marker linked *Co-1* gene on chromosome Pv01, and the OAB3<sub>450</sub> linked to *Co-5* gene on Pv07 (Young and Kelly, 1997). Other markers identified have included the CV542014 and TGA1.1 linked to the *Co-1*<sup>4</sup> allele on Pv01, (Goncalves-Vidigal et al., 2011), OQ41400 linked to *Co-2* gene on Pv11 (Young and Kelly, 1996), SW12 linked to *Co-3* gene on Pv04 (Miklas et al., 2000), and BM210 linked to *Co-5* gene on Pv07 (Campa et al., 2009). Many of the molecular markers are yet to be placed on the physical common bean map (Schmutz et al., 2014). Mapping of linked SNP markers in combination with the physical genome can help identifying the physical location of known, and yet to be discovered, anthracnose resistance genes, ultimately contributing to both the mapping of resistance genes, and improving the molecular tools necessary for marker assisted selection (MAS).

#### GENERAL OBJECTIVES

Colletotrichum lindemuthianum is a widespread pathogen with high variability. As such, efficient and effective methods for incorporating resistance into elite lines as well as identifying new sources of anthracnose resistance is vital to breeders worldwide. However, despite numerous studies investigating anthracnose resistance in common bean, there is still limited knowledge and underutilization of Andean sources of resistance. New sources of both Andean and Middle American resistance need to be identified and characterized to provide breeders with multiple options for pyramiding genes, in order to develop the most durable resistance. Therefore, the objectives of this study were to 1) identify and map new sources of anthracnose resistance in Andean beans and 2) fine map and identify new molecular markers for the detection of resistance to anthracnose race 73 in a black bean mapping population. In order to address these objectives, the research was divided into two main sections, each of which is represented by a chapter in this thesis. The objectives in the first chapter were to utilize the Andean Diversity Panel (ADP) – a compilation of approximately 230 lines of large-seeded dry beans preferred by both growers and consumers in Africa, United States, and Latin America – to 1) identify new sources of anthracnose resistance in a diverse panel of Andean beans, and 2) explore the genetic basis of the resistance using Genome-wide association analysis (GWAS). The objective in the second chapter was to use SNP markers to identify more tightly linked molecular markers and physically map the resistance locus to anthracnose race 73 in black beans that is widely deployed in the MSU breeding program.

APPENDIX

Differential Cultivar	Gene Pool	Host Genes	Binary Number
Michelite	Middle American	Co-11	1
MDRK	Andean	Co-1	2
Perry Marrow	Andean	Co-1 <sup>3</sup>	4
Cornell 49242	Middle American	Co-2	8
Widusa	Andean	Co-1 <sup>5</sup>	16
Kaboon	Andean	Co-1 <sup>2</sup>	32
Mexico 222	Middle American	Co-3	64
PI 207262	Middle American	Co-3 <sup>3</sup> , Co-4 <sup>3</sup>	128
ТО	Middle American	Co-4	256
TU	Middle American	Co-5	512
AB 136	Middle American	Co-6, Co-8	1024
G 2333	Middle American	Co-4 <sup>2</sup> , Co-5 <sup>2</sup> , Co-3 <sup>5</sup>	2048

Table 1. Anthracnose differential series, host gene pool, resistance genes, and the binary number of each cultivar used to characterize races of anthracnose in common bean.

Binary number 2n, where n is equivalent to the place of the cultivar within the series. The sum of the cultivars with susceptible reactions gives the binary number of a specific race (Pastor-Corrales, 1991). For example, race 73 is virulent on Mexico 222 [64], Cornell 49242 [8], and Michelite [1].

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# CHAPTER ONE: GENOME-WIDE ASSOCIATION STUDY OF ANTHRACNOSE RESISTANCE IN ANDEAN BEANS

## ABSTRACT

Anthracnose is a seed-borne disease of common bean (*Phaseolus vulgaris* L.) caused by the fungus Colletotrichum lindemuthianum, and the pathogen is cosmopolitan in distribution. It is one of the most economically important diseases of common bean, and can cause devastating yield losses in susceptible bean cultivars. The objectives of this study were: (i) identify new sources of anthracnose resistance in a diverse panel of Andean beans comprised of multiple seed types and market classes from the Americas, Africa, and Europe, and (ii) explore the genetic basis of this resistance using Genome-wide association mapping analysis (GWAS). Numerous resistant lines were identified within the 230 bean lines screened for eight races (7, 39, 55, 65, 73, 109, 2047, and 3481) of anthracnose included in the study. Twenty-eight lines were resistant to six out of the eight races screened, and one cultivar - Uyole98 was resistant to all eight races included in the study. Outputs from the GWAS indicated major QTL for resistance within Andean beans on three linkage groups: Pv01, Pv02, and Pv04 and two minor QTL were detected on Pv10 and Pv11. Candidate genes associated with the significant SNPs were detected on all five chromosomes. The information reported will provide breeders with new and diverse sources of resistance and genomic regions to target in development of markers linked to anthracnose resistance in Andean beans.

#### INTRODUCTION

Currently, there are over 800 million people in the world that are unable to obtain sufficient calories on a regular basis (Strange and Scott, 2005). If current population projections are maintained, by 2050 70% more food will be needed, with the majority of that increase in developing countries (Alexandratos and Bruinsma, 2012). To meet this increase in global food demand, the productivity of most food crops must increase in areas where yields are significantly lower than their potential. One specific component of production that has great potential for improving yields is incorporating disease resistance into food crops as more than 10% of food production is lost to plant diseases globally on an annual basis (Strange and Scott, 2005). Among the crops that suffer losses to diseases, grain legumes tend to be highly susceptible to many diseases.

The common bean (*Phaseolus vulgaris* L.) is the most important grain legume globally for direct human consumption and is particularly significant in many developing countries (Broughton et al., 2003). Historically, and still today, the common bean is grown and consumed in developing countries in Africa, Latin America, and Asia with eight of the top ten producing countries of dry beans considered as developing (Gepts et al., 2008). In 2013, FAO reported that common beans were grown on 29.1 million ha and produced over 22.8 million metric tons (FAO, 2014). However, these statistics are likely significant underestimations of actual production as common bean is largely a subsistence crop grown on small farms and consumed locally. In Latin America, a significant part of bean production is on farms less than 10 ha, often on marginal land with limited inputs. In Mexico for example, small holder farms (<5 ha) are responsible for over 65% of the total bean production in the country (Broughton et al., 2003). As such, beans play a major food security role in that country.

Beans are consumed in high numbers by many poorer countries due in large part to their high nutritional value. More than half of the developing world consumes insufficient amounts of micronutrients; two of the most common being iron and zinc. An average cup of beans supplies 15% of the recommended daily allowance of zinc, and 25% of the recommended daily allowance of iron, with some varieties containing even higher amounts (Cichy et al., 2009). Other nutritional benefits include calcium, dietary fiber, and vitamins (Leterme and Muñoz, 2002). In addition to supplying numerous micronutrients, rural farmers consume substantial quantities of beans every year due to it being a valuable source of dietary protein (Sathe, 2002). Beans complement carbohydrate-rich sources of food consumed in conjunction with beans such as rice and maize and are considered by many as a poor man's "meat." Nicaragua, one of Latin America's poorest countries, has an average pulse consumption of 25 kg/capita per year, with common beans accounting for 87% of that total (Leterme and Muñoz, 2002). Rwanda, in a similarly weak economic state has an even higher average yearly bean consumption rate of 40 kg/capita per year. When consumption was further investigated, it was found that lower income classes consumed as much as 20% more beans than the average statistics for their respective countries (Broughton et al., 2003).

In the tropics and subtropics, bean yields are greatly reduced as bean plants are susceptible to numerous diseases caused by fungal pathogens (Graham and Vance, 2003). Fungi are particularly problematic due to their prolific sporulation and ability to efficiently infect a field through wind and rain-splash. This increase in disease pressure is further exacerbated due to the tropical climate allowing for continuous cropping – resulting in further inoculum build-up without a winter season to reduce disease prevalence in the soil (Strange and Scott, 2005).

One of the most serious seed-borne diseases of common bean is anthracnose caused by the fungus Colletotrichum lindemuthianum (Sacc. & Magnus) Lams.-Scrib, and is cosmopolitan in distribution. It is one of the most economically important diseases of common bean (Melotto et al., 2000), and can cause devastation to farmers' fields, resulting in yield losses as high as 95% in susceptible bean cultivars (Guzman et al., 1979). This is of particular concern in developing countries, as populations with high amounts of poverty are often dependent on locally produced staples (Strange and Scott, 2005), and the failure of a crop could result in many people going without adequate food. Being a seed borne pathogen makes anthracnose especially problematic for small scale farmers of many third world countries who save their own seed from year to year (Ferreira et al., 2013). Transmission of anthracnose through the seed is very efficient (Kelly and Vallejo, 2004), which makes clean seed an essential component of anthracnose prevention around the world. For growers in the temperate regions, it is important to plant seed produced in semi-arid regions where anthracnose is not present. Effective clean seed programs have resulted in diminished cases of anthracnose in America and Europe (Pastor-Corrales and Tu, 1989). However, clean seed is not a viable option for many farmers in Latin America and Africa as clean seed programs require organized seed production in areas where the pathogen is not endemic (Ferreira et al., 2013). Thus, infected seeds of susceptible varieties still serve as a main source of disease inoculum in subsistence farming systems (Melotto et al., 2000).

Incorporating genetic resistance to anthracnose is the area of research and development that holds the most promise for reducing the effects of the pathogen in common bean. It is likely that resistance to *C. lindemuthianum* follows the gene-for-gene theory (Flor, 1947) in which a resistance gene in the plant corresponds with an avirulence gene in the pathogen to mediate an incompatible reaction. However, anthracnose is a highly variable – over 100 pathogenic races

have been reported globally – and thus finding durable resistance can be a challenge. One strategy to combat breakdown of major gene resistance is pyramiding multiple resistance genes to provide a more durable resistance. This is especially important for resistance against such a variable pathogen as *C. lindemuthianum* as resistance quickly breaks down single gene resistance. Further, the *C. lindemuthianum* pathogen becomes specialized on one of the gene pools of *P. vulgaris*, and thus pyramiding resistance genes from the alternative gene pool may prove useful (Young and Kelly, 1997). Therefore, developing cultivars with multiple resistance genes for more durable resistance should be of high importance to breeders. In a gene-for-gene system, resistance is triggered by the recognition of avirulent gene products produced by the pathogen by a specific gene product of the host plant. In *P. vulgaris*, as many as 40 known resistance genes (Co-genes) conferring resistance to anthracnose have been reported (González et al., 2015) but only a few are fine mapped to the bean genome. Fine mapping of these genes is needed if robust markers are to be developed to assist bean breeders develop durable resistance to anthracnose.

The majority of resistance genes are Middle American in origin. Kelly and Vallejo (2004) reported that of the known anthracnose resistance genes, only 10% were Andean in origin. This presents both complications and great potential. The relatively few anthracnose resistance genes currently present in Andean beans provides a challenge to bean breeders working to improve these seed types. Finding additional resistance sources in Andean beans would provide an additional reservoir of materials within this gene pool, and offer the possibility of pyramiding anthracnose resistance genes into Mesoamerican beans which could result in durable resistance.

The objective of this study was to utilize a subset of 226 lines from the Andean Diversity Panel (ADP) – a compilation of large-seeded dry beans preferred by many growers and

consumers around the world – to (i) screen a subset of APD panel against eight races of anthracnose to identify new sources of anthracnose resistance in Andean beans, and (ii) explore the genetic basis of the resistance using Genome-wide association mapping analysis (GWAS) to identify and fine map new genomic regions controlling resistance.

#### MATERIALS AND METHODS

A subset of 226 bean lines selected from the Andean Diversity Panel (ADP) developed by Cichy et al. (2015), was screened with eight different races of anthracnose in the greenhouse during Spring and Fall of 2014. Many of the 396 lines included in the ADP come from breeding programs in the U.S. and from African countries and South American countries where Andean beans originated (Tables S1 and S2). The subset of the ADP that was selected was based on ability to increase seed under Michigan growing conditions. The anthracnose races used in the study are named based on the standardized system of virulence of each race to the 12 differential bean cultivars (Table 1; Pastor-Corrales, 1991). The choice of races was made to include common cosmopolitan races, highly virulent Andean races, and highly virulent races across both gene pools. Race 7 is an Andean race that has been found in South America, and is also found in high frequency in the United States (Balardin et al., 1997; Kelly et al., 1994). Races 65 and 73 are both common Mesoamerican races in North, Central, and South America, with race 73 representing over 25% of all isolates identified in an anthracnose diversity study (Balardin et al., 1997). Race 39 is an Andean race that is known for being virulent to numerous Andean differential cultivars including Kaboon, Perry Marrow, and Michigan Dark Red Kidney (MDRK). Similarly, race 55 is also a highly virulent Andean race that in addition to the Andean cultivars Kaboon, Perry Marrow, and MDRK, race 55 is also virulent to the Andean differential

Widusa, resulting in susceptibility of all the differentials known to possess an allele of the *Co-1* locus (Oblessuc et al., 2014). Race 109 was included as it also is virulent to the Andean differential cultivars Kaboon and Perry Marrow. Lastly, highly virulent races 2047 and 3481 were included in the study as they are virulent across both gene pools (see Table 9.1 in Ferreira et al., 2013), and result in susceptibility of nearly the entire differential series. Race 2047 is virulent to all the Andean differential cultivars, whereas race 3481 is highly virulent on G2333 the most resistant source in the differential series (Table 1).

For each bean line included in the study, six seedlings were grown in trays containing standard potting soil in the Michigan State University greenhouses, East Lansing, MI. Inoculations were done by spraying a suspension of 1.2 x 10<sup>6</sup> *C. lindemuthianum* conidia ml<sup>-1</sup> onto the leaves and stems of seedling plants. Plants were then maintained under high humidity (>80%) in a mist chamber for a minimum of three days. Symptoms of anthracnose were observed on susceptible plants 8-10 days after initial inoculation and rated a 0-5 scale developed by Drijfhout and Davis (1989). Ratings were assessed as follows: 0, no symptoms observed; 1, pinpoint lesions present on stem and hypocotyl; 2, small surface lesions on stem and leaf veins; 3, large, sunken lesions present on stem; 4, lesions sunken to the center of the stem, wilting of chlorotic leaves; 5, plant killed by pathogen. Some ADP lines were heterogeneous mixtures for reaction to specific anthracnose races and were removed from the final analysis, and that data is reported in table S1. The data was retained and will be provided to the bean research community (Table S1 and S2). Correlations between races that clustered on the same region of a given chromosome based on GWAS was run using the following equation:

$$r_{X,Y} = \frac{COV_{X,Y}}{\sqrt{V_X V_Y}}$$

Where: *X*, *Y* are the anthracnose disease rating of the clustered races,  $r_{X,Y}$  is the correlation coefficient,  $COV_{X,Y}$  is the covariance between the two clustered anthracnose races, and  $\sqrt{V_X V_Y}$  is used to scale the covariance to vary between -1 and 1.

DNA was collected from young leaf tissues of ADP genotypes grown in the greenhouse at Michigan State University using a modified CTAB (Hexadecyltrimethyl ammonium bromide) extraction protocol (Doyle, 1987). The DNA concentrations were measured using a Nanodrop spectrophotometer, and its quality was checked on an agarose gel. The Andean panel was genotyped using an Illumina BARCBean6K\_3 BeadChip with 5398 SNPs (Hyten et al., 2010).

The population structure in the ADP was determined using principal component analysis (PCA) implemented in EIGENSTRAT (Price et al., 2006) as described in Kamfwa et al. (2015). After filtering for low quality and monomorphic SNPs as well as for minor allele frequency (MAF>0.02), a total of 4850 SNPs were retained for the PCA and association analysis. The kinship matrix developed using identical by descent method implemented in TASSEL was included in the association analysis to correct for cryptic relatedness. A Mixed Linear Model (MLM) (Zhang et al., 2010) was run in TASSEL to determine the SNP-trait associations. The MLM equation used in the analysis was as follows:

$$Y = X\alpha + P\beta + K\mu + \varepsilon$$

Where: Y is the phenotype of a genotype; X is the fixed effect of the SNP; P is the fixed effect of the population structure; K is the random effect of the relative kindship;  $\varepsilon$  is the error term and is assumed to be normally distributed with a mean of zero. The conservative Bonferonni corrected  $p = 1.0 \times 10^{-5}$  (for  $\alpha = 0.05$  and 4850 SNPs) was used to determine the significance threshold for SNPs.

The common bean genome (Schmutz et al., 2014) was browsed using Jbrowse on Phytozome v10 (Goodstein et al., 2012) to identify positional candidate genes associated with the significant SNPs. The functional annotation for the gene was then identified on Phytozome v10 in order to infer the possible role of the gene in conferring anthracnose resistance.

#### **RESULTS AND DISCUSSION**

Developing common bean cultivars with resistance to anthracnose is one of the most effective ways of controlling this important disease. In order to develop such varieties, identification of sources of resistance as well as understanding the underlying genetic basis of anthracnose resistance is critical. In the current study, resistance to eight races of *C*. *lindemuthianum* as well as the genetic basis of the resistance was investigated in a diverse group of Andean bean lines. Numerous resistant lines were identified within the 226 Andean bean lines screened for all eight races of anthracnose races (7, 39, 55, 65, 73, 109, 2047, and 3481) included in the study (Table 1.1: Table S1 ). In general, the anthracnose races not commonly associated with Andean beans (races 65, 73, and 3481) expressed the lowest level of virulence to the ADP. Race 3481, despite being a highly virulent race, was virulent on only 28.8% of the ADP, while races 65 and 73 were virulent to 36.7% and 34.5% respectively. It would appear that the majority of resistance within the ADP to these three races can be attributed to the *Co-1* locus and its alleles, as all three races were not virulent on the *Co-1* (MDRK), the *Co-1*<sup>3</sup> (Perry Marrow), or *Co-1*<sup>2</sup> (Kaboon) alleles at the Co-1 locus.

Conversely, the other 5 races of anthracnose were virulent to more than 50% of the cultivars screened. Resistance to virulent Andean races 39 and 55 was 41.6% and 41.2% respectively. Both races 39 and 55 are virulent to the majority of alleles at the *Co-1* locus, and

thus it can be assumed that resistance is provided by a locus less common within Andean beans. Resistance to races 7 and 109 were less prevalent than races 39 and 55 with only 37.6% of the ADP resistant to race 7 and 34.5% of the ADP resistant to race 109. The race most virulent to the ADP was race 2047, in which only 4.4% of the cultivars carried some level of resistance.

Within the ADP, certain lines possessed resistance to multiple anthracnose races. Of the 226 lines included in the study, 28 were resistant to six or more of the eight races included in the study (Table 1.2). Nineteen of the lines were bred in North America. The most resistant line identified was Uyole 98, a yellow bean variety from Tanzania, which was found to have resistance to all eight races of anthracnose. Previous work has also identified Uyole 98 as having resistance to another fungal pathogen, angular leaf spot (Fivawo and Msolla, 2011). Another cultivar of note was 'Red Hawk.' This cultivar was one of the 28 lines resistant to at least six races of anthracnose, and illustrates the potential value of gene pyramiding as it is known to possess the Andean resistance gene Co-1 and the Mesoamerican resistance gene Co-2 (Kelly et al., 1998)

GWAS results indicated that significant major QTL for resistance within Andean beans resided on three linkage groups. Races 65, 73, and 3481 all identified resistance on Pv01 (Figure 1.1). The resistance within the ADP to these three races showed a strong correlation expected from resistance mapping to the same region with correlation coefficients of  $r = 0.88^{**}$  between races 65 and 73,  $r = 0.64^{**}$  between races 65 and 3481, and  $r = 0.73^{**}$  between races 73 and 3481. Resistance to the virulent Andean races 39 and 55 was detected on Pv02 (Figure 1.2). The disease pattern of both of these races was also strongly correlated ( $r = 0.82^{**}$ ). Resistance to both anthracnose races 7 and 109 resided on Pv04 (Figure 1.3). However, unlike the other races that were strongly correlated, no correlation between races 7 and 109 (r = -0.04; p = 0.55) was

observed suggesting that either different loci on Pv04 confer resistance to these races or that resistance to race 7 within the ADP appears to residue on other linkage groups Pv10 and Pv11.

GWAS did not prove informative for determining resistance within the ADP to *C*. *lindemuthianum* race 2047. Only 10 lines within the ADP were resistant to the virulent race 2047, resulting in a low 4.4% level of resistance (Figure 1.1). It is likely that there was not sufficient resistance within the ADP for GWAS to identify meaningful associations between SNPs and the resistance trait, resulting in failure to identify any putative resistance loci (Figure S1).

The data suggest a correspondence with the major resistance genes Co-1 on Pv01 (Ferreira et al., 2013) and with the ANT02.1<sup>UC</sup> QTL or the Co-u gene on Pv02 (Oblessuc et al., 2014), and new sources of resistance on Pv04, Pv10, and Pv11. This work identifies both previously recognized resistance genes as well as resistance genes unknown to be present in Andean beans. Previous work has identified Co-1, Co-12, Co-13, Co-14, and Co-15 genes (Goncalves et al., 2010; Goncalves-Vidigal et al., 2008; 2009; 2012; Sousa et al., 2015), within the Andean gene pool. All the newly discovered resistance genes come from Brazilian landraces, and many condition resistance to anthracnose race 2047. Resistance to this virulent race was very rare within the panel, indicating that the sources of resistance discovered within the ADP are conditioned by other sources. The majority of anthracnose resistance genes in Andean beans reside on Pv01 and Pv04, making the identification of additional resistance sources critical for gene pyramiding.

## Linkage Group Pv01

The data for resistance to races 65, 73, and 3481 indicate a correspondence with the *Co-1* gene on Pv01 (Figure 1), which is known to condition resistance to anthracnose races 65 and 73

(Ferreira et al., 2013). Although no previous study has identified *Co-1* to condition resistance in common bean to race 3481, the *Co-1* gene is known to condition broad resistance (Goncalves-Vidigal et al., 2011). The *Co-1* gene is the known source of resistance in the differential cultivar MDRK, as well as the popular resistance source A193 widely used in Mexican breeding programs for anthracnose resistance (Mendoza et al., 2001).

The other differential cultivars of Andean origin that possess alleles at the Co-1 locus are Kaboon with the  $Co-l^2$  allele, Perry Marrow with  $Co-l^3$  allele and Widusa with  $Co-l^5$  allele (Goncalves-Vidigal and Kelly, 2006; Melotto and Kelly, 2000). The  $Co-1^4$  allele at the Co-1locus, which has been shown to confer resistance to 21 different races of anthracnose, including race 2047 (Goncalves-Vidigal et al., 2011) did not appear to be prevalent in the ADP. Only 10 accessions within the panel showed resistance to the highly virulent race 2047 that infects 11 of the 12 differential cultivars. This indicates that another allele at the Co-1 locus must also be conditioning resistance to the virulent race 3481 within the ADP. It is likely that the locus conditioning resistance to race 3481 in the ADP is the same as the Co-x gene identified in Jalo EEP558, which has been shown to condition resistance to the virulent C. lindemuthianum strain 100 (Geffroy et al., 2008). Strain 100 coincides with race 3993 when using the binary numbering system, which is differentiated from race 3481 by its virulence to the differential cultivar TU (512; Table 1). Other genes that condition disease resistance that have been identified in the vicinity of  $Co-1^4$  include the Ur-9 gene which confers resistance to common bean rust (Kelly et al., 2003), and the Phg-1 gene which confers resistance to angular leaf spot (Goncalves-Vidigal et al., 2011), but screening for these two pathogens was not conducted in this study.

Within this study, resistance to races 65, 73, and 3481, all associated significantly, and most strongly, with SNP ss715645251, indicating that the region plays an important role in

conferring anthracnose resistance. The SNP is located at 50.301532 Mb on the physical map, which falls within the region where the *Co-x* gene was shown to reside (Richard et al., 2014). As such, the region around the SNP was investigated for potential positional gene candidates. The SNP fell within the exon of gene Phvul.001G243800, which codes for a Leucine-rich repeat (LRR) receptor-like protein kinase, and was also identified as one of eight candidate genes by Richard et al. (2014). Kinases have previously been identified as playing an important role in the *COK-4* anthracnose resistance locus on Pv08 (Melotto et al., 2004). Kinases are known to play an important role in signal transduction, and receptor-like kinases carrying LRR motifs have been identified as an important component of race-specific disease resistance in rice and can be subject to adaptive selection (Wang et al., 1998). The identification of a LRR receptor-like protein kinases as a candidate gene, and its role in adaptive selection, supports prior literature indicating a co-evolution of common bean and anthracnose (Balardin and Kelly, 1998; Geffroy et al., 1999).

#### Linkage Group Pv02

The data from GWAS indicate that resistance to anthracnose races 39 and 55 resides on Pv02 (Figure 1.2) and appears to be associated with either the ANT02.1<sup>UC</sup> QRL (Oblessuc et al., 2014) or the *Co-u* gene on Pv02 (Geffroy et al., 2008). A major quantitative resistance loci (QRL) ANT02.1<sup>UC</sup> was identified as the source conferring resistance to races 38 and 55 in the Brazilian carioca genotype ICA-UNA (Oblessuc et al., 2014). In that study the molecular marker IAC255 was tightly linked to the QRL, which was less than 1 Mb away from SNP ss715648451, located at 48.606517 Mb on the physical map. SNP ss715648451 was the most significant SNP identified for resistance to both races 39 and 55 in the current study. This is a significant finding

as both races 39 and 55 are aggressive Andean races, and as such, identifying new sources of resistance within Andean beans is critical for future breeding.

Previously, the *Co-u* gene conditioning resistance to Tanzanian strains E4 and E42b in genotype BAT93 was reported in the vicinity of the I gene on Pv02 (Geffroy et al., 2008). In the absence of a physical map position for the Co-u gene we were not able to verify a direct association with resistance locus reported here. However, the most tightly linked SNPs (ss715648451, ss715648452, ss71639906) with resistance to anthracnose races 39 and 55 in our study were recently mapped by Bello et al. (2014) at 50 Kb from the cluster of NBS-LRR Rgenes residing near the I gene. Data would suggest that resistance identified to races 39 and 55 in the ADP is in fact the *Co-u* gene in the Mesoamerica genotype BAT93 previously reported by Geffroy et al. (2008). The most significant SNP ss715648451 was located within the exon of gene Phvul.002G328300, which codes for a Mitogen-activated protein kinase, which are known to play a role in disease response in Arabidopsis (Menke, 2004). Therefore, this gene could play an important role in initiating disease response in common bean. A second locus on Pv02 was recently reported to condition resistance to races 3, 19 and 449 in the Andean cultivar Xana (Campa et al., 2014) but based on a physical map position of 40.3666-42.5225 Mb, the two loci appear to be separate regions on Pv02.

#### Linkage Group Pv04

The data from GWAS indicate that resistance to anthracnose races 7 and 109 in the ADP resides within 85 Kb region on Pv04. The most significant SNP for race 7 was ss715642306 at 0.447165 Mb on the physical map, while the most significant SNP associated with race 109 was ss715649432 located at 0.532194 Mb on the physical map. Further, there was no correlation between resistance to anthracnose races 7 and 109 (r = -0.04), indicating that the two sources of
resistance could be separate resistance genes. Another factor that likely preventing correlation between the resistance of race 7 and 109 is that race 7 also showed moderate levels of resistance on Pv10 and Pv11.

One likely scenario is that one, or both, sources of resistance could be associated with the major Co-3 locus (Figure 1.3). The role of Co-3 locus in anthracnose resistance is well documented in the literature (Ferreira et al., 2013), and alleles have been identified in members of the differential series, Mexico 222 (Co-3) and PI 207262 (Co-3<sup>3</sup>) in addition to BAT93 (Co-9 renamed  $Co-3^3$ ), Ouro Negro (Co-10 renamed  $Co-3^4$ ) and Co-7 (renamed  $Co-3^5$ ; Sousa et al., 2014). Goncalves-Vidigal et al. (2012), have previously mapped the  $Co-3^4$  allele near the genomic marker g2303 located at 3.356300 Mb on the physical map, which is distant to, but within the same region, identified in this study. Further,  $Co-3^4$  was identified as conferring resistance to C. lindemuthianum race 7. Additionally, the Co-z resistance gene has been identified distally to the  $Co-3^4$ , and has been identified as an Andean source of resistance (Geffroy et al., 2000), potentially playing a role in resistance within the ADP. The Andean Co-15 gene in the Brazilian landrace Corinithiano has also been mapped in the region, as it has been found to be tightly linked to the genomic marker g2685 located at 9.078200 Mb on the physical map (Sousa et al., 2015). However, the resistance identified in the ADP is not likely Co-15, as Co-15 has been shown to confer resistance to race 2047, which was highly virulent on the ADP, with only 10 resistant lines in the entirety of the panel of 226 entries included in the study (Table 1.1). The results of this study are important for developing future breeding strategies as previous work on *Co-3* and its alleles has been largely reported in Mesoamerican germplasm.

Unlike resistance loci previously mapped, the significant SNPs associated with resistance to races 7 and 109 were not the same SNPs. In the case of race 7, SNPs ss715642306 and

ss715649436 were identified as significant. The first SNP falls within the gene Phvul.004G005800 while the later SNP is 2 Kb from the gene Phvul.004G006300. Both genes encode for Cytochrome P450. Cytochromes are known for playing a role in enzymatic complexes that catalyze redox reactions (Grant and Loake, 2000), which can trigger the plant hypersensitive disease resistance response (Delledonne et al., 2001). Hypersensitivity of common bean to anthracnose is commonly accepted as most R-genes trigger a hypersensitive response, making these genes likely candidates for playing a role in anthracnose resistance. Alternatively, SNP ss715649432 was identified as most strongly associated with resistance to race 109, which was also identified as a significant SNP for resistance to race 73. The SNP fell within the gene Phvul.004G006800, which encodes for the glycoprotein gp210 component of the nuclear pore complex. Although not as well recognized as other components of disease response, the nuclear pore complex has been found to condition disease resistance in *Arabidopsis* (Cheng et al., 2009).

## Linkage Group Pv10

The data from GWAS indicates moderate levels of resistance to anthracnose race 7 on Pv10, although not substantial enough to be considered significant with the conservative Bonferonni corrected p-value (Figure 1.3). No major Co genes have been identified on Pv10, other than a QTL for resistance to anthracnose race 7 but the location was not fine mapped (López et al., 2003). The region near the most strongly linked SNP ss715648754 ( $p = 1.77 \times 10^{-4}$ ), located at 3.784843 Mb, was investigated for potential positional gene candidates. The SNP fell 1.2 Kb from the gene Phvul.010G025500, which encodes for an N-terminal Toll/interleukin-1 receptor (TIR)-like domain (TNLs). This gene is one of many genes identified near the end of

Pv10 in a dense cluster of resistance-associated genes in the Andean genotype G19833 (Schmutz et al., 2014).

# Linkage Group Pv11

Results indicate that resistance to race 7 in the ADP is also present on Pv11, although not significant enough to be recognized with the conservative Bonferonni corrected p-value (Figure 1.3). Prior support exists for a QTL on Pv11 for resistance to race 385 (Cl43) in the Andean accession G19833 (López et al., 2003). This region appears to be distinct from the Co-2 gene cluster that is a common source of anthracnose race 7 resistance in Mesoamerican beans (Ferreira et al. 2013). Using BLASTN on Phytozome v10.2, the genetic marker Areoli9 utilized by Creusot et al. (1999) to characterize NBS-LRR resistant genes near the Co-2 gene, was located around 39.725 Mb on the physical map. The SNP ss715645476 identified in the ADP on Pv11 was located at 1.686 Mb on the physical map. The most strongly linked SNP ss715645476  $(p = 2.14 \times 10^{-5})$ , was located within the gene Phvul.011G021500, which encodes for a Phospholipid scramblase. The activity of this enzyme has been suggested to result in the outward translocation of phosphatidylserine from the cell – a major signal for macrophages to eliminate apoptotic cells, and is known to interact with cytochrome c (Collazo et al., 2006). It seems probable that this gene could play a complementary role in resistance to race 7 as the gene candidate on Pv04 encodes for cytochrome P450 suggesting that both genes are involved in programmed cell death to prevent further spread of disease. Support comes from prior genetic studies which show complementary gene action for anthracnose race 7 resistance in the common bean cultivars 'Xana' and 'Cornell 49242' on Pv04 and Pv11 (Campa et al., 2014).

#### CONCLUSION

A lack of information on specific SNP markers linked to the major resistance genes in the published literature prevents a final determination of co-localization between results from the GWAS and the presumed location of major anthracnose resistance gene. In this study, new sources of resistance were discovered on Pv02, Pv10, and Pv11, as well as what appears to be a unique location on Pv04. The physical position and the candidate genes identified in the current study will serve as a basis for developing functional markers. Identifying specific regions on the genome that condition resistance to this economically important disease will be useful in the continued effort of resistance breeding and the development of linked markers for gene pyramiding, ultimately resulting in more durable anthracnose resistance.

Information provided on resistance in this diverse group of Andean bean lines will be useful in future breeding efforts to develop anthracnose resistant depending on the prevailing races in a region. Finding resistance in adapted Andean lines with favorable agronomic and seed traits could have important implications and applications for breeders within target countries. Not only does it help maintain bean diversity through additional resistance options, but it also allows for a more rapid introgression of resistance into future Andean bean cultivars.

Identifying lines with anthracnose resistance that are already known to be accepted by farmers and consumers in different regions will result in not only suitable cultivars for production, but cultivars that farmers want to grow. By recognizing potential socio-economic implications of common bean improvement, this work could greatly increase the impact of new cultivars as consumer acceptance is a critical hurdle for any new variety. Breeding resistant cultivars for small scale farmers in target countries that depend on dry beans as a major source of both income and protein, should ultimately improve both their financial and food security.

APPENDIX

Race	Resistant Lines	Susceptible Lines	Heterogeneous	% resistant
			Lines	
7	85	134	7	37.6
39	94	130	2	41.6
55	93	132	1	41.2
65	143	68	15	63.3
73	148	66	12	65.5
109	78	142	6	34.5
2047	10	215	1	4.4
3481	161	62	3	71.2

Table 1.1. The prevalence of anthracnose resistance to eight races of *Colletotrichum lindemuthianum* within the 226 bean lines from the Andean Diversity Panel.

Variety		Anthracnose Race							
ID	Genotype	7	39	55	65	73	109	2047	3481
ADP0007	Bukoba	0.2	0.2	1.0	1.0	2.0	1.0	5.0	2.4
ADP0030	RHNo.6	0.3	1.0	2.0	0.2	1.0	5.0	5.0	0.3
ADP0111	Uyole98	2.2	0.0	1.6	1.2	1.2	1.0	2.0	0.0
ADP0112	Uyole96	1.0	2.0	1.2	0.2	0.3	3.0	3.0	0.0
ADP0113	OPS-RS4	5.0	1.5	1.4	0.4	0.5	1.0	3.6	1.0
ADP0116	A-800	0.5	0.7	1.8	1.5	0.6	0.0	5.0	4.2
ADP0121	Kranskop HR-1	5.0	1.0	1.0	0.2	1.0	0.0	4.8	0.3
ADP0211	G 4780	2.8	1.0	4.7	0.0	0.0	0.0	1.2	0.4
ADP0432	PR0637-134	3.6	1.0	1.0	0.3	0.5	1.0	1.3	1.0
ADP0463	PI353534-A	2.0	0.7	1.4	0.0	0.4	2.6	4.7	0.3
ADP0599	Isles	0.0	0.0	1.4	0.4	0.0	2.0	5.0	1.8
ADP0609	K-407	5.0	0.7	1.5	0.5	1.0	0.0	5.0	0.3
ADP0613	02-385-14	1.0	1.6	1.5	1.0	0.5	4.5	4.6	0.3
ADP0623	Drake	1.2	1.3	2.0	0.2	0.2	5.0	5.0	0.7
ADP0628	H9659-27-7	1.5	0.3	1.3	0.0	0.3	3.0	5.0	0.2
ADP0631	OAC Inferno	1.5	1.5	1.0	0.0	0.3	4.7	5.0	0.0
ADP0638	Red Hawk	1.3	0.5	1.5	0.0	1.3	3.3	5.0	0.7
ADP0639	Chinook 2000	0.3	0.3	1.0	0.0	1.2	5.0	5.0	0.2
ADP0650	K-42	5.0	2.0	1.8	0.0	1.0	0.0	4.5	0.0
ADP0653	USDK-CBB-15	0.0	0.2	0.5	0.0	0.0	3.3	5.0	0.0
ADP0656	Royal Red	5.0	1.7	1.0	0.2	0.2	0.0	5.0	0.8
ADP0657	Kardinal	4.3	2.0	1.5	0.2	0.2	0.0	4.8	0.5
ADP0658	Blush	4.5	0.0	0.8	0.0	0.0	0.0	4.7	0.2
ADP0660	Krimson	1.0	0.3	1.0	1.3	0.8	3.7	4.8	0.0
ADP0663	USCR-CBB-20	1.2	1.7	1.0	0.0	0.0	3.3	5.0	0.5
ADP0664	Silver Cloud	5.0	2.0	1.0	0.5	0.5	1.0	4.7	2.0
ADP0674	UCD0704	2.0	1.0	1.0	0.4	1.0	1.0	3.7	0.5
ADP0678	Hooter	4.2	2.0	1.5	0.0	0.0	1.0	3.3	0.2

Table 1.2. Twenty-eight lines within the 226 accessions from the Andean Diversity Panel with resistance to six or more of the eight races of anthracnose included in the study.

Values = 0 are most resistant, while values = 5 are most susceptible.

Anthracnose	SNP*	Chromosome	<b>SNP</b> Position	P-value**	$R^{2}*$	Minor Allele
Race			(Mb)		**	Frequency
7	ss715642306	Pv04	0.447165	6.37E-06	0.13	0.31
7	ss715649436	Pv04	0.487599	6.83E-06	0.12	0.31
39	ss715648452	Pv02	48.617342	4.60E-09	0.19	0.36
39	ss715648451	Pv02	48.606517	4.60E-09	0.19	0.36
55	ss715648451	Pv02	48.606517	3.26E-11	0.24	0.36
55	ss715648452	Pv02	48.617342	3.27E-11	0.24	0.36
65	ss715645251	Pv01	50.301532	4.04E-07	0.39	0.18
65	ss715646578	Pv01	48.340819	1.63E-06	0.14	0.37
73	ss715645251	Pv01	50.301532	3.44E-08	0.40	0.17
73	ss715645258	Pv01	50.155927	6.63E-07	0.16	0.28
109	ss715649432	Pv04	0.532194	3.88E-09	0.20	0.33
109	ss715640025	Pv04	0.212804	2.54E-05	0.12	0.33
3481	ss715645251	Pv01	50.301532	4.87E-10	0.47	0.19
3481	ss715646578	Pv01	48.340819	2.52E-05	0.10	0.36

Table 1.3. Chromosome, position, p-value, proportion of phenotypic variation explained ( $R^2$ ) and minor allele frequency of the two most significant SNPs for seven races of anthracnose resistance measured on 226 Andean bean genotypes.

\*SNP = Single Nucleotide Polymorphic code; \*\*P = significance level and E = exponential; \*\*\* $R^2$  = phenotypic variation explained by the SNP



Figure 1.1. QQ Plots and Manhattan Plots showing candidate SNPs and their P-values from GWAS using MLM for anthracnose resistance. From top to bottom include results for anthracnose resistance to A) race 65, B) race 73, and C) race 3481 on Pv01. Red line on Manhattan Plots is the significance threshold of P=1.03 x  $10^{-5}$  after Bonferonni correction of  $\alpha = 0.05$ .



Figure 1.2. QQ Plots and Manhattan Plots showing candidate SNPs and their P-values from GWAS using MLM for anthracnose resistance. From top to bottom include results for anthracnose resistance to A) race 39 and B) race 55 on Pv02. Red line on Manhattan Plots is the significance threshold of P=1.03 x  $10^{-5}$  after Bonferonni correction of  $\alpha = 0.05$ .



Figure 1.3. QQ Plots and Manhattan Plots showing candidate SNPs and their P-values from GWAS using MLM for anthracnose resistance. From top to bottom include results for anthracnose resistance to A) race 7 and B) race 109 on Pv04, with minor resistance loci on Pv10 and Pv11 for race 7. Red line on Manhattan Plots is the significance threshold of P=1.03 x  $10^{-5}$  after Bonferonni correction of  $\alpha = 0.05$ .

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# CHAPTER TWO: IDENTIFICATION OF *Co-1* ANTHRACNOSE RESISTANCE GENE IN MESOAMERICAN BEAN CULTIVAR JAGUAR

# ABSTRACT

Anthracnose is a seed-borne disease of common bean (*Phaseolus vulgaris* L.) caused by the fungus *Colletotrichum lindemuthianum*, and is a serious disease problem in Michigan, at times resulting in complete yield loss in susceptible cultivars. The black bean cultivar 'Jaguar' possesses a source of resistance to anthracnose race 73, which is widely deployed by the Michigan State University (MSU) bean breeding program. The objective of this study was to fine map the resistance to anthracnose race 73 in Jaguar using SNP markers, and to identify molecular markers for use in resistance breeding. Resistance to anthracnose was investigated in an F<sub>4:6</sub> recombinant inbred line (RIL) population developed from a cross between Jaguar and 'Puebla 152' (landrace cultivar known to be susceptible to race 73). A QTL study was conducted to determine the resistance in Jaguar, and a major QTL for the dominant resistant trait was identified at the *Co-1* locus. Using the Illumina BARCBean6K\_3 BeadChip, the physical location of the *Co-1* locus was mapped between 50.10 and 50.22Mb on chromosome Pv01, which should prove useful for breeding programs interested in using marker assisted selection for the *Co-1* gene.

#### INTRODUCTION

Traditionally, the United States has had a large role in global dry bean production, planting over 0.7 million hectares in 2015 (USDA-NASS, 2015). The U.S. is responsible for 6% of the world output (USDA-ERS, 2012) and is the 3<sup>rd</sup> largest exporter (Akibode and Maredia,

2011), with approximately 20% of U.S. bean production making its way to export markets. The states with the greatest production include North Dakota (38%), Michigan (14%), Nebraska (11%), Minnesota (10%), and Idaho (7%) (USDA-ERS, 2012). Michigan, in particular, has had a long history of producing dry beans, and has been a major producer since the late 19<sup>th</sup> century. The state illustrated its commitment to beans early in the 20<sup>th</sup> century by establishing the first dry bean breeding program in the US at MSU (Kelly and Cichy, 2013). To date, Michigan is still the leading producer of black beans and the second leading producer of navy beans in the country, with dry beans being produced on over 1,000 Michigan farms (USDA-ERS, 2012).

Michigan is known for having a humid growing season, and most black and navy beans are grown under rain fed conditions. The high humidity presents ideal growing conditions for many fungal pathogens. One pathogen that is especially prevalent in common bean production areas in Michigan is anthracnose. The seed-borne, fungal pathogen is caused by *Colletotrichum lindemuthianum* (Sacc. & Magnus) Lams.-Scrib, and can cause devastation to commercial fields planted to susceptible cultivars. Anthracnose is one of the most economically important diseases of common bean (Melotto et al., 2000), and failure to manage the disease can result in financial hardship for farmers. Clean seed programs have proven effective for reducing anthracnose prevalence in temperate regions (Pastor-Corrales and Tu, 1989); however, access to certified clean seed is not always easily available, and farmers may decide to replant seed from previous years. In such cases, anthracnose can become a serious issue as transmission through the seed is very effective (Kelly and Vallejo, 2004).

Three races (7, 65, 73) of anthracnose have been previously characterized in Michigan (Kelly et al., 1994; Balardin and Kelly, 1996) The more persistent race 73, which has been found to be the most prevalent race across the Americas (Balardin et al., 1997), and race 65, which has

been previously identified in Ontario, Canada (Tu, 1984) are both largely associated with the Mesoamerican genepool. Race 73, in particular, has been a problem for Michigan bean growers over the past 20 years as many of the new upright cultivars lack resistance (Kelly et al., 2014). The race was first discovered in Michigan in 1993, and differed from previous anthracnose races in the state as race 73 was able to overcome the *Are* (*Co-2*) gene that was widely deployed in the Mesoamerican navy bean cultivars grown in the state at the time (Kelly et al., 1994; Young and Kelly, 1996).

Today, the bean breeding program at MSU utilizes the same resistance source for anthracnose races 73 and 65 in many black bean cultivars released in Michigan, including the cultivar Jaguar. Jaguar is known to have resistance to anthracnose races 73 and 65 (Kelly et al., 2001), but the underlying genetic location of the actual resistance has never been fully ascertained. In general, resistance deployed in the MSU breeding program to prevent race 73 has largely been assumed to be controlled by the *Co-1* locus (Kelly et al., 2001; 2014), and with the recent release of the bean genome and the BARCBean6K\_3 BeadChip, more concrete information on the underlying genetic location is obtainable. Identifying the specific source of resistance and markers tightly linked to the gene(s) would greatly facilitate introgression of the trait into resistance gene pyramids. The goal of this study was to determine the basis of the resistance to anthracnose race 73 in the cultivar Jaguar using SNP markers, and to identify molecular markers more tightly linked to the resistance that could be used in breeding programs for more effective marker assisted selection.

## MATERIALS AND METHODS

## Plant material

The mapping population consisting of 95 F<sub>4:6</sub> recombinant inbred lines (RILs) was derived from the cross Jaguar x Puebla 152. The population was developed by artificial hybridization between the two parents to create an initial F<sub>1</sub> hybrid followed by single seed descent (SSD) from the F<sub>2</sub> until the F<sub>4</sub> generation. Seed was advanced as mass selected rows for two generations until the F<sub>4:6</sub> generation at the Saginaw Valley Research and Extension Center in Frankenmuth, MI. Jaguar is a black bean cultivar derived from a cross between MSU breeding lines B90211 and N90616, known to possess resistance to anthracnose race 73 (Kelly et al., 2001). The same anthracnose resistance source was deployed in the recently released cultivar 'Zenith' (Kelly et al., 2014). The resistance can be traced back to the navy bean cultivar 'Sanilac' (Andersen et al., 1960; Young and Kelly, 1996), which inherited its resistance from the navy bean 'Emerson 847' (Cardenas et al., 1964). Puebla 152 is a black bean landrace from Mexico that is known to be susceptible to anthracnose race 73.

#### Greenhouse design and DNA isolation

Six seedlings of each RIL were grown in trays containing standard potting soil in the MSU greenhouses, East Lansing, MI. Inoculation of anthracnose race 73 was performed by spraying a suspension of  $1.2 \times 10^6$  *C. lindemuthianum* conidia ml<sup>-1</sup> onto the leaves and stems of the seedling plants. Plants were then maintained under high humidity (>80%) in a mist chamber for three days. Symptoms of anthracnose were observed on each plant 8 days after initial inoculation using a categorical scale of disease absent or present as anticipated of a qualitative trait. Plants without any symptoms as well as very minor necrotic lesions were scored as resistant, while plants with large, sunken lesions and dead plants were scored as susceptible.

DNA was collected from young leaf tissues of RIL genotypes grown in the greenhouse at MSU using a modified CTAB (Hexadecyltrimethyl ammonium bromide) extraction protocol (Doyle, 1987). The DNA concentrations were measured using a Nanodrop spectrophotometer, and its quality was checked on an agarose gel.

#### SNP analysis

The RIL population, along with the parents, was genotyped using an Illumina BARCBean6K\_3 BeadChip with 5398 SNPs (Hyten et al., 2010). The SNP-based genetic map was developed using JoinMap 4 (Van Ooijen, 2006). The SNP genotyping data for the population were manually inspected in Excel, and SNPs with no calls, those that were monomorphic between parents, and any in which the parents were heterozygous were eliminated. The markers were ordered on the map and the genetic distances between the markers were determined using the regression mapping algorithm and Kosambi's mapping function. The LOD range was between a minimum of 2 and a maximum of 10, with the remaining parameters left at JoinMap defaults for linkage analysis. Win QTL Cartographer V2.5\_011 (Wang et al., 2012) was utilized to conduct the quantitative trait loci (QTL) analysis. The Linkage map was drawn using Mapchart 2.3 for Windows (Voorrips, 2002).

#### Molecular marker analysis

Gene-based markers developed by McConnell et al. (2010) were investigated for polymorphism associated with susceptibility and resistance to anthracnose in the parents. Markers included in the study were based on proximity to the region identified as significant in the SNP analysis. In an effort to identify any markers that were co-segregating with the anthracnose resistance gene, bulked segregant analysis was utilized (Michelmore et al., 1991). Both parents, along with contrasting resistant and susceptible bulks, were amplified using the

thermocycler PTC 100. PCR reactions were performed as described by McConnell et al. (2010) with annealing temperatures determined for each marker according to primer nucleotide composition. Restriction enzymes were used as indicated in supplemental table 1 (McConnell et al., 2010). PCR products were resolved on agarose gel containing ethidium bromide, run in 1x TAE buffer, and visualized under UV light. Markers analyzed included g2504, g1404, g1224, g499, g1367, g2562, and g683, which spanned the region of 47.640 Mb to 51.619 Mb on Pv01.

## **RESULTS AND DISCUSSION**

## Phenotypic Distribution

The observed segregation of resistance within the RIL population did not conform to the anticipated 1:1 ratio for a major qualitative trait. Within the population, there were significantly more lines displaying anthracnose resistance than susceptibility ( $\chi^2 = 12.9$ ; p = 0.0003). This could be the result of segregation distortion due to small population size used, as this has been reported in other common bean RIL populations (Ochoa et al., 2006). Despite the distortion from the expected ratio, resistance appears to be controlled by a single locus.

# QTL Identification

The resulting map included 1131 SNP markers across all 11 linkage groups, with limited coverage on chromosomes Pv05 and Pv08. The total map size was 747.4 cM with an average distance between markers of 1.1 cM. Thirteen SNP markers that exhibited significant association  $(Pr(F) \le 0.01\%)$  with resistance to *C. lindemuthianum* race 73 were identified on Pv01 (Table 2.1). The phenotypic variation ( $R^2$ ) explained by the most significant SNP ss715645258 was 69.4%. The significant QTL conferring anthracnose resistance within the RIL population was identified on the long arm of Pv01. The largest LOD score of 36.1 was identified on the genetic

map between SNPs ss715645262 and ss715645252 (Figure 2.1). These flanking SNPs are located on Pv01 at 50.099818 and 50.222584 Mb respectively (Figure 2.2)

# Significant SNPs across populations

An unexpected advantage of evaluating the Jaguar/ Puebla 152 RIL population and the Andean Diversity Panel (ADP) for resistance to race 73, was the possibility of detecting resistant loci at the same genomic location in these contrasting genetic populations. Comparing results from different genetic populations provides a useful verification of map locations (Arahana et al., 2001). The probability that both studies would identify the same significant SNPs by chance is equal to the product of the significance criterion for each population, and as such, the identification of the same SNPs are highly significant. The two most significant SNPs identified in the ADP conferring resistance to anthracnose race 73 were ss715645258 and ss715645251 at the physical locations of 50.155927 and 50.301532 Mb respectively. These two SNPs were also identified as highly significant within the RIL population, with the highest R<sup>2</sup> values of all the significant SNPs ( $R^2 = 69.4$  and 67.8; Table 2.1). Further, the region of 50.155927 and 50.301532 Mb overlaps significantly with the region of 50.099818 and 50.222584 Mb identified as the region with the greatest LOD score detected in the RIL population. Since the Co-1 locus (original A gene) is widely reported in Andean bean germplasm (Young and Kelly, 1996) and has now been mapped in the ADP to the same region as the QTL for resistance in Jaguar, we conclude that the gene conditioning resistance to race 73 in the Mesoamerican black bean cultivar Jaguar is the same *Co-1* gene.

# Comparison with previous studies

The physical location of 50.099818 and 50.222548 Mb on Pv01 defined by the range with the greatest LOD score in the RIL population is very close to the physical location of the

*Co-1* locus described in the literature. Five alleles, *Co-1* to *Co-1*<sup>5</sup> have been reported at the *Co-1* locus (Melotto and Kelly, 2000; Goncalves-Vidigal and Kelly, 2006; Goncalves-Vidigal et al., 2011) in addition to *Co-w* and *Co-x* resistance genes identified in the same region of Pv01 (Geffroy et al., 2008). Richard et al. (2014) identified the region spanning 50.264307 to 50.322583 Mb as harboring the *Co-x* gene, previously proposed by Geffroy et al. (2008) as the resistant source to anthracnose strain 100 in the Andean genotype Jalo EEP558. This region was reported as being at least 190 kb from the previously reported *Co-1*<sup>4</sup> allele based on the position of the linked marker CV542014 (Goncalves-Vidigal et al., 2011). Similarly, the findings of this study identified the resistance source for anthracnose race 73 in Jaguar to be at least 41.7 kb on the proximal side of *Co-x*, which happens to be the opposite side of *Co-x* gene from where the *Co-1*<sup>4</sup> allele was located (50.63-50.65 Mb) based on synteny mapping with the *Rhg 1* gene in soybean (Richard et al., 2014).

Race-specific anthracnose resistance has been reported in the same region of Pv01 in numerous Andean genotypes including MDRK, JaloEEP558, Kaboon, Andecha, Xana, and AND277 (Melotto and Kelly, 2000; Rodríguez-Suárez et al., 2007; Geffroy et al., 2008; Campa et al., 2009; Goncalves-Vidigal et al., 2011; Campa et al., 2014). No segregation was detected in an allelism test of 200 progeny from the F<sub>2</sub> population developed from MDRK (*Co-1*) x JaloEEP558 (*Co-x*) inoculated with race 73 (Vallejo et al., 2003). Thus, the possibility that *Co-x* is the same *Co-1* allele should be considered. Similar to the findings of the current study, Young and Kelly (1997) identified anthracnose race 73 as avirulent to the *Co-1* locus and independent from the *Co-3*, *Co-4* and *Co-5* loci, but they were unable to determine the actual relationship of the different resistance genes or alleles. The specific physical locations of numerous resistant alleles have not been determined in prior studies due to limited genomic coverage available with

marker technologies used in the past. The *Co-x* gene proposed by Geffroy et al. (2008) was the first locus on Pv01 to be fine mapped in the cultivar JaloEEP558 (Richard et al., 2014). In the RIL study we used race 73 to detect the QTL on Pv01. In the ADP study race 73 identified the same resistance SNPs and region as did races 65 and 3481on Pv01. Richard et al. (2014) used strain 100 which they characterized on the differentials as race 3993 (Table S1 in Richard et al., 2014) to detect the *Co-x* in JaloEEP558. Since race 3993 differs from race 3481 at one avirulence gene (Avr Co-5) that recognizes the *Co-5* gene on Pv07, we assume that races 3481 and 3993 should detect the same resistance region of Pv01 as do races 65 and 73 which differ for another avirulence gene (Avr Co-2) that recognizes the *Co-2* gene on Pv11. In addition the region identified as the location of the *Co-1*<sup>2</sup> allele in the navy bean cultivar 'Bolt' (Vazin et al., 2014). This region should be further investigated to help determine which genes are alleles, and which ones may be separate sources of resistance.

It is possible that the resistance that has been identified as race-specific and falls within a 410 kb region between the  $Co-1^4$  marker and the region identified in the current study on the long arm of Pv01 is a resistance cluster. Across many different plant species, clusters of resistance genes have been identified and described (Michelmore and Meyers, 1998) and clustered resistance regions are also common in *P. vulgaris* genome (Kelly et al., 2003). Some previously identified anthracnose resistance loci have been identified as being comprised of numerous resistance genes. The *Co-4* locus on Pv08 of common bean for example, has been shown to contain a cluster of 18 *COK-4* anthracnose resistance genes spanning a 325 kb region (Oblessuc et al., 2015). In addition, resistance to multiple different diseases have been mapped in the vicinity of the *Co-1* locus, including resistance genes for rust (*Ur-9*) and angular leaf spot

(*Phg-1*) resistance (Jung et al., 1998; Goncalves-Vidigal et al., 2011). It is probable then, that the *Co-1* resistance locus is another anthracnose resistance cluster, similar to that of the *Co-4* locus on Pv08. The *Co-1* locus could be comprised of a cluster of linked resistance genes conditioning resistance to specific races of anthracnose, which has been proposed in the published literature (Ferreira et al., 2013; Campa et al., 2014).

Previous workers have identified molecular markers linked to Co-1 (Young and Kelly, 1997; Mendoza et al., 2001; Goncalves-Vidigal et al., 2011; Vallejo and Kelly, 2008). However, many of these markers have limited overall usefulness for marker assisted breeding as they have not been able to be reproduced in different laboratories and many markers are linked in repulsion phase. The marker  $SE_{ACT}/M_{CCA}$  is a co-dominant sequence tagged site (STS) that is not allele specific. However, the marker is still nearly 10 cM from the Co-1 gene, and is only useful for selection within Andean beans (Vallejo and Kelly, 2008). The CV542014 marker used by Goncalves-Vidigal et al. (2011) to tag the  $Co-l^4$  allele was monomorphic between Jaguar and Puebla 152 parents. In an attempt to develop more useful marker information from the region, the area directly surrounding the flanking SNPs was investigated using gene-based markers developed by McConnell et al. (2010). Many of the selected markers (g2504, g1404, g1224, g499, g1367, g2562, g683 and CV542014) had been shown to be linked to the  $Co-1^4$  allele (Goncalves-Vidigal et al., 2011). A lack of polymorphism between parents and bulks for these gene-based markers was observed and prevented their further development as linked markers (Figure 2.3).

## CONCLUSIONS

The resistance deployed in the MSU breeding program has largely been assumed to be controlled by the *Co-1* locus (Kelly et al., 2001; 2014) and that assumption was confirmed in this study. A major putative QTL for resistance to anthracnose in common bean was identified on Pv01 between SNPs ss715645252 and ss715645262 at the physical distances of 50.222584 and 50.099818 Mb respectively. It is likely that this region corresponds to the major *Co-1* resistance cluster, as well as the *Co-x* resistance gene. The total combined distance of the resistance region identified in this study and the *Co-x* region identified by Richard et al. (2015) is 223 kb, which is less than the 325 kb range described by Oblessuc et al. (2015) for the 18 copies of the COK-4 gene located at the *Co-4* locus on Pv08.

The improved genetic coverage provided by SNP markers in this study has great potential for the development of useful molecular markers. The g-markers utilized in the region were not close enough to the region of interest for fine mapping and would not be useful for marker assisted selection. However, an additional study is being conducted using InDel markers with physical positions near the significant SNPs in an effort to identify markers closely associated with the *Co-1* gene that would be useful for marker assisted breeding. Identification of PCR-based markers are especially useful for the continued effort of breeders in third world countries as they can be utilized in labs with limited resources.

APPENDIX

SNP marker	SNP position (Mb)	Pr (F)*	$\mathbb{R}^2$
ss715649883	37.652001	0.000000002	0.3202
ss715645856	49.617274	0.000000055	0.2726
ss715645287	49.793139	0.000000000	0.5828
ss715645285	49.853879	0.000000000	0.6447
ss715645284	49.862290	0.000000000	0.5986
ss715645280	49.895862	0.000000000	0.6709
ss715645275	49.953420	0.000000000	0.6333
ss715645270	50.014600	0.000000000	0.6397
ss715645262	50.099818	0.000000000	0.6112
ss715645259	50.130201	0.000000000	0.6714
ss715645258	50.155987	0.000000000	0.6935
ss715645252	50.222584	0.000000000	0.6531
ss715645251	50.301592	0.000000000	0.6779
55/1JU <del>4</del> JZJ1	50.501572	0.00000000	0.0772

Table 2.1. Thirteen SNP markers, location and physical position on chromosome Pv01 used to identify major QTL for resistance to anthracnose race 73 in Jaguar x Puebla 152 black bean RIL population

\*significant at Pr(F) = 0.01%

**PV01** 



Figure 2.1. Bean chromosome Pv01 in which anthracnose race 73 resistance was directly located with graphical representation of LOD values. AR = anthracnose resistance.



Figure 2.2. Mapping of *Co-1* gene on chromosome Pv01. a) Genetic map location of *Co-1* on Pv01 based on the 95 RILs in Jaguar x Puebla 152 RIL population expressed in centiMorgans. b) Physical location of SNP markers flanking *Co-1*.



Figure 2.3. Electrophoretic analysis of amplification products of gene-based markers g499 and g1367. J = Jaguar; P = Puebla 152; R = resistant bulk; S = susceptible bulk; L = 100bp ladder

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