ANGULAR LEAF SPOT AND ASCOCHYTA DISEASE RESISTANCE IN COMMON BEAN

Characterization and Application for Breeding

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Summary

Common bean (*Phaseolus vulgaris* L.) is the most important grain legume for human consumption and an important food security crop. Its affordable and nutrient-dense grains provide protein and micronutrients such as iron and zinc for millions of people in Latin America and Eastern Africa, where consumption can reach up to 66 kg annually [1]. In these regions, common beans are often cultivated by smallholder farmers as a subsistence crop under sub-optimal conditions. Consequently, on farm yields throughout the tropics are only a fraction (around 17 to 23%) of the yield that has been achieved under experimental conditions [2]. Globally, one of the most limiting factors to crop productivity are plant diseases, which account for the loss of 20 to 30% of global crop production, with highest losses occurring in already food insecure regions in the tropics [3] where common beans often are staple foods.

The common bean crop is heavily affected by plant diseases, with angular leaf spot (ALS), caused by *Pseudocercospora griseola* being one of the most devastating fungal diseases, responsible for yield losses of up to 80% [4]. Another fungal disease, Ascochyta caused by *Boeremia diversispora*, has been locally important in the tropical highlands but is emerging in many new locations throughout the tropics and temperate zones. Currently available common bean cultivars are often ineffective in resisting these two diseases and the only possibility for farmers to combat them is the application of pesticides. However, pesticides are expensive and rarely available for smallholder farmers. A more sustainable and feasible solution for disease control is offered by resistance breeding, which is facilitated by a detailed knowledge of the genetic control of inherent disease resistance.

This thesis presents a meticulous investigation into the genetic control of disease resistance to the fungal common bean diseases ALS and Ascochyta. Breeding for the two diseases has been ongoing for many years [2, 5], but has been challenged by the high diversity of the causal agent in the case of ALS, and the lack of resistance in the common bean gene pool in the case of Ascochyta. The goal of this research is to identify the genetic determinants of resistance against different pathotypes of *P. griseola* and to verify Ascochyta resistance transferred from the sister species *Phaseolus dumosos* to common bean. In addition, we aimed to establish tools for the implementation of these findings within molecular plant breeding programs.

In **chapter 1**, the importance of common bean as a food crop, the effect of plant pathogens on food security and the detailed mechanisms of how plant disease resistance is functioning are described. **Chapter 2** reviews previous investigations of the ALS disease in common bean. Since the first ALS studies were conducted, genomic tools have evolved substantially allowing new insights. Through the availability of a reference genome sequence in common bean, genetic marker positions can be compared and candidate genes within marker intervals can be identified. This chapter updates previous studies and provides the base for future ALS studies.

Chapter 3 describes the pathotype-specific ALS response of common bean. A diversity panel, containing the best available resistant common bean varieties, was assembled and tested against a diverse collection of ALS pathotypes from two continents. In Colombia, the panel was tested at two field locations and in the greenhouse with four pathogen isolates. To compare the resistance effective against pathogen populations between Africa and South America, the same panel was tested in Uganda in a field and in a greenhouse trial. Genome-wide association studies (GWAS) allowed the identification of two resistance loci effective against specific pathotypes: The resistance locus on chromosome 8 was effective in all trials in Uganda and Colombia, while the resistance locus on chromosome 4 was effective against one particular pathogen strain in Colombia. The locus on chromosome 8 was further dissected by clustering haplotypes according to their genotypic information and analyzing the haplotype-specific effect on ALS resistance. Of the eleven haplotypes at the locus, one haplotype conferred broad-spectrum resistance and six further haplotypes conferred strain-specific resistance. Molecular markers co-segregating with resistance loci or haplotypes will facilitate breeding for pathotype-specific ALS resistance in Colombia and Uganda.

Chapter 4 describes an applied breeding project, where we aimed at pyramiding five ALS resistance loci into a common bean line exhibiting good agronomic characteristics. Four resistant common bean lines and two elite bean lines were crossed in a sophisticated scheme and subjected to frequent genotypic and phenotypic selections. In an advanced generation, we have found seven genotypes that were homozygous for all five resistance loci and 84 genotypes that contained all five resistance loci, but at least one of them in a heterozygous state. In future experiments, their progenies will be tested for ALS resistance against a broad variety of pathotypes and selected for good grain- and agronomic characteristics. Common bean lines combining multiple resistance loci are expected to show superior resistance and may offer durable disease resistance.

Chapter 5 describes an investigation into the Ascochyta resistance of interspecific lines available at CIAT. These putative Ascochyta-resistant interspecific lines were crossed to elite common bean breeding lines and their progenies tested for Ascochyta resistance in greenhouse and field trials. While the tests in the greenhouse showed good segregation for resistance, the partially inbred lines did not show the expected resistance under field conditions. In spite of this, some lines with repeated low disease scores were found in field evaluations, which will be of use for future breeding activities.

In **chapter 6**, the future of common bean as a food security crop is discussed and an outlook on the importance of common bean and plant diseases is given. Furthermore, the approaches taken in this thesis are critically reviewed, possible future investigations are suggested and the implications of our findings for common bean breeding are discussed.

Diseases will continue to be an important constraint of common bean production in the tropics, given the favorable conditions for tropical pathogens in the future. Global warming will increase their range, globalization will facilitate their spread and the increasing prevalence of monocultures will increase their population sizes. To face the projected increased threat by bean pathogens, it is crucial for common bean breeders to have an overview of the available sources of resistance and their effectiveness in different geographic locations as presented for ALS in this thesis. Additionally, within the frame of this thesis, we worked towards the establishment of ALS and Ascochyta resistant germplasm with acceptable grain- and agronomic characteristics that will be introduced to the breeding pipeline to facilitate the transfer of resistance to elite breeding varieties.

In conclusion, this thesis contributes valuable tools and germplasm to the common bean breeding community to facilitate incorporation of disease resistance in their breeding programs. The resulting disease resistant cultivars will reduce the yield gap on smallholder as well as industrial farmers' fields and therefore contribute to local and global food security.

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2. Beebe, S. E. (2012). Common bean breeding in the tropics. In J. Janick (Ed.), *Plant Breeding Reviews volume 36* (pp. 357–426). Hoboken NJ, USA: Wiley-Blackwell.
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Zusammenfassung

Die Gartenbohne (*Phaseolus vulgaris* L.), nachfolgend Bohne genannt, ist die wichtigste Hülsenfrucht für den menschlichen Verzehr und leistet einen wichtigen Beitrag zur Ernährungssicherheit. Die kostengünstigen und nährstoffreichen Bohnen sind ein wichtiger Lieferant von Protein und Mikronährstoffen wie Eisen und Zink für Millionen von Menschen in Lateinamerika und Ostafrika. In diesen Regionen kann der Pro-Kopf-Verbrauch bis zu 66 kg pro Jahr betragen [1]. Bohnen werden von Kleinbauern oft als Subsistenzkultur unter suboptimalen Bedingungen angebaut. Deshalb wird im Feld nur ein Bruchteil der Erträge erreicht (etwa 17 bis 23%), die unter experimentellen Bedingungen erzielt werden [2]. Einer der grössten ertragslimitierenden Faktoren sind Pflanzenkrankheiten, welche etwa 20 bis 30 % der weltweiten Produktionsverluste ausmachen [3]. Deutlich höher sind die Verluste in Regionen, in welchen die Ernährungssicherheit kritisch ist [3] und in welchen Bohnen oftmals ein Grundnahrungsmittel sind.

Eine der verheerendsten Pilzkrankheiten in Bohnen, welche zu Ertragsausfällen von bis zu 80% führen kann, ist Angular Leaf Spot (ALS), verursacht durch *Pseudocercospora griseola* [4]. Eine weitere Pilzerkrankung, Ascochyta verursacht durch *Boeremia diversispora*, war ursprünglich im tropischen Hochland verbreitet. Neuerdings tritt Ascochyta aber an vielen neuen Orten in den Tropen und gemäßigten Zonen auf. Die derzeit verfügbaren Bohnensorten sind anfällig gegen diese beiden Krankheiten, was zu erheblichen Ernteausfällen führen kann. Die einzige Möglichkeit für Landwirte, die Bohnenpathogene zu bekämpfen, ist die Anwendung von Pestiziden. Pestizide sind jedoch teuer und für Kleinbauern selten verfügbar. Eine nachhaltigere und praktikablere Lösung zur Krankheitsbekämpfung bietet die Resistenzzüchtung, die durch detaillierte Kenntnisse der genetischen Determinanten der inhärenten Krankheitsresistenz ermöglicht wird.

Diese Arbeit stellt eine umfangreiche Untersuchung der genetischen Kontrolle der Krankheitsresistenz gegen die Bohnenpilzkrankheiten ALS und Ascochyta dar. Die Resistenzzüchtung gegen die beiden Krankheiten läuft seit vielen Jahren [2, 5], wurde aber durch die hohe Pathogendiversität bei ALS und dem Mangel an Resistenzen im Bohnengenpool bei Ascochyta erschwert. Das Ziel dieser Arbeit ist es, die genetischen Determinanten der Resistenz gegen verschiedene Pathotypen von *P. griseola* zu identifizieren und die Ascochyta-Resistenz zu überprüfen, die von der Schwesterart *Phaseolus dumosos* auf die Bohne übertragen wurde. Ein weiteres Ziel der Arbeit ist es, Instrumente für die Umsetzung dieser Erkenntnisse in molekularen Pflanzenzüchtungsprogrammen zu etablieren.

In **Kapitel 1** werden die Bedeutung der Bohne als Nahrungspflanze, der Einfluss von Pflanzenpathogenen auf die Ernährungssicherheit und die detaillierten Mechanismen der die Resistenz gegen Pflanzenkrankheiten beschrieben.

Kapitel 2 gibt einen Überblick über frühere Untersuchungen der ALS-Krankheit in Bohnen. Seit den ersten ALS-Studien haben sich die genomischen Technologien erheblich weiterentwickelt und neue Erkenntnisse ermöglicht. Durch die Verfügbarkeit einer Referenzgenomsequenz der Bohne können zum Beispiel Markerpositionen verglichen und Kandidatengene innerhalb von Markerintervallen identifiziert werden. Dieses Kapitel aktualisiert frühere Studien und kann deshalb als Grundlage für zukünftige ALS-Studien dienen.

Kapitel 3 beschreibt die pathotypspezifische ALS-Reaktion der Bohne. Ein Diversitätspanel bestehend aus den besten resistenten Bohnen, die verfügbar waren, wurde zusammengestellt und gegen eine Vielzahl von Pathotypen aus zwei Kontinenten getestet. In Kolumbien wurde das Panel an zwei Feldstandorten und im Gewächshaus mit vier Pathotypen getestet. Um die Resistenz gegen Pathogenpopulationen auf einem weiteren Kontinent zu vergleichen, wurde das gleiche Panel in Uganda in einem Feld- und einem Gewächshausversuch getestet. Genomweite Assoziations-studien (GWAS) ermöglichten die Identifizierung von zwei Resistenzloki, die gegen bestimmte Pathotypen wirksam sind: Der Resistenzlokus auf Chromosom 8 war in allen Experimenten wirksam, während der Resistenzlokus auf Chromosom 4 gegen einen bestimmten Pathotyp wirksam war. Der Lokus auf Chromosom 8 wurde weiter seziert, indem Haplotypen entsprechend ihrer genotypischen Informationen zusammengefasst wurden und der haplotypspezifische Effekt auf die ALS-Resistenz analysiert wurde. Von den elf Haplotypen am Lokus verlieh ein Haplotyp Breitspektrum-Resistenz und sechs weitere Haplotypen eine pathotyp-spezifische Resistenz. Die gefundenen molekularen Marker, die mit Resistenzloki oder Haplotypen ko-segregieren, werden die Züchtung für pathotypspezifische ALS-Resistenzen in Kolumbien und Uganda erleichtern.

Kapitel 4 beschreibt ein angewandtes Züchtungsprojekt, bei dem versucht wurde fünf ALS-Resistenzloki in einer Bohnenlinie zu vereinen, die gute agronomische Eigenschaften und einen gewünschten Samentyp aufweist. Vier resistente Bohnenlinien und zwei Elite-Bohnenlinien wurden in einem ausgeklügelten Schema gekreuzt und häufigen genotypischen und phänotypischen Selektionen unterzogen. In einer fortgeschrittenen Generation haben wir sieben Genotypen gefunden, die für alle fünf Resistenzorte homozygot waren, und 84 Genotypen, die alle fünf Resistenloki enthielten, aber mindestens einer von ihnen in einem heterozygoten Zustand. In zukünftigen Experimenten werden ihre Nachkommen auf ALS-Resistenz gegen eine Vielzahl von Pathotypen getestet und Individuen selektiert die gute agronomische Eigenschaften aufweisen. Es wird erwartet, dass Bohnenlinien, die mehrere Resistenzloki kombinieren, eine überlegene und dauerhafte Resistenz gegen Krankheiten bieten können.

In **Kapitel 5** wurde die Ascochyta-Resistenz von den vermeintlich Ascochyta-resistenten, interspezifischen Linien untersucht, die am CIAT verfügbar waren. Die inter-spezifischen Linien wurden mit Elite-Bohnenzuchtlinien gekreuzt und ihre Nachkommen in Gewächshaus- und Feldversuchen auf Ascochyta-Resistenz getestet. Während die durch Kreuzung und anschliessende Inzucht entstandenen Linien im Gewächshaus eine gute Segregation der Resistenz zeigten, konnten wir die erwartete Resistenz unter Feld-bedingungen nicht nachweisen. Jedoch wurden einige Linien mit wiederholt niedrigen Ascochytabefall gefunden, welche für zukünftige Züchtungsaktivitäten von Nutzen sein werden.

In **Kapitel 6** werden die in dieser Arbeit verwendeten Ansätze kritisch hinterfragt, mögliche zukünftige Untersuchungen vorgeschlagen und die Auswirkungen unserer Erkenntnisse auf die Bohnenzüchtung diskutiert. Abschliessend wird kurz die Zukunft der Bohne als Nahrungsmittel diskutiert und ein Ausblick auf die Bedeutung von Bohnen und Pflanzenkrankheiten gegeben.

Bohnenkrankheiten werden angesichts der günstigen Bedingungen für tropische Pflanzenpathogene in Zukunft weiterhin eine wichtige Einschränkung der Bohnenproduktion in den Tropen sein. Die globale Erwärmung wird das Habitat von tropischen Pflanzenpathogenen vergrössern, die Globalisierung ihre Verbreitung fördern und die zunehmende Prävalenz von Monokulturen ihre Populationszahlen erhöhen. Um der prognostizierten erhöhten Bedrohung durch Pflanzenpathogene zu begegnen, ist es für die Bohnenzüchter von entscheidender Bedeutung, einen Überblick über die verfügbaren Resistenzquellen und deren Wirksamkeit an verschiedenen geografischen Standorten zu haben, wie sie für ALS in dieser Arbeit vorgestellt werden. Im Rahmen dieser Arbeit haben wir zudem an der Etablierung von ALS und Ascochyta resistenten Bohnenlinien mit akzeptablen agronomischen Charakteristiken gearbeitet, welche die Übertragung von Krankheitsresistenzen auf Elite-Zuchtsorten erleichtern.

Zusammenfassend lässt sich sagen, dass diese Arbeit wertvolle Werkzeuge und Zuchtmaterial für die Bohnenzuchtgemeinschaft beiträgt, um die Aufnahme von Krankheitsresistenzen in ihr Zuchtprogramm zu erleichtern. Die daraus resultierenden krankheitsresistenten Sorten werden die Ertragslücke auf den Feldern von Klein- und Grossbauern verringern und somit zur lokalen und globalen Ernährungssicherheit beitragen.

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Chapter 1

General Introduction

The contribution of plant breeding to achieve sustainability and food security on smallholder farms

Although today we are in the comfortable situation where food production is keeping pace with the demand globally, this is not the case in some regions and it may not be the case in the future, as the demand for food is expected to increase 25-70% by 2050 in relation to 2014 [1]. Also before the 1960s, food was scarce, harvests poor and famines common throughout the world, when the so-called 'green revolution' began with the industrialization of agriculture. Besides mechanization and the invention of the Haber-Bosch process that allowed the production of unprecedented amounts of nitrogen fertilizer [2], plant breeding played a crucial part in the green revolution [3]. The wheat breeder Norman Borlaug is largely considered the father of the green revolution, for his advancement in breeding disease and logging resistant wheat cultivars that could be planted in higher densities and were responsive to fertilizer application [4]. These improved cultivars exhibited a reduced height, which allowed them to partition more energy into the grain instead of height growth, resulting in a higher harvest-index. The green revolution production strategy, which consisted of planting improved wheat varieties in combination with the management package, improved yields tremendously. The same strategy was later implemented in rice and maize and widely adopted throughout the world and facilitated the feeding of the 7.6 billion people on planet earth in 2017 [5, 6].

Even though more people are overweight than underweight today, regions with a high percentage of undernutrition prevail, especially in the tropics [7]. In 2016, a third of the population in Eastern Africa and 11% of the world's population was considered chronically undernourished [8]. Developing countries, mostly in Africa, that already now experience high levels of food insecurity, are expected to carry most of the burden of the predicted population increase of 2.3 billion people from 2009 to 2050 [9]. This will make the undernutrition and malnutrition problems even more pressing and solutions to increase crop production in these countries are urgently needed.

To find solutions to enhance food security for developing countries that are frequently affected by food insecurity, it is important to consider how their food is being produced. In developing countries in the tropics, a large percentage of the population engages in agriculture as smallholder farmers [10]. Smallholder agriculture consists of a small plot of land that produces a diverse set of cash and subsistence crops [11]. Farming is usually the main income for these smallholder farming families and they largely depend on their crops for food security, which makes them very vulnerable to disturbances such as droughts, pest outbreaks, climate change and conflicts [8]. Globally, around 20-30% of crop yield is lost because of plant pathogens and pests [12]. Highest losses occur in food-deficient regions that have fast-growing populations, where usually smallholder farmers are the predominant food producers [13]. While the growing conditions are favorable in the tropics with two or even three growing seasons a year, these regions have the highest pressure from diseases and pests [14]. In addition to the conductive environment for diseases, smallholder farmers

are rarely trained in best farming practices, and do not have the means to ensure favorable development of crops through irrigation, application of fertilizer or pesticides [15, 16]. In developing countries, agrochemicals are further problematic for several reasons: firstly, the availability of agrochemicals is not always a given and if they are available, they are often of poor quality or contain more toxic active ingredients than their counterparts used in the developed nations [14, 17]. Secondly, safe handling of agrochemicals is not always practiced with missing labels, missing protective gear and no training on how to handle the chemicals a common occurrence. This poses a health risk for consumers and farmers applying the agrochemicals. Lastly, in Africa the main problem is affordability and availability of agrochemicals, while Latin America and Asia have one of the highest pesticide use per hectare of arable land [18]. The mis- and over-use of these chemicals have been associated with several health concerns and effects on newborns and adults [19-21]. Even though pesticides would offer means for disease control, given their expensiveness and effects on human health, a more sustainable and feasible method to stabilize yield on smallholder farms is needed.

One such more sustainable alternative is offered by plant breeding. Resistance breeding based on naturally occurring genetic diversity can offer a more sustainable and feasible solution to increase crop productivity on smallholder farms. By exploiting natural variation, resistances and tolerances existing within the gene pool of crop species, new varieties can be bred to better cope with the challenging conditions encountered on smallholder farms and hence increase food security for people that most rely on the productivity of their fields. This thesis contributes to a sustainable increase in crop productivity by studying fungal diseases affecting common bean (*Phaseolus vulgaris* L.), an important food security crop in the tropics, and establishing tools for the implementation of the findings within molecular plant breeding programs.

Molecular plant breeding

Plant breeding is the science of adapting the genetics of plants into an intended direction to usually increase yield, nutritional content, or abiotic and biotic stress tolerance [3]. The extent to which plants can be changed largely depends on the genetic and phenotypic diversity of a crop species [22]. Sophisticated breeding strategies have been developed depending on the reproductive strategy of crop species, but they are all based on a common principle. Through the crossing of two genotypes, traits are reshuffled and among the hundreds or thousands of variants produced in the cross, variants outperforming the parents or combining desirable characteristics may be chosen [23, 24]. To select the best variants, plant breeding depended for centuries on the trained breeder's eye.

With the establishment of molecular plant breeding techniques, breeders were given an aid to support the selection process, because relying entirely on phenotypic selection is laborious and not feasible for all traits [25]. Many traits are affected by environmental variation and repeated trials in multiple years are needed to obtain a reliable measure [26]. Other examples where molecular plant breeding is advantageous are traits that can only be measured destructively or traits which depend on a specific environmental condition in order to be measurable, such as disease resistance or drought [25]. Most important however is that breeders need to improve many traits simultaneously and it is impossible to measure or test all of them [27]. In addition, genotyping costs have reached a level where they are highly competitive if not more economic than phenotypic tests [28-30].

To make a trait accessible for molecular breeding through marker-assisted selection (MAS), that traits' genetic base needs to be investigated and molecular markers co-segregating with the trait of interest established [31]. Molecular markers allow genetic polymorphisms to be rendered visible through a range of techniques from random amplification of DNA to highly specific single nucleotide polymorphism (SNP) based markers [32]. These markers allow the tracking of the presence of certain traits indirectly in breeding material and do not rely on suitable testing environments for traits to be expressed. Furthermore, molecular makers allow trait testing at seed or seedling stage to remove individuals without the trait of interest and therefore greatly reduce breeding population sizes.

Initially, MAS focused on qualitative genes that show Mendelian segregation and have major phenotypic effects. These traits can be categorized (i.e. presence-absence, resistant-susceptible), and are usually controlled by one or a few genes and show weak environmental interaction [3]. Molecular markers co-segregating with the trait of interest can be found by linkage mapping, whereas segregating populations are tested with hundreds of molecular markers to find significant marker-trait associations [33]. With the increase of available, high-density molecular markers and more precise phenotyping methods, quantitative traits became accessible through quantitative trait locus (QTL) mapping [34]. QTL mapping, similar to co-segregation studies, relies on artificial populations segregating for the trait of interest to calculate marker-trait associations. The plunge of the cost for DNA sequencing in the last decade has resulted in the generation of even larger genetic resources, not only for model plant species but also for a wide variety of crop species [35]. This unprecedented density of SNP markers has allowed investigations into the extent of historical recombination in panels of diverse genotypes relevant for breeding through genome-wide association studies (GWAS) [36]. In combination with the increasing availability of reference genome sequences, molecular markers can be mapped to the genome and genes in the surrounding areas can be screened to find candidates for the causal gene affecting the trait [37]. While MAS methods proved successful to introgress genetically simple traits, their use was limited when multiple genetic loci were involved each with small effects [38]. Emerging models for genomic selection (GS) overcome this limitation by estimating phenotypic traits based on genotypic information only, which allows selection of the best progenies and the prediction of the phenotypic variance of progenies of different crossing partners [39, 40]. In addition to the selection aids MAS and GS, genetic engineering and site-directed mutagenesis tools allow the manipulation of the genetic content of desired cultivars directly without the dependence on recombination for reshuffling traits. These new plant breeding techniques are rapidly developing and are becoming readily available also for minor crops [41], but whether the products generated by these techniques find their way onto the table will depend on the public perception of these tools and governmental regulations [42-44].

Molecular plant breeding techniques have evolved tremendously in the last decade and, in combination with phenotyping techniques that have made similar advances [45], are increasingly used to support plant breeders in making their selections. The optimal breeding strategy depends on the target trait and not all traits are equally suited for MAS or GS. While GS is more suitable for highly quantitative traits such as yield or grain composition, MAS is the preferred strategy for traits determined by a few genes that are laborious to test, which is often the case for plant disease resistance [46, 47]. Evaluating breeding material for disease resistance requires large greenhouse spaces or a disease-conductive climate in the field. Furthermore, disease evaluations are not always possible because of biosafety

regulations that prohibit the release and exchange of pathogens [48]. New cultivars should contain resistance to several diseases with ideally multiple genes for each disease. Hence, having a functional MAS scheme is highly valuable for breeders as it allows them to concentrate their efforts and resources into promising plant genotypes.

Plant disease resistance mechanisms and the challenge of durable resistance

Unlike abiotic stresses that are comparable across continents, pathogen populations causing biotic stresses are variable, constantly evolving and genetically different at different locations [49-53]. In natural populations, there is a continuous arms race between pathogens and host plants: pathogens overcome resistance, resistant plants become more abundant, pathogens evolve to cause an epidemic on the abundant, previously resistant plants and the cycle continues [54]. To be on the winning side of this arms race, the host and pathogen species need to have the capacity to evolve, which depends on the genetic diversity of the population, that again depends on population sizes, gene flow, recombination, selection and ultimately on mutation [54]. Through industrialization of agriculture, the genetic diversity of the crops in the fields was manipulated, which affected the evolution of crop pathogens. Growing the same few varieties densely packed in monocultures over large areas across continents, increased yield, but decreased genetic diversity [55-57]. By deliberately selecting varieties, we halted or at least slowed down the natural evolution of the plants and in this sense made it easier for a pathogen to spread, once it has overcome the resistance [58].

On a molecular level, plant defense against pathogens is usually described as a two-layer process: The first layer is called pathogen-associated molecular pattern-triggered immunity (PTI) and consists of recognition of patterns that are shared among many pathogens. The second layer called effector-triggered immunity (ETI) then recognizes effectors that are secreted by specialized pathogens. A zigzag model has been proposed to explain the action of the two mechanisms [59]: Upon encountering a pathogen, the plant recognizes commonly occurring microbial or pathogen-associated pattern (MAMPs or PAMPs), which triggers a PTI defense response in the plant to halt pathogen growth. PTI uses transmembrane receptors that recognize MAMPs or PAMPs shared by many pathogens [60]. If the first layer of defense is not sufficient to prevent host colonization, the pathogen starts to secrete effector proteins to suppress the defense of the PTI. Plants have evolved a mechanism to recognize these effectors and protect themselves through ETI. Hypersensitive response is often observed as a mode of defense after ETI, but the mechanisms that stop pathogen growth are not always well understood but can involve transcriptional reprogramming, reactive oxygen species and the salicylic acid pathway among others [59, 61, 62]. In ETI, genes coding for effectors released by the pathogen are named *avr* genes and the corresponding genes in the plant that are recognizing the effectors are called *R* genes. If both, *R* and *avr* genes are present in a plant-pathogen interaction, the plants are resistant but if one of them is missing, the plants are susceptible [63]. Pathogen isolates with a mutation that avoids recognition of its effector by the host plant or pathogen isolates that gained a new effector through horizontal gene transfer and therefore avoid the plant's ETI, have an advantage and will increase in abundance. Similarly, a host plant harboring a new version of the *R* receptor which recognizes the newly acquired pathogen effector will be resistant again and become more abundant, until the cycle repeats itself and a new pathogen isolate overcoming the resistance appears [59]. The above mentioned

zigzag model has been challenged by new findings that suggest a continuous surveillance system rather than the two-layered PTI and ETI system [64- 66].

In contrast to the highly dynamic host-pathogen system described above, another resistance mechanism, non-host resistance, is considered the most durable and efficient resistance to pathogens, since most plants are non-hosts to most pathogens. The mechanisms of non-host resistance are not as well understood as host resistance and are thought to be mediated through multiple pathways involving PTI and ETI among others [67-69].

Common bean – an important and diverse food security crop in Eastern Africa and Latin America

Common bean is an important source of proteins and micronutrients in Eastern Africa and Latin America. Because of their affordable prices, high protein levels and complementary amino acid spectrum to cereals, dry beans are a good substitute for meat, giving rise to the term 'meat of the poor'. In Uganda, common beans and posho (a polenta-like maize dish) are the cheapest foods to survive on and a survey in the Ugandan newspaper 'New Vision' found that over 70% of schools serve beans and posho every day [70]. Not surprisingly, the highest common bean consumptions per capita are found in Uganda, Rwanda and Kenya, with consumption reaching up to 66 kg annually per person in some areas [71]. Besides Eastern Africa, common beans are a staple food in many countries in Latin America and are becoming increasingly popular in developed countries because of their good nutritional properties. Estimating the actual production of common bean is difficult because grain legume species are not distinguished in the FAO statistics. Considering that common beans are the predominant grain legume produced in the Americas and Eastern Africa, the biggest producer in the last ten years were Brazil, USA, Mexico followed by Tanzania, Uganda, Kenya and Ethiopia [72].

Common beans grow well in the tropical and temperate zones and have an approximate growing season of 60 - 120 days throughout which they require 300 - 500 mm of water [73]. As a member of the legume family, they can assimilate nitrogen through their associated rhizobia [74]. Common beans are a very versatile crop and based on their growth habit, can be categorized as climber or bush types, with intermediate types existing [75, 76]. The entire bean plant can be utilized, with the leaves being eaten as vegetables or fed to livestock, the immature pods eaten as snap beans and the mature seeds eaten as dry beans [77]. The latter is especially important for smallholder farmers because these seed can be stored over a long period.

One of the most remarkable traits of common bean is their diversity, of which a fraction is represented within the 37,000 common bean samples maintained at the Centro Internacional de Agricultura Tropical (CIAT) headquarters in Colombia [78]. Common bean seeds come in different sizes, with different patterns and in colors ranging from black to white to red to yellow (Figure 1). The high diversity of the common bean crop can be attributed to its complex evolutionary and domestication history. Genetic studies have located the origin of the wild progenitors of the common bean crop to Mexico, from where they spread across central America [79]. A small founding population migrated to South America and led to the divergence of the wild Andean and the wild Mesoamerican common bean gene pool approximately 165,000 years ago. In the process of transitioning from Central America to South America, the Andean common beans underwent a strong genetic

bottleneck that was followed by a rapid expansion across the continent [80]. From these wild progenitors, common beans were domesticated at least twice, in Mesoamerica and the Andean region, which resulted in the domesticated small-seeded Mesoamerican and the large-seeded Andean gene pools [81-83]. The earliest traces of domesticated common beans date approximately 10,000 years back [83, 84]. Traditionally common beans were grown in association with maize and squash, with these three crops being known as the three sisters. Originally from Mexico, the three sister association planting was widely adopted by Native Americans [85, 86]. Upon the discovery of the Americas by Columbus, common beans were transferred to Europe, Africa and Asia where secondary centers of diversification exist [87, 88]. In the newly reached territories, common beans were readily adapted and preferences for different grain types developed. For the Americas, in the Andean zone large red or mottled common beans are preferred while in Brazil and central America small black, red or carioca types are preferred [89, 90]. In Africa, the diversity is even higher and common beans are planted in variety mixtures and strong local grain preferences exist within countries [91]. General trends were observed in Kenya where mostly Andean common beans are grown, while in neighboring Ethiopia mostly Mesoamerican common beans are grown [87]. In Europe, where common bean breeding as vegetables was initiated, snap beans remain the predominant form of bean consumption up to now [72, 92].

Figure 1: Picture representing part of the phenotypic grain diversity that can be found in the common bean crop. The grain types represented here were collected during field visits of the common bean breeding program at CIAT Colombia.

In addition to *P. vulgaris*, the *Phaseolus* genus contains over 50 species of which four more are domesticated [93]. Three of these can be crossed to common bean and hence represent the extended gene pool of common bean [94]. Domesticated members of the secondary gene pool, which consists of *Phaseolus dumosus* Macfady (year-long bean) and

Phaseolus coccineus L. (runner bean), are generally cross-compatible with common bean [95, 96]. The domesticated tertiary gene pool consists of *Phaseolus acutifolius* A. Gray (terpary bean), which can be crossed to common bean but because of post-zygotic incompatibilities requires embryo rescue to be viable [97, 98]. The extended gene pool species all originated in the Americas, but have adapted to different environments and hence have developed distinct traits, missing in the common bean gene pool, that can be taken advantage of in common bean improvement [99].

Common bean breeding for the tropics

Common bean breeding has a long tradition due to their popularity and importance. Initially, farmers selected good grain types and used them as seeds in the following planting seasons. Up to today, countless farmer-selected varieties, so-called landraces, are cultivated [100-104]. Systematic common bean breeding began in the nineteenth century in Europe and the USA and focused on breeding stringlessness in snap beans as well as resistance to fungal, bacterial and viral diseases [105, 106]. Because of the self-pollinating, homozygous nature of common bean, smallholder farmers obtain seeds mostly by saving part of last year's harvest or from grains bought on the market [107]. Common bean breeding and seed production are therefore of relatively low commercial interest compared to crops where farmers loose substantial performance by reusing seeds because of hybrid technology or the high frequency of seed-borne diseases [108, 109]. With the exception of snap bean, which has received substantial interest from breeding companies due to their high commercial value [92], common bean breeding is currently conducted mostly by public institutions including universities, national agricultural research programs and CIAT. Common bean breeding is very laborious because it needs to be conducted for each grain type separately (i.e. red kidney bean, navy bean, pinto bean) and usually breeding programs run activities for several grain types in parallel.

Desirable plant characteristics differ depending on the mode of cultivation. On large industrialized farms, where common beans grow with high agrochemical input in relatively favorable conditions, yield is the most important trait next to canning quality, disease resistance and a bush type growth habit that can be mechanically harvested [110]. On smallholder farms, crops are usually exposed to various stresses, therefore, cultivars need to be able to grow well in sub-optimal conditions and harbor resistances against biotic stresses [111]. A high yielding cultivar is of no use here, as long as it cannot achieve its potential due to stresses. Hence breeding for abiotic and biotic stresses is of highest importance. Typical breeding aims are tolerance to low soil fertility, tolerance to heat and drought stress, improved nutritional content (especially iron and zinc), and resistance to various insect pests as well as fungal, bacterial and viral diseases [111]. As discussed above, common beans have a large genetic diversity due to their complex evolutionary history and their multiple centers of diversification. This diversity can be harnessed and manipulated in the breeding process. In addition, interspecific crosses have successfully transferred traits which have not been found in the *P. vulgaris* gene pool before [99]. For example, breeders have successfully transferred drought, cold and heat tolerance from terpary bean and disease resistance from terpary, year-long and runner bean to common bean [112-114].

Several molecular breeding resources have become available for common bean in the last two decades. Two reference genome sequences are available for the Andean line 'Chauca Chuga' (G19833) [80] and the Mesoamerican line BAT 93 [115] and several genotypes have been resequenced [116]. Common bean with its diploid, self-pollinating

nature and the relatively small genome size of 587 Mbp [80] is easily accessible to genetic and genomic analyses. Geneticists have produced a large amount of data in the last years, but their incorporation in common bean breeding is only slowly picking up because of the few available molecular markers that are specific in other than the experimental populations they were found in [47, 117]. With the increasing availability of highly specific molecular markers, genetic resources will complement the breeder's eye and speed up breeding efficiency in this important food-security crop.

Angular leaf spot and Ascochyta in common bean

The wild ancestor of common bean evolved in a tropical environment together with various pathogens. As a consequence, common beans today are challenged by several fungal, viral, bacterial and insect pests that attack at various stages of their life, sometimes with devastating effects on yield [111, 118]. Fungal pathogens cause high yield losses with the globally most important foliar fungal common bean diseases being anthracnosis (caused by *Colletotrichum lindemuthianum* (Sacc. & Magnus) Lams.-Scrib), rust (caused by *Uromyces appendiculatus* F. Strauss (sin. *U. phaseoli* G. Winter)) and angular leaf spot (ALS, caused by *Pseudocercospora griseola* (Sacc.) Crous & U. Braun). Additionally, there are locally important diseases such as Ascochyta (caused by *Boeremia diversispora* (Bubák) Aveskamp, Gruyter & Verkley) that show high incidences in the tropical highlands and are expanding their range out of the tropics towards Europe. In this thesis, resistance to the two reoccurring and devastating foliar fungal common bean diseases ALS and Ascochyta (Figure 2) is studied.

Figure 2: Characteristic symptoms caused by the fungal common bean diseases angular leaf spot (left) and Ascochyta (right).

The common bean disease ALS is one of the most important and abundant diseases in Africa and Latin America and can cause yield losses of 31-80% [119, 120]. The causal agent, *P. griseola*, is an ascomycete fungus belonging to the class of Dothideomycetes [121]. The genus *Pseudocercospora* is an anamorph of the genus

Mycosphaerella, the largest genus of plant pathogenic fungi, hence no sexual life cycle is known for *P. griseola* [122, 123]. ALS can be transmitted through seeds, but the most important source of primary inoculation is infected plant-debris in the field [124]. Pathogen spread is favored by conditions of alternating temperature and humidity [125]. After germination of the spore, the fungus enters the leaf through the stomata where it grows intercellularly and causes neighboring cells to disintegrate and desiccate, which can be visually detected as lesions [126]. Lesions can occur on stems, pods and leaves of common bean and are delimited by the leaf veins giving them an angular shape. If the infestation is severe, premature defoliation of the plants occurs. Because of its devastating effect on bean yields, resistance breeding for ALS is a major focus of common bean breeding in the tropics. These breeding efforts are challenged by the high diversity of the causal agent *P. griseola* and the pathotype-specific reaction of ALS resistance that requires a different breeding strategy for each location [125, 127, 128].

Ascochyta was considered a minor disease until recently but it is now an important disease occurring in the cooler humid climates found at higher altitudes in the tropics and in temperate regions [129-131]. The causal agent of Ascochyta in common bean, previously known as *P. exigua* var. *diversispora* or *Ascochyta phaseolorum*, was re-classified according to molecular phylogeny and named *Boeremia diversispora* [132-134]. The genus *Boeremia* belongs to the Didymellaceae family within the Dothideomycetes class [133]. Ascochyta is characterized by dark concentric lesions on foliage, pods and stems and is especially important on snap beans, where the lesions on pods lower the market value [119, 131]. Ascochyta has been reported to cause yield losses of 41-75%, although these estimates may be on the higher end as a natural epidemic of white leaf spot (caused by *Pseudocercosporella albida* (Matta & Belliard)) was encountered during field trials that probably contributed to the yield loss [119]. Research on Ascochyta in common bean is still in its infancy with a few early investigations published in the 1980s and 90s [119, 131, 135]. Recently, several publications have emphasized the increasing importance of this disease for Europe and Africa [129, 130, 136]. Resistance to Ascochyta has not been found in common bean but has been frequently observed in members of the secondary gene pool. All 119 gene bank accessions of *P. dumosus* tested and 74 of the 103 tested accessions of *P. coccineus* were found to be resistant to Ascochyta in field trials [131]. Several efforts have been made to transfer Ascochyta resistance to common beans, but achieving strong resistance has proven difficult [135].

Thesis goals

This doctoral thesis aims at investigating the resistance of common bean against the fungal diseases ALS and Ascochyta. In addition, it aims at establishing germplasm and tools to support resistance breeding in tropical and subtropical areas where ALS and Ascochyta are recurring and devastating common bean diseases.

After a broad introduction into the topic in **chapter 1**, **chapter 2** aims at summarizing the last 40 years of ALS resistance studies. Genetic tools have evolved substantially in this time and this review aims at comparing previously found resistance loci with the help of new genetic tools, mainly the reference genome.

ALS resistance is reported to be highly pathotype-specific, but for the five ALS resistance loci described in common bean, little is known about their effectiveness against different pathotypes on different continents. **Chapter 3** aimed at obtaining a broader understanding of the sources of ALS resistance, the genetic determinants of resistance and the effectiveness of resistance against different pathotypes on two continents. In addition, we aimed at developing molecular markers that co-segregate with resistance loci, which can be incorporated in the MAS breeding scheme of common bean breeding programs.

To make ALS resistant varieties with resistance to a broad spectrum of ALS pathotypes available to farmers as soon as possible, a breeding project has been initiated and is described in **chapter 4**. We aim to combine five well-characterized resistance loci of Andean and Mesoamerican origin into a common bean line that exhibits desired agronomic qualities, mainly good grain types and a bush type growth habit.

Ascochyta resistance breeding is not routinely conducted, mostly because of the absence of strong resistance in the common bean gene pool. At CIAT, Ascochyta resistance was introgressed into common bean from its sister species *P. dumosus*. In **chapter 5**, we aimed at determining the genetic causes of resistance in interspecific, Ascochyta-resistant lines by crossing them to elite climbing common bean lines and testing their progenies for resistance in field and greenhouse trials.

To conclude, **chapter 6** discusses the findings and implication of the conducted studies for common bean production and pathology research.

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Chapter 2

A Review of Angular Leaf Spot Resistance in Common Bean

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Abstract

Angular leaf spot (ALS), caused by *Pseudocercospora griseola*, is one of the most devastating diseases of common bean (*Phaseolus vulgaris* L.) in tropical and subtropical production areas. Breeding for ALS resistance is difficult due to the extensive virulence diversity of *P. griseola* and the recurrent appearance of new virulent races. Five major loci, *Phg-1* to *Phg*-5, conferring ALS resistance have been named, and markers tightly linked to these loci have been reported. Quantitative trait loci (QTLs) have also been described, but the validation of some QTLs is still pending. The *Phg-1*, *Phg-4*, and *Phg-5* loci are from common bean cultivars of the Andean gene pool, whereas *Phg-2* and *Phg-3* are from beans of the Mesoamerican gene pool. The reference genome of common bean and high-throughput sequencing technologies are enabling the development of molecular markers closely linked to the *Phg* loci, more accurate mapping of the resistance loci, and the comparison of their genomic positions. The objective of this report is to provide a comprehensive review of ALS resistance in common bean. Furthermore, we are reporting three case studies of ALS resistance breeding in Latin America and Africa. This review will serve as a reference for future resistance mapping studies and as a guide for the selection of resistance loci in breeding programs aiming to develop common bean cultivars with durable ALS resistance.

Introduction

Common bean (*Phaseolus vulgaris* L.), which includes dry and snap beans, is the world's most important grain legume for direct human consumption and an important source of protein, fiber, calories, and vital micronutrients, particularly for millions of people in Latin America and Eastern and Southern Africa [1]. Frequent consumption of dry beans combined with cereals ensures a balanced diet of essential amino acids and other nutrients that contribute to alleviating malnutrition and preventing cardiovascular disease, diabetes, and certain types of cancer [1-3].

The Americas are the largest common bean-producing region, and Brazil is the world's largest producer and consumer [4, 5]. Africa, where common bean was introduced after the discovery of the Americas, is second in production, and the consumption in several African countries, up to 66 kg person^{-1} yr^{-1}, is greater than that in Latin America [1, 4-6].

Numerous infectious diseases caused by fungi, viruses, bacteria, and nematodes represent major limitations to common bean production throughout the world [7, 8]. Angular leaf spot (ALS), a disease caused by *Pseudocercospora griseola* (Sacc.) Crous & Braun [previously referred to as *Phaeoisariopsis griseola* (Sacc.) Ferrari], was until the 1980s considered to be of minor importance in Latin America [9]. However, in the mid-1980s, ALS began to be considered a significant constraint to common bean production in Brazil, Central America, and Eastern and Southern Africa [9-12]. Currently, ALS is one of the most recurring and devastating diseases of common beans in Latin America and Africa, the most important production areas of the world [6, 10, 13-16]. Angular leaf spot has also been reported to occur sporadically in countries of the temperate climate zone, including the United States and Canada and was recently reported for the first time in northern Spain [17-19].

Yield losses caused by ALS can reach up to 80% [9, 20, 21]. Although fungicides are an option for the control of ALS, they are often expensive or not readily available to smallholder farmers, the predominant producers of common beans in the tropics. Cultivars with resistance to *P. griseola* offer a cost-effective, easy-to-use, and environmentally friendly management strategy [11]. However, development of common bean cultivars with effective ALS resistance is difficult due to the broad and changing virulence diversity of the ALS pathogen that renders varieties that are resistant in one year or location susceptible in another [11, 22].

Several sources of ALS resistance have been identified among the primary and secondary gene pools of *P. vulgaris* [11, 23-27]. Resistance to the ALS pathogen is mainly conferred by single dominant resistance genes (hereafter also referred to as loci) but recent studies also indicate a more quantitative nature of resistance and associated quantitative trait loci (QTLs) have been found. To date, five ALS resistance loci have been approved by the Bean Improvement Cooperative (BIC) Genetics Committee (http://arsftfbean.uprm.edu/bic/wp-contentontent/uploads/2018/04/Bean_Genes_List_2017.pdf) that maintains the guidelines for the nomenclature of disease resistance genes in common bean. These include three dominant and independent *Phg* loci named *Phg-1*, *Phg-2*, and *Phg-3* and two major QTL named *Phg-4* and *Phg-5* [28-36].

New technologies, including the reference genome of common bean [37] and high-throughput sequencing, facilitate the development of different types of molecular markers that are tightly linked to these loci and provide new insight into the relationship between

existing and newly discovered disease resistance loci. This review aims to (i) discuss the virulence diversity of *P. griseola* and its impact on disease resistance breeding, (ii) review the current knowledge of ALS resistance, (iii) comment on how new genomic resources could facilitate and accelerate ALS research, including gene discovery and the development of highly accurate molecular markers, and (iv) present three case studies of ALS resistance breeding in Brazil, Colombia, and Uganda.

Pseudocercospora griseola the causal agent of the angular leaf spot disease

The ALS pathogen belongs to the class Dothideomycetes, the largest and most diverse class of ascomycete fungi, which contains many important plant pathogens, endophytes, and saprobes [16, 38]. Although *P. griseola* can be transmitted through seeds, the most frequent source of primary inoculum to initiate ALS disease under natural conditions is the presence of plant debris infected with the pathogen [13]. *Pseudocercospora griseola* is considered a fastidious pathogen [13], yet it grows and produces spores on artificial culture media. Lyophilization has been successfully used for the long-term storage of spores [39]. The response of common bean germplasm to *P. griseola* is usually evaluated using a disease severity scale ranging from 1 to 9, where scores of 1 to 3 are considered resistant, 4 to 6 are intermediate, and 6 to 9 are susceptible [40].

The ALS pathogen is known for its extensive virulence diversity [11, 12, 15, 22, 41-43]. In the early 1980s, the Bean Program of CIAT, Cali, Colombia, developed a set of 12 common bean differential cultivars: six Andean and six Mesoamerican. To standardize races of *P. griseola* (Table 1), a binary code was implemented and launched in 1995 during the first ALS workshop [44]. The set of differential cultivars has been extensively used throughout the world and has permitted the comparison of races of the ALS pathogen between locations in single regions and across countries and continents.

Characterization of the virulence phenotype (known as race) of isolates of *P. griseola* on Andean and Mesoamerican common bean differential cultivars has resulted in the separation of these isolates into two distinct virulence groups [45]. Isolates obtained from large-seeded bean cultivars of the Andean gene pool from Ecuador, Colombia, and Argentina were virulent only on Andean differential bean cultivars, and these races are referred to as Andean. Isolates from small- and medium-seeded Mesoamerican cultivars from Central America, Brazil, Bolivia, and Argentina were virulent on both Mesoamerican and Andean ALS differential cultivars and are referred to as Mesoamerican [11, 22, 42, 45-47]. Similar studies using differential cultivars and molecular techniques have revealed that the virulence and genetic diversity of two other bean diseases, anthracnose (*Colletotrichum lindemuthianum*) and rust (*Uromyces appendiculatus*), also segregate into two distinct groups that mirror the diversity of their common bean host [46, 48, 49].

Some of the most aggressive isolates of *P. griseola*, belonging to race 63-63, that are virulent on all Andean and Mesoamerican differential cultivars were first observed in Latin America. These races were recurrently found in Brazil, Argentina, and Central America [15, 22, 47, 50]. Later, these races were infrequently found in Africa [51-53]. Although Andean and Mesoamerican races have been found in the Americas and Africa, their predominance on these continents differ. Initially, Andean races infecting only Andean differential cultivars were the predominant races in Africa [12, 42]. Shortly thereafter, the first reports of races that infected mainly Andean differentials but also a few

Mesoamerican differential cultivars appeared. These races were termed Afro-Andean [51-54]. Using molecular markers to analyze the diversity of Afro-Andean races, Mahuku et al. [51] suggested that the Afro-Andean races belonged to the Andean group, disputing the existence of the Afro-Andean group. Recently, Serrato-Diaz et al. (personal communication, 2018) further investigated the population structure of *P. griseola* by sequencing four nuclear genes to construct a phylogenetic tree. The authors found that isolates from Puerto Rico, Honduras, and Guatemala clustered in the Mesoamerican clade, whereas the Tanzanian isolates clustered into three clades: Mesoamerican, Andean, and Afro-Andean. Their findings also suggest a more diverse population structure than the previously reported Mesoamerican and Andean groups, but further investigations are required to determine the classification of pathogen isolates.

Table 1: Set of 12 common bean differential cultivars and binary code used to designate races of *Pseudocercospora griseola*. Each race is assigned two numbers based on the summation of the binary numbers of the susceptible Andean and Mesoamerican cultivars, respectively. The example in the table shows how a Mesoamerican isolate of *P. griseola* was characterized as race 25-39.

Differential cultivars	Seed size	Common bean race	Resistance gene/ Chromosome	Binary value	Reaction and binary value of susceptible cultivars
Andean differential cultivars					
Don Timoteo	Large	Chile	Unknown	1	Susceptible - 1
G 11796	Large	Peru	Unknown	2	Resistant
Bolon Bayo	Large	Peru	Unknown	4	Resistant
Montcalm	Large	Nueva Granada	Unknown	8	Susceptible - 8
Amendoin	Large	Nueva Granada	Unknown	16	Susceptible - 16
G5686	Large	Nueva Granada	*Phg-4*/Pv04; *Phg-5*^{2}/Pv10	32	Resistant
					Andean binary value (1+8+16) = 25
Mesoamerican differential cultivars					
PAN 72	Small	Mesoamerica	Unknown	1	Susceptible - 1
G2858	Medium	Durango	Unknown	2	Susceptible - 2
Flor De Mayo	Small	Jalisco	Unknown	4	Susceptible - 4
Mexico 54	Medium	Jalisco	*Phg-2*/Pv08	8	Resistant
BAT 332	Small	Mesoamerica	*Phg-2*^{2}/Pv08	16	Resistant
Cornell 49-242	Small	Mesoamerica	Not named	32	Susceptible - 32
					Mesoamerican binary value (1+2+4+32) = 39
					Race = 25-39

Although differential cultivars provide a simple and effective method for studying pathogen virulence diversity, some or all differential cultivars may become susceptible to new virulent isolates due to changes in the virulence spectra of pathogens over time and space. In fact, the virulence diversity of *P. griseola* appears to have changed already, and many new isolates found in Latin America and Africa were virulent on all differential cultivars [15, 47, 50, 52, 53, 55, 56]. Because of the increased occurrence of isolates of race 63-63, efforts are underway to develop a new set of differential cultivars, and in 2015, new candidates were proposed during the Common Bean Disease Workshop in Skukuza, South Africa. However, before a new set of differential cultivars can become available,

they need to be tested for their reaction to *P. griseola* isolates from different countries and, in particular, for reactions to isolates of race 63-63.

Because of the high virulence diversity of *P. griseola* and the great potential for overcoming resistance, a successful resistance breeding strategy requires a sound understanding of the virulence diversity and evolution of this pathogen. Based on what is known about parallel evolution between gene pools of the common bean host and pathogen, proposed strategies for durable ALS resistance include pyramiding of Andean and Mesoamerican resistance genes or using Mesoamerican resistance sources in areas where predominantly Andean isolates exist, and vice versa [11, 42].

Overview of major loci conditioning resistance to *P. griseola*

Since the beginning of the 1980s, many studies have reported new sources and loci conferring resistance to ALS, as well as molecular markers linked to these loci. However, many of these publications did not include appropriate physical linkage information or allelism tests and thus could not be validated. Only repeatedly characterized loci or QTLs for which linked molecular markers are available can be submitted for acceptance to the BIC Genetics Committee. The currently approved ALS resistance loci include three dominant and independent loci, *Phg-1*, *Phg-2*, and *Phg-3*, as well as two major QTLs, *Phg-4* and *Phg-5* (Table 2).

Table 2: Named and mapped angular leaf spot resistance genes in common bean. Resistance loci are stated with their new name accepted by the Bean Improvement Cooperative Genetics Committee and their originally published name. Table modified from Souza et al. [60]

Locus symbol		Resistance Source	Gene Pool†	Chromosome	Pathogen race	Reference
New	**Original**					
Phg-1	*Phg-1*	AND 277	A	Pv01	63-23	[28, 32]
Phg-2	*Phg-2*	Mexico 54	MA	Pv08	63-19	[61, 62]
Phg-2^{2}	–	BAT 332	MA	Pv08	63-39	[31]
Phg-3	*Phg-ON*	Ouro Negro	MA	Pv04	63-39	[30, 33]
Phg-4	*Phg*$_{G5686A}$ ALS4.1^{GS,UC}	G5686	A	Pv04	31-0 31-0	[26, 36]
Phg-5	ALS10.1^{DG,UC}	CAL 143	A	Pv10	0-39 Field	[34, 35]
	ALS10.1^{DG,UC,GS}	G5686	A	Pv10	31-0	[36]

†A, Andean; MA, Mesoamerican

Methods of characterizing ALS resistance loci have changed over time. Initial work with random amplification of polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), and restriction fragment length polymorphism (RFLP) markers was followed by sequence characterized amplified region (SCAR), simple sequence repeat (SSR), and single-nucleotide polymorphism (SNP) marker systems [57-59]. The publication of the common bean reference genome [37] has permitted mapping and

comparison of the positions of most SCAR, SSR, and SNP markers (Figure 1). In this section, progress in ALS resistance characterization, focusing mainly on genetic mapping studies and remapping molecular markers linked to ALS resistance genes on the reference genome of common bean, is discussed. For details on markers, see supplemental table S1.

Phg-1

Origin: The *Phg-1* locus was reported on chromosome Pv01 and is tightly linked to the anthracnose resistance locus *Co-1*^{4} in cultivar AND 277 [32].

Molecular Markers: The *Phg-1* and *Co-1*^{4} loci are tightly linked (0.0 cM) to each other on chromosome Pv01 [32]. Two molecular markers, CV542014^{450} and TGA1.1^{570}, flanking the *Co-1*^{4}*/Phg-1* loci were identified as linked at 0.7 and 1.3 cM, respectively.

Alleles: No alleles were reported.

Breeding value: The Andean cultivar AND 277, which was obtained from a cross of Andean cultivars G21720 x BAT 1386, is an important ALS resistance source that has been used in breeding programs in Brazil and southern Africa [12, 28, 63]. AND 277 was reported to be resistant to *P. griseola* and *C. lindemuthianum* under field conditions during two years of evaluations in Malawi [12]. The ALS-resistant Andean cultivar CAL143, derived from a cross of G12229 x AND 277, may carry *Phg-1* present in the AND 277 parent; however, there are no studies supporting this assertion [12]. CAL 143 is a high-yielding variety that has a strong level of resistance to ALS, rust, and halo blight (*Pseudomonas syringae* pv. *phaseolicola*) under field and greenhouse conditions [34, 64, 65].

Phg-2

Origin: The *Phg-2* locus was discovered in Mesoamerican cultivar Mexico 54 as a single dominant resistance locus on chromosome Pv08 [62].

Molecular Markers: Sartorato et al. [62] reported the RAPD markers OPN02 and OPE04, linked to *Phg-2* in Mexico 54 at 5.9 and 11.8 cM, respectively. The SCAR marker SN02, which was developed based on the OPN02$_{890}$ fragment, shows polymorphisms identical to those of the original mapping population [29, 61]. The SN02 marker was however not polymorphic in other evaluated populations using Mexico 54 as the resistant parent [31]. The polymerase chain reaction (PCR) marker g796, which is highly specific for Mexico 54, was found at 3 cM distance [66].

Alleles: In addition to Mexico 54, other ALS resistant Mesoamerican cultivars contain a resistance locus that maps to the lower end of chromosome Pv08. These include Cornell 49-242, MAR 2, G10474, BAT 332, and G10909. Physical position analysis using the common bean reference genome sequence [37] indicated that the ALS resistance in these cultivars may be conferred by alleles of *Phg-2* [60]. However, this information requires verification.

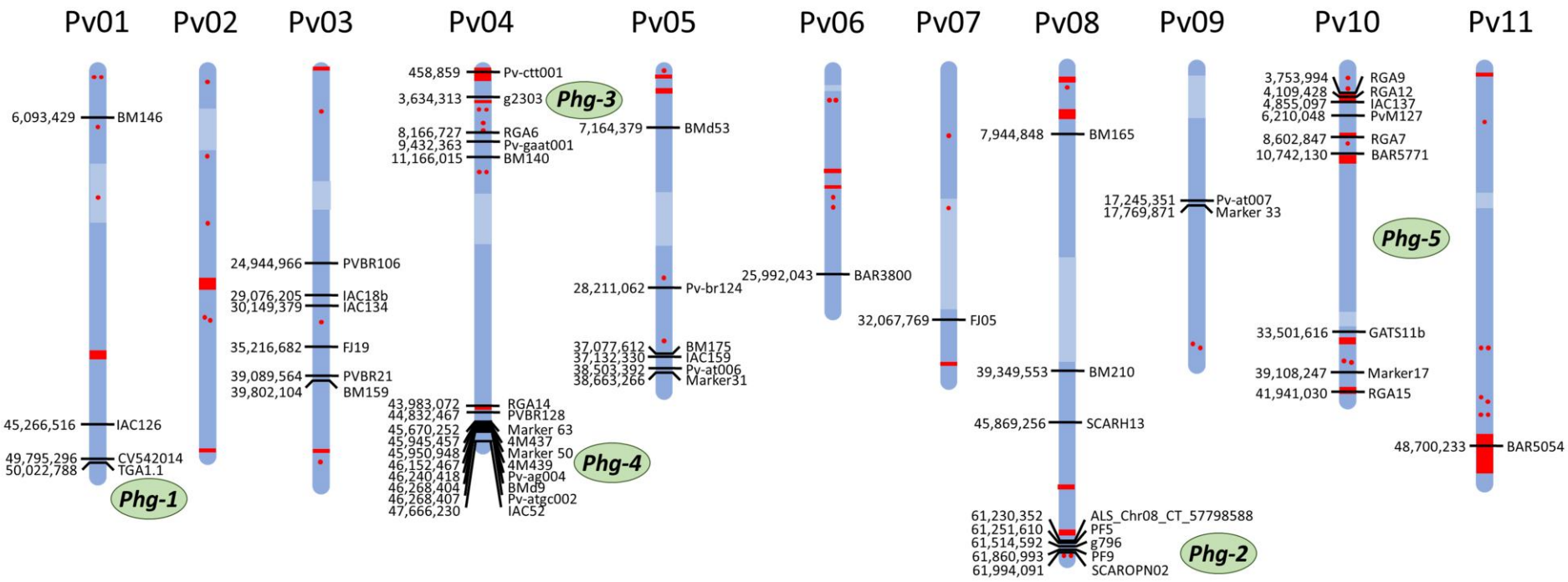

Figure 1: Genetic map showing positions of reported markers tagging ALS resistance loci. Markers linked to ALS resistance loci are shown with their location mapped on the *Phaseolus vulgaris* reference genome v2.1 [89]. The ALS resistance loci approved by the BIC Genetics Committee and their approximate positions are marked in green on the right side of the chromosome. Centromere regions are shown in light blue as reported in the reference genome [37]. Resistance genes, containing an ARC domain (PF00931) are marked in red, with points if there are less than three genes and with bands if there are three or more genes. A summary of markers linked to resistance loci and their primer sequences is given in the supplementary table S1.

In cultivar Cornell 49-242, a single dominant resistance locus was found to control resistance to race 31-17 [67, 68]. The $OPN02_{890}$ and $OPE04_{650}$ RAPD markers linked to *Phg-2* in Mexico 54 were also found to be linked to the resistance locus in Cornell 49-242 at 3.2 and 12.5 cM, respectively [67]. Given that the same markers are linked to ALS resistance in Mexico 54 and Cornell 49-242, these loci might be allelic [67]. $OPE04_{650}$ was also found to be linked at 5.8 cM to a single dominant resistance locus in MAR 2 conferring resistance to ALS race 63-39 [69].

In the common bean genotype G10909, two genes on chromosomes Pv04 and Pv08 confer resistance to the highly virulent race 63-63 [27]. The resistance gene $Phg_{G10909B}$ on chromosome Pv08 was found to cosegregate with SCAR markers $PF13_{310}$, $PF9_{260}$, and $OPE04_{709}$ at 4.9, 7.4, and 9.9 cM, respectively [27].

In the BAT 332 cultivar, a single and dominant gene linked to RAPD markers $OPAA07_{950}$ and $OPAO12_{950}$ at 5.10 and 5.83 cM, respectively, confers resistance to race 61-41 [70]. Additionally, an allelism test between Mexico 54 and BAT 332 inoculated with race 63-39 showed no segregation, which is an indication that the loci conferring ALS resistance in Mexico 54 and BAT 332 are allelic [31]. The angular leaf spot resistance locus in BAT 332 has been designated as the $Phg\text{-}2^2$ allele and is presently the only allele of *Phg-2* officially accepted by the BIC Genetics Committee.

A single dominant resistance locus linked to the codominant SCAR marker PF5, positioned 5.0 cM from the resistance locus, has been reported in the Mesoamerican accession G10474. The gene-pool-specific PF5 marker can be used to transfer resistance from G10474 to Andean common bean cultivars [71]. In addition, another highly specific marker, ALS_Chr08_CT_57798588, found through whole-genome sequencing of G10474, can be used in marker-assisted selection (MAS) [72].

In summary, the known physical positions of linked markers suggest that the ALS resistance genes in MAR 2, Cornell 49-242, G10474, and G10909 are either alleles of *Phg-2* from Mexico 54 or they may represent different loci within a resistance gene cluster. Nonetheless, allelism studies have only confirmed that the resistance loci in BAT 332 and MAR 2 are allelic to *Phg-2* [31, 68]. Further genomic characterization of the *Phg-2* locus is necessary to clarify allelic relationships or the presence of potentially different genes within the locus.

Breeding value: The *Phg-2* locus found in Mexico 54 and its potential alleles present in various Mesoamerican cultivars confer the broadest known resistance and are present in several bean cultivars used in ALS resistance breeding. Mexico 54 has been extensively used in breeding and research because of its good resistance to African *P. griseola* isolates, though the cultivar was resistant to only 5 out of 19 Colombian races tested [73-76]. The putative *Phg-2* allele of G10474 confers broad resistance to most races of *P. griseola* and is extensively used in breeding at CIAT. G10474 was found to be resistant to most races screened under greenhouse conditions, with only races from Haiti and South Africa able to cause disease in this cultivar [71, 73].

Phg-3

Origin: The *Phg-3* ALS resistance locus was first reported in the Mesoamerican common bean cultivar Ouro Negro [30]. A later study reported that *Phg-3* co-segregates and is tighly linked (0.0cM) with the anthracnose resistance locus *Co-3*^{4} (previously named *Co-10*) on chromosome Pv04 [33].

Molecular Markers: Gonçalves-Vidigal et al. [33] reported that the *Phg-3*/*Co-3*^{4} resistance loci are tightly linked to the marker g2303, at 0.0 cM on Pv04, enabling the use of MAS to transfer the cluster to commercial bean cultivars.

Alleles: The resistance locus *Phg*$_{G10909A}$ in the Mesoamerican cultivar G10909 is located on chromosome Pv04, 13 cM from marker Pv-gaat001 [27]. *Phg*$_{G10909A}$ and *Phg-3* are in the same region, though no allelism tests between these loci have been conducted.

Breeding value: Ouro Negro is a highly productive black-seeded Mesoamerican cultivar with desirable agronomic and cooking characteristics that was selected from CIAT accession G3680, also known as Honduras 35 [77]. The *Phg-3* ALS, *Co-3*^{4} anthracnose, and *Ur-14* rust resistance alleles present in Ouro Negro are very important for common bean breeding programs in Brazil [33, 77, 78], conferring resistance to at least 21 *C. lindemuthianum* races and seven *P. griseola* races, including highly virulent race 63-63 [33, 79-83].

Phg-4

Origin: The *Phg-4* gene, previously named *Phg*$_{G5686A}$, was discovered in the cultivar G5686 inoculated with *P. griseola* race 31-0 [26]. Keller et al. [36] used a fine-mapping approach to characterize and delimit the G5686 QTL on chromosome Pv04 and named it ALS4.1$^{GS, UC}$. Because of the consistent and significant effects of this major locus across different environments and populations [26, 36], the BIC Genetics Committee has approved the name *Phg-4* for ALS4.1 [60].

Molecular Markers: The *Phg-4* locus was found to be linked, at 0.0 cM, to the microsatellite marker Pv-ag004 from G5686 [26]. Keller et al. [36] used fine mapping to investigate the *Phg-4* locus in detail, delimiting it to a 418 kb genomic region between markers Marker63 and 4M439. The delimited region contained 36 genes, including 11 serine/threonine protein kinases arranged in a repetitive array, which are promising candidate genes for ALS resistance. Single nucleotide polymorphism-based markers highly specific to *Phg-4* in G5686 are available on several genotyping platforms [36, 72].

Alleles: Using a CAL 143 (resistant) x IAC-UNA (susceptible) cross, Oblessuc et al. [34] reported two adjacent QTLs, ALS4.1$^{GS, UC}$ and ALS4.2$^{GS, UC}$, delimited by marker intervals IAC52 to BMd9 and PVBR92 to Pv-gaat001, respectively. Although these two QTLs were reported as being close to the *Phg-4* locus, they have not been approved by the BIC Genetics Committee as alleles of *Phg-4*.

Breeding value: G5686 has been inoculated with >500 isolates of *P. griseola* from 27 countries and found to be one of the most resistant genotypes. G5686 is currently being used in breeding line development at CIAT.

Phg-5

Origin: The resistance locus *Phg-5* on chromosome Pv10 has been found in two different Andean common bean cultivars: CAL 143 and G5686. The *Phg-5* locus was first reported in a CAL 143 x IAC-UNA recombinant inbred line (RIL) population evaluated in the field under natural infection and in the greenhouse inoculated with *P. griseola* race 0-39 [34]. *Phg-5*, previously named QTL ALS10.1, exhibited a strong effect in both environments. Keller et al. [36] confirmed the presence of *Phg-5* in common bean accession G5686. However, besides a rough positional analysis, there is no evidence that *Phg-5* in CAL 143 and G5686 represent the same gene or are allelic.

Molecular Markers: The closely linked markers GATS11b and IAC137 flank the *Phg-5* locus in CAL 143 [34]. Although the two markers are closely linked, the physical positions of GATS11b (33.50 Mb) and IAC137 (4.86 Mb) are very far apart as this range coincides with the centromeric region of chromosome Pv10, which shows little recombination [37]. Oblessuc et al. [35] increased the marker density around the *Phg*-5 locus and identified the marker ATA220, which coincided with the peak LOD score but did not map to the common bean reference genome. Investigation of the transcriptional modulation of the *Phg-5* region revealed an enrichment of genes involved in plant–pathogen interactions, and seven of the 323 genes located in the core region were found to be differentially regulated after infection [84]. Keller et al. [36] reported a minor QTL linked to Marker17 on chromosome Pv10 in G5686, which was also designated as *Phg-5*.

Alleles: In a G19833 x DOR364 cross, Lopez et al. [85] reported four resistance gene analogs on chromosome Pv10 linked to ALS resistance. Another minor QTL was found in AND 277 on chromosome Pv10, associated with marker BAR5771, conferring resistance to race 1-21 [86].

Breeding value: CAL 143 has been used extensively in breeding, and it is a popular variety that has been released in several African countries, including under the name of Lyambai in Zambia. G5686 is one of the most resistant Andean genotypes known and carries the resistance loci *Phg-5* and *Phg-4* [26, 36].

Other reported angular leaf spot resistance loci tagged by markers

Besides the five well-characterized ALS resistance loci (*Phg-1* to *Phg-5*), other loci have been reported, but they showed either a weak effect on resistance or there is not sufficient evidence to validate their existence and to assign them a *Phg* symbol.

Chromosome Pv01: In phenotypic evaluations under field conditions of a Jalo EEP 558 (resistant) x Small White (susceptible) cross, Teixeira et al. [87] found an ALS QTL linked to marker BM146 of Jalo EEP 558, which they reported to be on chromosome Pv05. However, this marker has been mapped to the upper end of chromosome Pv01 on the reference genome, with *Phg-1* positioned at the lower end of the same chromosome.

Chromosome Pv03: Using an RIL population from a CAL 143 × IAC-UNA cross, Oblessuc et al. [34] found a minor QTL on chromosome Pv03 flanked by markers PVBR21 and FJ19. These authors also reported an ALS resistance locus on chromosome Pv02 flanked by markers IAC134 and IAC18b. However, these markers have been mapped to

chromosome Pv03 of the reference genome, in proximity to other reported markers (Figure 1). The resistant allele at this locus is derived from CAL 143.

Chromosome Pv05: Oblessuc et al. [34] discovered two QTLs on chromosome Pv05 in the CAL 143 x IAC-UNA cross. The QTL ALS5.1^{UC} is flanked by markers BMd53 and FJ05, and QTL ALS5.2^{UC}, which exhibited a strong effect under greenhouse conditions, is flanked by markers BM175 and IAC261. By mapping the abovementioned markers onto the reference genome, BMd53 and BM175 were confirmed to be located on chromosome Pv05; but FJ05 and IAC261 were mapped to chromosomes Pv07 and Pv01, respectively (Figure 1). In another study using RILs from an AND 277 (resistant) x SEA 5 cross (susceptible) inoculated with race 1-21, a minor QTL was found associated with marker IAC159 on chromosome Pv05 [86]. Quantitative trait locus mapping in G5686 x Sprite revealed a minor QTL in the same genomic region that explained 3.7% of the variance associated with Marker 31 [36].

Chromosome Pv06: In the AND 277 x SEA 5 RIL population, another minor QTL associated with marker BAR3800 was found on chromosome Pv06 [86].

Chromosome Pv08: An ALS resistance QTL was found on the upper arm of chromosome Pv08, opposite to *Phg-2*. Teixeira et al. [87] found markers BM210 and BM165 to be linked to ALS resistance in Jalo EEP 558. Marker BM165 was initially reported to be on chromosome Pv05, though it maps to chromosome Pv08. Another resistance locus in AND 277 was reported linked to the RAPD marker OPH13$_{490}$ at 5.5 cM [28]. This marker was converted into a SCAR marker and named SCARH13 [88]. Although the authors initially thought the locus they have found was *Phg-1* located on chromosome Pv01, the reverse primer of marker SCARH13 mapped on chromosome Pv08 of the reference genome, while the forward primer did not map (Supplementary table S1).

Chromosome Pv09: A study of a G5686 (resistant) x Sprite (susceptible) cross revealed a locus named *Phg$_{G5686C}$* on chromosome Pv09 linked, at 12.1 cM, to marker Pv-at007 [26]. This locus was confirmed as a minor QTL explaining 1.7% of the variance linked to Marker 33 [36].

Chromosome Pv11: Bassi et al. [86] reported a major QTL conferring ALS resistance on chromosome Pv11, explaining 26.5% of the observed phenotypic variance using RIL of AND 277 x SEA 5. The marker BAR5054 that was associated with ALS resistance, was also associated with susceptibility to powdery mildew (*Erysiphe polygoni*), in AND 277 [86].

Segregation and allelism studies

In addition to the abovementioned studies reporting molecular markers linked to ALS resistance loci, numerous segregation studies have been conducted. In these studies, resistance sources were crossed to susceptible cultivars or to other resistance sources, and segregation ratios were analyzed to draw conclusions about the genetic architecture or allelism of resistance loci [27, 31, 68, 90]. These studies were often inconclusive and for several reasons did not always support the results from published genetic studies. First, ALS resistance is often quantitative and the classification of ALS responses into resistant and susceptible categories is not adequate because the score distribution of resistant and susceptible plants does not segregate into two clear groups [34, 64, 87]. Second, when

evaluations are conducted using single F_2 plants, errors can be introduced during phenotyping or because of hybridization problems. Finally, distinct interaction between the resistance loci in common bean and isolates of *P. griseola* exists; thus, the resistance loci that are observed are contingent on the isolate of *P. griseola* that is used. Taken together, the diversity of pathogen isolates and technical and statistical issues of most published allelism studies render these results difficult to interpret, thus limiting the knowledge gained from such studies.

Outlook on genetic characterization of angular leaf spot resistance

Methods to genetically characterize ALS resistance have changed substantially in recent years. For instance, publication of the reference genome of common bean [37] has allowed for the assignment of positions for most SCAR, SSR, and SNP markers and also allowed for comparison of loci obtained in different studies. For several genotypes, resistance loci have been mapped to similar positions on the genome. Although mapping studies are often complemented by allelism and segregation analyses, some allelism studies are difficult to interpret and frequently report more and different genes involved in resistance. Overall, additional studies are needed to resolve these discrepancies.

The identification of resistance genes will be a major goal for geneticists to understand the nature of defense genes and to define haplotypes for marker design to aid in breeding. In this respect, the locus best characterized to date is *Phg-5*, where expression of candidate genes has been investigated [84]. However, fine mapping has been hindered by the partial localization of the core QTL region to a pericentromeric region that spans several megabases where little recombination occurs. In contrast, the *Phg-4* locus has been mapped to a much smaller region in which 36 genes have been annotated [36]. Although whole genome sequencing data of G5686 is available, the repetitive nature of the *Phg-4* region poses a barrier to de novo assembly and to correct identification of all copies of potential resistance genes and polymorphisms. Other technologies, such as bacterial artificial chromosome (BAC) sequencing, 10x sequencing (www.10xgenomics.com), PacBio (www.pacb.com) and Oxford Nanopore (www.nanoporetech.com), are promising for overcoming the issues of short read assemblies and will allow to assemble repetitive genomic regions more accurately.

Next-generation sequencing technologies have reduced sequencing costs and allowed agricultural research scientists to perform a wide variety of applications, such as high-throughput genotyping by sequencing (GBS), whole-genome sequencing (WGS), genome-wide association studies (GWAS), and genomic selection. So far, most ALS resistance studies published have been conducted on biparental mapping populations with associated markers that often were polymorphic only in segregating populations from crosses between Andean and Mesoamerican cultivars. Genome-wide association studies will allow for finding resistance loci in a more diverse genetic background than biparental mapping, where allelic diversity is limited.

Genome-wide association studies have been used to explore the genetic basis of disease resistance, to identify new genomic regions controlling resistance, and to find molecular markers associated with resistance in common bean [91-93]. Perseguini et al. [91] using GWAS detected 17 and 11 significant marker–trait associations, on a 0.05 significance level, for ALS and anthracnose resistance loci, respectively. Significantly, associated markers were distributed on most chromosomes of the genome.

These authors reported that their results indicated a quantitative and complex inheritance pattern for the ALS and anthracnose diseases of common bean. Conversely, Nay et al. [94] conducted GWAS in a large common bean panel and tested it with a mixture of five races of *P. griseola* in the field in Darien, Colombia, and with one race (63-47) in the greenhouse. A single ALS resistance locus on chromosome Pv08 was conferring resistance in the field and the greenhouse trials. The GWAS results in this study suggested a qualitative nature of ALS resistance, and a SNP that clearly distinguished resistant from susceptible bean lines was reported [94]. More GWAS studies are underway to evaluate the pathotype-specific effect of resistance against prevalent races of *P. griseola* in Colombia and Uganda (M.M. Nay, unpublished data, 2018). These studies will give insights into the effectiveness of ALS resistance loci on different continents. Furthermore, the high-density marker data obtained using GBS or WGS will enable the selection of markers that specifically tag ALS resistance loci, unlike previously developed markers that were effective in two-parental study populations but have been ineffective when used in breeding materials.

Breeding of angular leaf spot resistant cultivars

Conventional ALS resistance breeding is based on the selection of resistant common bean lines under field conditions using natural or artificial inoculation. Characterization of ALS resistance loci has advanced in recent years, and molecular markers linked to resistance alleles in some of the most resistant donor genotypes are available. Moreover, MAS allows for the selection of resistant lines from segregating populations using genetic analysis instead of phenotypic screening (reviewed in Miklas et al. [95]). Conversely, DNA-based selection techniques, such as marker-assisted and genomic selection, have not been routinely applied in common bean breeding, which still depends heavily on traditional techniques. In the next sections, we review some of the ALS resistance breeding strategies implemented in different breeding programs in the tropics, where ALS is a recurrent and severe disease.

Breeding for angular leaf spot resistance at CIAT headquarters in Cali, Colombia

Breeding for resistance to ALS has long been a major objective of the bean program at CIAT in Cali, Colombia. Large collections of 22,832 wild and cultivated common beans have been screened in the field using local pathogen isolates, and highly resistant sources such as G10909, G10474, and G5686 have been identified [11, 25]. Pre-breeding lines of the Mesoamerican small red-seeded grain class were created by crosses and backcrosses with identified resistance sources, and ALS resistance was introgressed into elite breeding germplasm by subsequent crossing and selection under field conditions.

In the Andean bean breeding program, resistance sources AND 277 and CAL 143 of the Andean gene pool were used for introgressing ALS resistance. Recently, transfer of the ALS resistance of Mesoamerican origin into Andean bush types was attempted through MAS by crossing Andean elite lines with ALS-resistant Mesoamerican pre-breeding lines [96]. A new, highly specific marker based on the whole-genome sequence of G10474 is now available, and it has been validated to tag *Phg-2* specifically in G10474 and another ALS resistant genotype, but not in other Mesoamerican breeding lines [72]. Another project at CIAT is aimed at pyramiding five QTLs from the resistance sources AND 277, G5686, G10474, and G10909 and combining them with the well-accepted grain quality of CAL 143

and KAT B1. Single nucleotide polymorphism markers that tag ALS resistance loci were established at a commercial genotyping service provider, allowing for high-throughput screening of the progeny of crosses.

Taken together, efforts to generate ALS-resistant breeding lines at CIAT, Colombia, are ongoing due to the persistent need in Africa and other regions. Better molecular markers that are more specific, easier to use on a large scale, and tag different resistance loci are becoming available and should increase the efficiency of ALS resistance breeding. Despite the availability of molecular tools, phenotypic evaluations under field conditions will remain an important method and the final quality check for new varieties.

Breeding for angular leaf spot resistance in Brazil - Recurrent selection strategy

Brazil is the largest producer and the largest consumer of common bean in the world [5]. With the expansion of irrigated areas in Brazil, common bean has become a highly valuable crop that can be grown all year. The increase in production has also resulted in an increase in the incidence of common bean diseases, with ALS causing significant crop damage [97, 98]. As the *P. griseola* races occurring in Brazil are some of the most aggressive, it is difficult to obtain common bean lines with durable resistance [50, 99-103].

One of the methods used for breeding of durable ALS-resistant cultivars in Brazil is recurrent selection [104]. In 1998, a phenotypic recurrent selection program was initiated in the state of Minas Gerais with the goal of obtaining elite lines that accumulate important ALS resistance alleles and also exhibit high seed yield and a carioca grain type, the market class preferred by Brazilian consumers [105, 106]. The breeding program involved a circulating diallel cross of seven carioca-seeded lines and 10 sources of ALS resistance from different market classes including Andean and Mesoamerican common bean cultivars (AN 512561, AND 277, Ouro Negro, Compuesto Negro Chimaltenango, CAL 143, MAR 2, MAR 1, G5686, MA 4.137, and Jalo). In 2011, the progress of the breeding program was evaluated, and the five best lines from Cycles I to VII were evaluated in three locations in Minas Gerais (T.L.P.O. Souza, unpublished data, 2018). The mean ALS severity scores of the lines from each recurrent selection cycle ranged from 6.2 in cycle I in Lambari to 3.2 in cycle III in Patos de Minas. In addition, the interactions of lines x locations were significant for ALS severity, which suggested that the prevalent races of *P. griseola* differ among locations and/or that environmental conditions favor the development of the disease differently among locations. On average, in the three environments, the genetic progress for ALS severity estimated by the cycle of selection was −2.9% and for seed yield was 1.8%, confirming the efficiency of a recurrent selection program. The chosen strategy, therefore, appears to have been successful in accumulating alleles for ALS resistance and for high seed yield.

Furthermore, the 27 highest performing lines from different cycles were tested in different years in the final field trials for agronomic performance evaluation of elite common bean lines conducted by Universidade Federal de Lavras–EMBRAPA in Minas Gerais. The lines MAIV 18-529 and MAIV 18-524 were selected as the most promising, and they have been used as parents (ALS elite resistance sources) in elite crosses in different breeding programs in Brazil.

Breeding for angular leaf spot resistance in East Africa

Angular leaf spot is one of the most important diseases of common bean in East Africa [11], with annual yield losses in Africa up to 384,200 metric tons, as estimated by Wortmann et al. [6]. In fact, the disease has been associated with up to 50% yield losses among released bean varieties in Uganda [11, 31, 107]. Moreover, progress in breeding for ALS resistance has been slow, mainly because of the high diversity of ALS pathogen races found in East Africa [53, 56, 108, 109]. Research on ALS in East Africa has focused on the identification and genetic characterization of new sources of resistance in local landraces and released varieties [75, 108-110]. In these studies, Mexico 54 was found to be resistant to most races of *P. griseola*, and this cultivar is the most common source of ALS resistance in breeding in Uganda and East Africa [31, 66, 74-76].

Marker-assisted selection can improve breeding efficiency by facilitating introgression of resistance loci into elite cultivars, and it allows pyramiding of resistance loci for more durable resistance. In the 2000 to 2010 period, the National Agricultural Research System (NARS) common bean breeding programs in East and Central Africa had little access to MAS facilities and hence collaborated with CIAT, which had established a simple but fully functional MAS laboratory at the National Agricultural Research Laboratories (NARKL) at Kawanda in Uganda. This laboratory was and is still used for MAS projects by CIAT in collaboration with NARS for targeting disease resistance, including resistance to ALS. For example, the SCAR marker $OPE4_{709}$, which tags *Phg-2* from Mexico 54, was established to select potentially resistant progeny containing *Phg-2*. Using this marker, the prevalence of the *Phg-2* locus in advanced lines was assessed, and it was found that 60% of lines from Rwanda and 13% of lines from Uganda harbor the resistance allele. The lines from Rwanda had previously undergone selection at an ALS hotspot in Rubona, which explains the relatively high frequency of lines carrying the *Phg-2* resistance locus. Given the high diversity of *P. griseola* races found in Africa [53, 56, 108, 109], more than one resistance gene will be required to confer durable ALS resistance to a wide range of races. Ddamulira et al. [76] pyramided resistance genes from AND 277 (*Phg-1*), Mexico 54 (*Phg-2*), and G5686 (*Phg-4*, *Phg-5*, and Phg_{G5686C}) and introgressed the resistance into the susceptible cultivars Kanyebwa (local landrace) and CAL 96. The presence of the resistance loci was verified by molecular markers, and the resulting population showed reduced ALS symptom severity compared with single crosses when inoculated with race 61-63 [76]. Supported by MAS, breeding at CIAT Uganda focuses on combining ALS resistance with resistance to other stresses, and in one study, six genes for resistance to anthracnose, *Pythium*, viruses, and ALS were pyramided [111].

Angular leaf spot studies at CIAT Uganda have heavily relied on student projects, but the obtained knowledge with regard to genes, markers, and new resistant germplasm is used in systematic breeding, supporting phenotypic selection in germplasm development. However, reliance on Mexico 54 as the major source of resistance has resulted in less-than-adequate progress in the desired market classes. This might be caused by the undesired characteristics introduced through crossing the small-seeded Mesoamerican cultivar Mexico 54 with large-seeded Andean beans, which is the preferred grain type in East Africa. Identification of effective resistance sources from the Andean gene pool will reduce the linkage drag and accelerate breeding progress.

Outlook on breeding for resistance to angular leaf spot

Breeding common bean cultivars with durable resistance to *P. griseola* will continue to be a high priority in many countries of Latin America and Africa, where ALS is a recurring disease. However, durable resistance is difficult to achieve due to the extensive virulence diversity of the ALS pathogen and its capacity to produce new virulent strains. In this review, we examined the components of a durable strategy for resistance to ALS, which includes a review of the virulence diversity of the ALS pathogen, the existing situation of ALS resistance loci, and molecular markers linked to these loci for use in resistance breeding. We also consider how new technologies could facilitate and accelerate ALS resistance breeding but also disease resistance breeding in common bean in general. Different populations of the ALS pathogen have co-evolved separately with Andean and Mesoamerican common beans, such that there are distinct Andean and Mesoamerican races of the ALS pathogen. A similar pattern was observed in the common bean diseases anthracnose and rust. For rust, combining Andean and Mesoamerican resistance genes in single cultivars has resulted in resistance to all known races of the rust pathogen [112].

These results have important implications for the development and deployment of ALS resistant varieties. Even though there are three Andean and two Mesoamerican ALS resistance loci known there are no common bean cultivars grown by farmers that combine Andean and Mesoamerican resistance loci and would allow evaluating whether this combination of genes confers broad and durable resistance. The extensive virulence diversity of the ALS pathogen suggests that common bean cultivars with single genes for resistance to ALS will likely succumb to new virulent races of the ALS pathogen in the future. This has frequently been observed in common bean cultivars harboring single genes for resistance to the rust and anthracnose pathogens [113-115]. In addition, there is ample evidence that the virulence diversity of the ALS pathogen is changing. New races that overcome the resistance of all differential cultivars have been found in Brazil, Central America, and, more recently, in Africa. This situation calls for a breeding strategy based on a broad diversity of quantitative and qualitative resistance, which may confer broad-spectrum and durable resistance [116]. Breeding, however, is currently based on a few well-characterized resistance loci: the Mesoamerican Mexico 54 with *Phg-2* in Africa, the Mesoamerican Ouro Negro with *Phg-3* in Brazil, the Andean G5686 with *Phg-4*, and the Mesoamerican G10474 likely with *Phg-2* in Colombia. These single resistance genes are easy to transfer to new commercial cultivars, but they are also at risk of losing their resistance to new virulence races of the ALS pathogen. Thus, there is a need to discover new Andean and Mesoamerican ALS resistance loci to broaden the genetic base of common bean against the highly virulent ALS pathogen. There is also the need to learn about the spectrum of resistance of each of the ALS resistance genes by challenging them with a broad diversity of Andean and Mesoamerican races of the pathogen, and to combine (pyramid) various effective genes into commercial cultivars. Releasing and disseminating new superior varieties will stabilize bean productivity in vulnerable populations and will positively affect the livelihoods of the producers that depend on this crop for nutrition and income.

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Supplementary material

Table S1. **Molecular markers associated with angular leaf spot resistance in common bean**. Positions given are from the common bean reference genome version 2.1 [89]. Primers were mapped on the reference genome as paired end reads using bowtie2 with a maximum insert size of 2500bp, or if not succcessful, as single reads.

Marker linked with ALS resistance	Polymorphism type	Marker type	Chr	Prev. Chr.§	Starting position of the molecular marker	End postion of the molecular marker	Resistant parent	A/M	Susceptible parent	A/M	Reference	Primer fwd	Primer rev	Dist. (cM)	reported gene name/QTL	pathogen race used in study
BM146	Indel	SSR	1	5	6'093'429	6'093'688	Jalo EEP 558	A	Small white	M	[87]	GAGATGAGTCCTTTCCCTACCC	TGCAGACACAATTTATGAAGGC			natural inoculation
IAC126	Indel	SSR	1	5	11'665'032	11'665'194	CAL 143	A	IAC-UNA	M	[34]	TTTTTCTCGTTTTACTTTTATCTG	TACTGATTTATTTCTTCCACTTC			natural field and race 0–39
CV542014^{450}	Indel	STS	1		49'795'296	49'795'615	AND 277	A	Ouro Negro / Ruda	M	[32]	CACTTTCCACTGACGGATTTGAACC	GCACAAGGACAAGTGGTCTGG	0.7	*Phg-1*	63-23
TGA1.1^{570}	Indel	STS	1		50'022'788	50'023'315	AND 277	A	Ouro Negro / Ruda	M	[32]	CAGAGGATGCTTCTCACGGT	AAGCCATGGATCCCATTTG	1.3	*Phg-1*	63-23
PVBR106	Indel	SSR	3		24'944'966	24'945'153	CAL 143	A	IAC-UNA	M	[34]	CAACAAACAAGGCTGAAAAACA	AAAAAGAGAGGAGAGAGAAGAGAG		ALS2.1^{UC}	natural field and race 0–39
IAC18b	Indel	SSR	3	2	29'076'205	29'076'459	CAL 143	A	IAC-UNA	M	[34]	AACTTGTTGCCAGAATGACT	CACAACTCCCCTTTATGCTA			natural field and race 0–39
IAC134	Indel	SSR	3	2	30'149'147	30'149'379	CAL 143	A	IAC-UNA	M	[34]	TGGAAACAGCAGAGCGATACT	TTGGTCCCTAAATTCTTCTCAT			natural field and race 0–39
FJ19	Indel	SSR	3		35'216'517	35'216'682	CAL 143	A	IAC-UNA	M	[34]	ATGTTAGTGCCTTATTTCTCT	AAGGTAGGGTTGGGATTGT			natural field and race 0–39
PVBR21	Indel	SSR	3		39'089'564	39'089'806	CAL 143	A	IAC-UNA	M	[34]	GAAGAACCGCAAGTAGAGAAGT	TAACATCAGACGCCGACGA			natural field and race 0–39
BM159	Indel	SSR	3		39'802'104	39'802'278	CAL 143	A	IAC-UNA	M	[34]	GGTGCTGTTGCTGCTGTTAT	GGGAGATGTGGTAAGATAATGAAA		ALS3.1^{UC}	natural field and race 0–39
Pv-ctt001	Indel	SSR	4		458'859	458'998	G5686	A	Sprite	A	[26]	GAGGGTGTTTCACTATTGTCACTGC	TTCATGGATGGTGGAGGAACAG	17	*Phg*$_{G5686B}$	31-0
g2303	presence/absence	STS	4		3'634'313	3'634'313	Ouro Negro	M	AND 277	A	[33]	GGGGCGGAATCAGGTTCACCA	GGTTTAGGACAACTAATGAGAGTGATGTACCGTGT	0.0	*Phg-ON*	63-39
RGA6*		RGA	4		8'166'727	8'167'194	G19833	A	DOR364	M	[85]	GGIGGIGTIGGIAAIACIAC	GAAGCAIGCGATGTCIAGGAA			
Pv-gaat001	Indel	SSR	4		9'432'363	9'432'503	CAL 142	A	IAC-UNA	M	[34]	AAGGATGGGTTCCGTGCTTG	CACGGTACACGAAACCATGCTATC		ALS4.2	natural field and race 0–39
Pv-gaat001	Indel	SCAR	4		9'432'363	9'432'503	G10909	M	Sprite	A	[27]	AAGGATGGGTTCCGTGCTTG	CACGGTACACGAAACCATGCTATC	13	*Phg*$_{G10909A}$	63-63
BM140	Indel	SSR	4		11'166'015	11'166'193	CAL 143	A	IAC-UNA	M	[34]	TGCACAACACACATTTAGTGAC	CCTACCAAGATTGATTTATGGG			natural field and race 0–39
RGA14*		RGA	4		43'983'072	43'982'830	G19833	A	DOR364	M	[85]					
Marker63	SNP	Tms	4		45'670'275		G5686	A	Sprite	A	[36]	GAAATGAGGCTAACAAGGAGGTT[A/G]	AGCCTGCAAGATTTTCAGATATCCT		ALS4.1$^{GS, UC}$ (ALS4.1$^{EC, DG, GS, UC}$)	31-0
4M437	SNP	HRM	4		45'945'457	45'945'749	G5686	A	Sprite	A	[36]	CACAGAACCAACAGTTTCTAAGC	GGGTGCAACCAAGAGTTTT		ALS4.1$^{GS, UC}$ (ALS4.1$^{EC, DG, GS, UC}$)	31-0
Marker50	SNP	Tms	4		45'950'948		G5686	A	Sprite	A	[36]	CCAGGTAAATAGGGTAATGAAGTTG[G/T]	ACTCATGAGATTGTGTATGGCCAACC		ALS4.1$^{GS, UC}$ (ALS4.1$^{EC, DG, GS, UC}$)	31-0
MAS_ALS4b	SNP	LGC genomics KASP	4		45'974'437		G5686	A			[36]				ALS4.1$^{GS, UC}$	31-0
4M439	SNP	HRM	4		46'152'467	46'152'748	G5686	A	Sprite	A	[36]	TGTGGATCTCCACCTAGCAG	CTGCTCTTAGAACTTTGGAGATTC		ALS4.1$^{GS, UC}$ (ALS4.1$^{EC, DG, GS, UC}$)	31-0
Pv-ag004	Indel	SSR	4		46'240'418	46'240'670	G5686	A	Sprite	A	[26]	TTGATGACGTGGATGCATTGC	AAAGGGCTAGGGAGAGTAAGTTGG	0.0	*Phg*$_{G5686A}$	31-0
BMd9	Indel	SSR	4		46'268'404	46'268'519	CAL 143	A	IAC-UNA	M	[34]	TATGACACCACTGGCCATACA	CACTGCGACATGAGAGAAAGA		ALS4.1	natural field and race 0–39
Pv-atgc002	Indel	SSR	4		46'268'407	46'268'526	CAL 143	A	IAC-UNA	M	[34]	AGCTTTCACACTATGACACCACTGG	TGCGACATGAGAGAAAGACACGG		ALS4.1$^{GS, UC}$	natural field and race 0–39
IAC52	Indel	SSR	4		47'666'230	47'666'422	CAL 143	A	IAC-UNA	M	[34]	TGCATGTATGTAGGCGGTTTA	GTGGCTTTTGCTTTTGTAGTCA		ALS4.1$^{GS, UC}$	natural field and race 0–39
Pv-atct001	Indel	SSR	4?		Chr02:13'350'370	Chr04:46'233'047	ESAL 550	A	Carioca MG	M	[117]	CAATTAAAACTCAACCAACCCAAATA	TTTCCCGCCTAGAATATGTGAGA	7.6		natural inoculation
OPM02$_{460C}$		RAPD	4				Ouro Negro	M	US Pinto et al. 111	M	[30]	ACAACGCCTC		5.3		63-39
OPAA19$_{600C}$		RAPD	4				Ouro Negro	M	US Pinto et al. 111	M	[30]	TGAGGCGTGT		10		63-39
OBA16$_{669}$ / OBA16$_{583}$		RAPD	4				Ouro Negro	M	Ruda	M	[83]			10.4		31-55 , 63-31
OAD09$_{3210}$		RAPD	4				Ouro Negro	M	Ruda	M	[83]			13.9		31-55 , 63-31
SCARM02	Indel	SCAR	4		NM	NM	Ouro Negro	M	TO	M	[88]	CAACGCCTCATTAAATTGGA	CGCCTCTAAACGGGAGAAAC	5.3	*Phg-ON*	
SCARBA16	Indel	SCAR	4		NM	NM	Ouro Negro	M	TO	M	[88]	TTCCACGTCTATTTTGCATCA	CACGCATCACGCAGAACT	7.1	*Phg-ON*	
SCARAA19	Indel	SCAR	4		NM	NM	Ouro Negro	M	TO	M	[88]	TGAGGCGTGTCAATGGATATAA	GAGGCGTGTTGATAATTCTGG	10.1	*Phg-ON*	
PVBR92	Indel	STS	4		NM	NM	CAL 143	A	IAC-UNA	M	[34]	AATATTGGGGGCAACATTCA	GCCATCAACAGAACCGATCA		ALS4.2$^{GS, UC}$	natural field and race 0–39
BMd53	Indel	SSR	5		7'164'379	7'164'467	CAL 143	A	IAC-UNA	M	[34]	TGCTGACCAAGGAAATTCAG	GGAGGAGGCTTAAGCACAAA			natural field and race 0–39
Pv-br124	Indel	SSR	5		28'211'062	28'211'198	CAL 143	A	IAC-UNA	M	[34]	CCTAAAAACCAGGTGCGAGA	TGGGAAACCTAGCCAAACAC		ALS5.1^{UC}	natural field and race 0–39
BM175	Indel	SSR	5		37'077'612	37'077'765	CAL 143	A	IAC-UNA	M	[34]	CAACAGTTAAAGGTCGTCAAATT	CCACTCTTAGCATCAACTGGA			natural field and race 0–39
IAC159	Indel	SSR	5		37'132'330	37'132'620	AND277	A	SEA 5	M	[86]	AAAACCCAACCAAACAT	ACGCCAATAAATCTAAAG			1-21
Pv-at006	Indel	SSR	5		38'503'392	38'503'503	CAL 143	A	IAC-UNA	M	[34]	CCGTTGCCTGTATTTCCCCAT	CGTGTGAAGTCATCTGGAGTGGTC		ALS5.2^{UC}	natural field and race 0–39
Marker31	SNP	Tms	5		38'663'266		G5686	A	Sprite	A	[36]	TTCAACACCAAAGACATTCAAACTA[A/G]	GGTGTTCCTCATTTTCTGCTTCCTATT		ALS5.1^{UC,GS}	31-0
BAR3800	SNP	VeraCode (Illumina)	6		25'992'043		AND277	A	SEA 5	M	[86]	ATCCGAGTCCATGTCAAGCTGAGTTGA[T/C]CCCAGTCCATTTCGTGTTGAATGAG				1-21
FJ05	Indel	SSR	7	5	32'067'769	32'067'950	CAL 143	A	IAC-UNA	M	[34]	AAGAAACAGAAACAATAAAAAC	TTTCCATTTATTTTCAGTCACA			natural field and race 0–39
BM165	Indel	SSR	8	5	7'944'848	7'945'005	Jalo EEP 558	A	Small white	M	[87]	TCAAATCCCACACATGATCG	TTCTTTCATTCATATTATTCCGTTCA			natural inoculation
BM210	Indel	SSR	8		39'349'553	39'349'702	Jalo EEP 558	A	Small white	M	[87]	ACCACTGCAATCCTCATCTTTG	CCCTCATCCTCCATTCTTATCG			natural inoculation
SCARH13	presence/absence	SCAR	8	1	NM	45'869'256	AND 277	A	Vermelho		[88]	GACGCCACACCCATTATGTT	GCCACACAGATGGAGCTTTA	5.6	*Phg-1*	
PF5	Indel	SCAR	8		61'251'610	NM	G10474	M	Sprite	A	[71]	CTTGTTCTGAGTCATTTACCTTGC	GAATTCACAGTCCAAACTACTCTAATC	5		63-63
g796	Indel	SCAR	8		61'514'592	61'514'800	Mexico 54	M	Amy	A	[66]	GAGAAACTACGGGCTGTTTTACCC	AGTTAAGACCGTTCTGAAGCTTC	3	*Phg-2*	63-39
PF9$_{260}$	Indel	SCAR	8		61'860'933	NM	G10909	M	Sprite	A	[27]	ATTCAACTTTTCTACATTTTCTAATGG	AACATATGATTTTGATTTAGTTTTTCC	7.4	*Phg*$_{G10909B}$	63-63
SCAROPN02	presence/absence	SCAR	8		61'994'091	61'994'987	Mexico 54	M	Ruda	M	[27]	ACCAGGGGCAACATACTATG	CTGTTCATAATGCCCCTGGT			
ALS_Chr08_CT_57798588	SNP	KASP	8		61'230'352						[72]					
PF13$_{310}$	Indel	SCAR	8		NM	61'251'599	G10909	M	Sprite	A	[27]	GAATTCACAGTGCAAACTACTC	TTAACTTCATACTTGTTCTGAGTCA	4.9	*Phg*$_{G10909B}$	63-63
OPN02$_{890}$		RAPD	8				Mexico 54	M	Ruda	M	[61]	ACCAGGGGCA		5.9		63-19
OPN02$_{890}$		RAPD	8				Cornell 49-242	M	Ruda	M	[67]	ACCAGGGGCA		3.2	*Phg-2*	63-19
OPAC14$_{2400}$		RAPD	8				Mexico 54	M	Ruda	M	[61]	GTCGGTTGTC		6.6		63-19
OPE04$_{709}$		RAPD	8				G10909	M	Sprite	A	[27]	GTGACATGCC		9.9	*Phg*$_{G10909B}$	63-63
OPE04$_{650}$		RAPD	8				Mexico 54	M	Ruda	M	[61]	GTGACATGCC		11.8		63-19
OPE04$_{650}$		RAPD	8				Cornell 49-242	M	Ruda	M	[67]	GTGACATGCC		12.5	*Phg-2*	31-17
OPE04$_{500}$		RAPD	8				MAR-2	M	Ruda	M	[69]	GTGACATGCC		5.8		63-39
OPAA07$_{950}$		RAPD	8				BAT 332	M	Ruda	M	[70]	CTACGCTCAC		5.1		61-41
OPAO12$_{950}$		RAPD	8				BAT 332	M	Ruda	M	[70]	TCCCGGTCTC		5.83		61-41
Pv-at007	Indel	SSR	9		17'245'351	17'245'527	G5686	A	Sprite	A	[26]	AGTTAAATTATACGAGGTTAGCCTAAATC	CATTCCCTTCACACATTCACCG	12.1	*Phg*$_{G5686C}$	31-0
Marker33	SNP	Tms	9		17'769'871	17'769'918	G5686	A	Sprite	A	[36]	CCACAGTCCCATTTCAGTCAG[A/G]	GTTTCTAGTGGTGAGTTTGTTGTTGTCA		ALS9.1^{GS}	31-0
RGA9*		RGA	10		3'753'994	3'753'749	G19833	A	DOR364	M	[85]	GAIGTNTTYTTITCITTYAGIGG	IGGGTCIACGTCGTAGAAIACIGG			
RGA12*		RGA	10		4'109'428	4'109'667	G19833	A	DOR364	M	[85]	GAIGTNTTYTTITCITTYAGIGG	IGGGTCIACGTCGTAGAAIACIGG			
IAC137	Indel	SSR	10		4'855'097	4'855'249	CAL 143	A	IAC-UNA	M	[34]	GCAGCAGCAAATACAAC	CCCCTAAACAATACAGC		ALS10.1^{DG,UC}	natural field and race 0–39
PvM127	Indel	SSR	10		6'210'048	6'210'194	CAL 143	A	IAC-UNA	M	[35]	AACTTTCTTTGACCCTCTC	GCTTTGTCTTGTTCTTCCA		ALS10.1^{DG,UC}	natural field and race 0–39
RGA7*		RGA	10		8'602'847	8'603'326	G19833	A	DOR364	M	[85]	GGIGGIGTIGGIAAIACIAC	GAAGCAIGCGATGTCIAGGAA			
BAR5771	SNP	VeraCode (Illumina)	10		10'742'130		AND277	A	SEA 5	M	[86]	GTAAAGCTCTGGAAGTAGTAGCGGA[G/A]CCATCGAGTTGTGAATACCACCGCCAAA				1-21
GATS11b	Indel	SSR	10		33'501'616	33'501'699	CAL 143	A	IAC-UNA	M	[34]	CCCACACATTGGTGCTAGTG	AGCGCAATGCTACTCGAAAT	6	ALS10.1^{DG,UC}	natural field and race 0–39
Marker17	SNP	Tms	10		39'108'247		G5686	A	Sprite	A	[36]	AGCAGCAGAATTCTGCAATC[G/T]	GGTTTTCTGGTTTTGGGTGGTAAATG		ALS10.1^{DG,UC,GS}	31-0
RGA15*		RGA	10		41'941'030	41'941'272	G19833	A	DOR364	M	[85]	GAIGTNTTYTTITCITTYAGIGG	IGGGTCIACGTCGTAGAAIACIGG			
ATA220	Indel	SSR	10				CAL 143	A	IAC-UNA	M	[35]			0.6	ALS10.1^{DG,UC}	natural field and race 0–39
BAR5054	SNP	VeraCode (Illumina)	11		48'700'233		AND 277	A	SEA 5	M	[86]	ATTGCATAGCTGATTGTTGATTCGCACACATTCTT				1-21
OPH13$_{490}$		RAPD	11				AND 277	A	Ruda	M	[28]	GACGCCACAC		5.5	*Phg-1*	63-23

§For markers that were reported on a different chromosome in the original publications, the originally reported chromosome is given in addition.*RGA Markers were amplified with degenerate primers, and their position determined by blasting the protein sequences given in Lopez et al. [85] to the reference genome. Abbreviations: Andean or Mesoamerican (A/M), Chromosme (Chr), Disease resistance gene analogs (RGA), Genetic distance (Dist.), Kompetitive allele specific PCR (KASP), Not Mapped (NM), Previous (Prev), Random Amplification of polymorphic DNA (RAPD), Single nucleotide polymorphism (SNP), Simple Sequence Repeat (SSR), Melting temperature shift (Tms).

Chapter 3:

Haplotypes at the *Phg-2* locus are determining pathotype-specificity of angular leaf spot resistance in common bean

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Abstract

Angular leaf spot (ALS) is one of the most devastating diseases of common bean (*Phaseolus vulgaris* L.) and causes serious yield losses worldwide. ALS resistance is reportedly pathotype-specific, but little is known about the efficacy of resistance loci against different pathotypes.

Here, we report on ALS resistance evaluations of 316 bean lines under greenhouse and field conditions at multiple sites in Colombia and Uganda. Surprisingly, genome-wide association studies revealed only two of the five previously described resistance loci to be significantly associated with ALS resistance. *Phg-2* on chromosome eight was crucial for ALS resistance in all trials, while the resistance locus *Phg-4* on chromosome 4 was effective against one particular pathotype. Further dissection of *Phg-2* uncovered an unprecedented diversity of functional haplotypes for a resistance locus in common bean. DNA sequence-based clustering identified eleven haplotype groups at *Phg-2*. One haplotype group conferred broad-spectrum ALS resistance, six showed pathotype-specific effects, and the remaining seven did not exhibit clear resistance patterns.

Our research highlights the importance of ALS pathotype-specificity for durable resistance management strategies in common bean. Molecular markers co-segregating with resistance loci and haplotypes will increase breeding efficiency for ALS resistance and allow to react faster to future changes in pathogen pressure and composition.

Introduction

Plant diseases can cause substantial loss of crop yields with detrimental effects on food security [1, 2]. In Latin America and Africa, for example, common bean (*Phaseolus vulgaris* L.) is one of the most important crops and particularly valued for its protein and micronutrient content. However, common bean production is frequently reduced by pathogen attacks with angular leaf spot (ALS), caused by *Pseudocercospora griseola* (Sacc.) Crous and Braun [3], being one of the most devastating common bean diseases in the tropics and subtropics. ALS has been reported to cause yield losses of up to 80% [4-9]. In the tropics and subtropics, common beans are mostly cultivated by smallholder farmers with limited possibilities to protect their crops from diseases or adverse climatic conditions and, therefore, depend on resistant common bean varieties to maintain stable yields [10].

Common bean germplasm can be divided into two gene pools, the Andean and the Mesoamerican gene pool [11, 12]. The latter, genetically more diverse Mesoamerican gene pool has been reported to contain more and stronger ALS resistance sources [13]. Breeding for ALS resistance is challenged by the high genetic diversity of the pathogen and the recurrent appearance of new *P. griseola* pathotypes [14-16]. To categorize pathotypes, they are tested for their ability to infect six Andean and six Mesoamerican common bean lines with distinct resistance patterns (also referred to as differentials), in order to determine their race [13]. The ALS pathogen co-evolved within the two common bean gene pools into Andean races, only causing disease on Andean beans, and Mesoamerican races, showing a higher specificity for Mesoamerican beans but also attacking beans of the Andean gene pool [11, 12, 16-18]. Resistance in common bean has been reported to be pathotype-specific with large differences of the effectiveness in different locations and continents [16, 19-21].

Previous ALS resistance studies defined five repeatedly characterized resistance loci, in addition to several minor resistance sources (reviewed in [13]): *Phg-1* was found in the line AND 277, closely linked to the anthracnosis resistance locus *Co-1*^{4} at the lower end of chromosome (Chr) 1 [22, 23]. *Phg-2* was found on Chr 8 in the Mesoamerican lines Mexico 54, with potential resistant alleles in Cornell 49–424, BAT 332, MAR 2, G10474, and G10909 [24-29]. The *Phg-3* locus was found in Ouro Negro on the lower arm of Chr 4 and *Phg-4* in G5686 on the upper arm [30-32]. *Phg-5*was found in the lines CAL 143 and G5686 on Chr 10 [32, 33]. Besides these well-characterized major resistance loci, indications for quantitative resistance were reported [32-35].

All the above-mentioned studies were conducted in bi-parental mapping populations, limiting the allelic diversity in the population to the two parental alleles. The establishment of such mapping populations is laborious, and the resistance loci found in such experiments may only be effective in the original background due to epistatic effects [36, 37]. In addition, bi-parental mapping studies were often tested for ALS resistance with a single pathotype or at a single field location, even though the pathotype-specific resistance reaction of *P. griseola* is well described [16, 20, 23, 29-31, 33, 35, 38]. Hence, little is known about the range of effectiveness and the interaction of different ALS resistance loci in common bean in different environments with possibly different pathotypes. Furthermore, all previous mapping studies were conducted with Latin American pathotypes, and it is unknown whether the same resistance loci are effective against pathotypes from Africa.

Genome-wide association studies (GWAS) in panels specifically assembled to contain breeding germplasm with phenotypic variability for the trait of interest can overcome the above-mentioned limitations of bi-parental mapping populations. This type of analysis became possible through technological advancements, particularly in next generation sequencing, which allows to genotype hundreds of individuals at a sufficiently high marker density to cover the linkage disequilibrium blocks and to find trait-specific single nucleotide polymorphisms (SNPs) for breeding. By testing a diversity panel with different pathotypes, GWAS enables the identification of pathotype-specific resistance loci as has been recently demonstrated for anthracnose [39].

The main objective of this study was to gain a broader understanding of ALS resistance sources, the resistance loci they contain, and their effectiveness against different pathotypes on two continents. Specifically, we aimed at i) assembling a panel consisting of the currently available ALS resistance sources, ii) evaluating its resistance against multiple ALS pathotypes under greenhouse and field conditions, and iii) identifying pathotype- and field location-specific resistance loci and haplotypes through genotyping by sequencing (GBS) and GWAS.

Materials and methods

Plant material

An association mapping panel of 316 common bean lines, named extended BALSIT (extBALSIT), was used for ALS resistance evaluations and GWAS. ExtBALSIT included the Bean ALS International Trial (BALSIT) panel consisting of 55 lines, complemented with previously characterized resistance sources [22-33], CIAT breeding material with phenotypic variability for ALS response and susceptible checks. The panel included 124 large-seeded Andean beans, 129 small-seeded Mesoamerican, and 63 lines from inter-gene pool crosses. The 316 common bean lines of the extBALSIT panel were multiplied, out of which 264 lines received phytosanitary certificates and were shipped from Colombia to Uganda for ALS-resistance evaluation.

Evaluation of angular leaf spot disease resistance

The extBALSIT panel was evaluated for ALS resistance in the greenhouse with single-spore *P. griseola*isolates and in the field with mixes of isolates. Highly pathogenic Mesoamerican and Andean races were chosen for the greenhouse experiments. Isolates belonging to races 63–63, 63–47, 30–0, and 13–63 were used in Colombia and race 61–63 in Uganda. In the field, inoculations were conducted with pathogen isolates previously collected at the respective field sites in Colombia and different districts in Uganda (Supplementary table 1) Disease severity was evaluated with the CIAT standard scale ranging from 1 (no disease symptoms) to 9 (very severe disease symptoms and defoliation) [40].

Greenhouse experiments were conducted at CIAT headquarters in Colombia (Cali) and at CIAT in Uganda (Kawanda). Three and five seeds of each common bean line were planted per pot under well-watered conditions in Colombia and Uganda, respectively. In Colombia, primary leaves were treated with Elosal (Bayer Crop Science, Monheim am Rhein, Germany) eight days after sowing, to prevent powdery mildew infections, and urea

was added before inoculations. For each pathotype, two replicates in time were screened with each replicate containing one pot per line of the extBALSIT panel. Pathogen isolates were grown in V_8 medium [41] for 8–20 days before inoculation, depending on growth rate of the isolate. Inoculum was prepared according to the CIAT manual [41] and spray-inoculated on trifoliate leaves of 17-day-old plants in Colombia, and 21-day-old plants in Uganda. After inoculation, plants were transferred to a humidity chamber for four days in Colombia, while in Uganda, they were covered with a plastic bag for three days to increase humidity. Ten days after inoculation, plant disease scores were evaluated four times within a week, usually on days 10, 12, 14, and 17. Because of the slow disease progression in Uganda, an additional evaluation was conducted 21 days after inoculation.

Field experiments were conducted during the rainy season in October 2016 and 2017 in Darien (N3 53'31'' W76 31'0,'' 1,491 m a.s.l.) and Quilichao (N3 04'22" W76 29'55," 991 m a.s.l.), Colombia, and in May 2018 in Kawanda (N0 24'11" E32 31'54," 1,178 m a.s.l.), Uganda. Common bean lines were evaluated as single rows in Colombia and in a randomized complete block design with two replicates in Uganda. The rows measured 2.5–3 m in Colombia and 5 m in Uganda, the distance between rows measured 0.6 m, and seeds were sown with a density of 10 seeds/m. Susceptible and resistant checks were added every eight rows, and a border of susceptible checks was planted to favor spread of the disease. Plants were inoculated three times in a weekly interval using a backpack sprayer, starting approximately 20 days after planting when the third trifoliate leaf of most plants was fully extended (stage V4, according to van Schoonhoven and Pastor-Corrales [40]). ALS symptoms on leaves were also evaluated three times in a weekly interval and started at the appearance of the first disease symptoms approximately 40 days after inoculation. Pods were evaluated at the mid-pod fill stage, approximately 3 weeks after the last foliar evaluation (exact dates are given in Supplementary table 2). Phenotypic data of the extBALSIT panel is available on dataverse.org (https://doi.org/10.7910/DVN/U2BAWN).

Inoculum was prepared according to [41], as a mixture of five, six, and five single-spore pathogen isolates in Darien, Quilichao, and Kawanda, respectively (Supplementary table 1). The isolates in Uganda did not sporulate well and a precise ad justment of the spore concentration was not possible. Therefore, fungal mycelium of 70 petri dishes was scraped off and diluted in water for the first inoculation and 35 petri dishes for the subsequent inoculations.

DNA extraction and genotyping

For genotyping, three emerging trifoliate leaves were sampled and used for DNA extraction following a urea–phenol–chloroform–isoamylalcohol protocol reported by [42]. DNA quality was checked by agarose gel electrophoresis and quantified by absorption of fluoresce using PicoGreen to stain double stranded DNA (Molecular Probes Inc., Eugene OR, USA). The common bean lines of the extBALSIT panel were subjected to GBS according to [43] with the following modifications: adaptor concentrations were 6 ng/µl, digestion per reaction was conducted with 0.5 µl restriction enzyme ApeKI (50 U/µl, New England Biolabs [NEB], Ipswich MA, USA), ligation with 0.5 µl ligase (20 U/µl, Promega, Madison WI, USA) and 3 µl buffer per sample, filled up with ddH_2O to reach the target reaction volume. After adapter ligation, the 96 samples were pooled and cleaned with a PCR Clean-Up System (Promega), according to the manufacturer's protocol. For each pool, PCR was conducted in duplicate and merged afterwards. Each PCR reaction with a total volume of 50 µl contained 1x buffer (10 mM Tris-HCl pH 8.8, 50 mM KCl, 0.8% [v/v]

Nonidet P40 [Fermentas, Waltham MA, USA]), 2 mM $MgCl_2$, 0.1% bovine serum albumin, 1% polyvinylpyrrolidone, 0.016 μM of each primer, 0.4 mM dNTP, 0.3 μl TAQ polymerase (Sigma-Aldrich, St. Louis MN, USA), and 2 μl DNA template. Primers used for amplification were the following: forward PCR_Primer1_Short: AATGATACGGCGA-CCACCGAGATCTACACTCTTTCCCTACACGACGC and reverse PCR_Primer2.1.i7: AAGCAGAAGACGGCATACGAGATGTCGATTGTGACTGGAGTTCAGATGTGTG. Each library containing 96 individually barcoded genotypes was sequenced by 150 bp single end sequencing on a single lane of the Illumina HiSeq Instrument (Illumina, San Diego CA, USA) at Hudson Alpha sequencing facility (Huntsville AL, USA). For SNP calling, the NGSEP pipeline [44] was used with the following quality criteria: a minimum quality score of Q40, scores in at least 220 of the 316 common bean lines, a minor allele frequency exceeding 5%, and a heterozygosity rate below 6%. Subsequently, heterozygous data points were removed. Genomic positions of SNPs and candidate genes were inferred according to the v2.1 of the *P. vulgaris* reference genome [45]. Genotypic information of the extBALSIT panel is available on dataverse.org (https://doi.org/10.7910/DVN/U2BAWN).

Genome-wide association studies

Genotype to phenotype associations were identified with TASSEL 5 [46]. For greenhouse and field trials, mean ALS scores from the last evaluation of the trial were used. A mixed linear model was implemented using principal component analysis (PCA) with the first two principal components to correct for population structure and the K matrix to correct for kinship [46]. Within TASSEL, the kinship was calculated using the centered identity-by-state (IBS) method, P3D was implemented for variance component analysis, and no compression was used [47, 48]. The significance threshold was adjusted with the Bonferroni correction. TASSEL output and phenotypic data were analyzed and plotted using RStudio (version 3.4.4) with the packages qqman, ggplot2, reshape2, and psych [49].

Haplotype analysis at the *Phg-2* locus

In order to group the haplotypes at the *Phg-2* locus on Chr 8, SNPs located in the interval of significant associations (i.e., from position 61,150,549–62,934,224 bp in the reference genome sequence) were clustered using a hierarchical clustering method implemented in R. The 276 common bean lines with less than 50% missing SNP data in the interval were retained for analysis. The genotype matrix was translated to numeric values, Euclidian distance between the common bean lines calculated and hierarchical clustering according to the Ward.D2 method was performed [50, 51]. The resulting dendrogram was cut to group the haplotypes into eleven groups. The haplotype groups were named Andean or Mesoamerican, according to the gene pool of the lines from which the haplotypes originated. To evaluate the effect of the haplotypes, the disease scores of each haplotype for each experiment were plotted in R.

Results

Angular leaf spot resistance is highly location- and pathotype-specific

Evaluation of the extBALSIT panel for ALS resistance revealed trial-specific frequency distributions of ALSscores (Figure 1, Supplementary figure 1). Differences were observed between continents, locations, greenhouse and field experiments, and different pathotypes. For most trials, a continuous distribution of disease scores was found, only in the greenhouse experiment with pathotype COL 30–0, the histogram clearly differentiated resistant and susceptible lines, indicating major gene resistance. Twenty-seven lines were found resistant (ALS score ≤3 on a 1 to 9 scale) in all 6 trials conducted in Colombia, 43 were resistant in the 2 trials conducted in Uganda, and 2 (AAB 8–2, G6727) were resistant in all experiments. The differences between the continents were also notable: of the 46 most resistant lines in Colombia (average ALS leaf score over all experiments ≤3), only 15 had an average score of ≤3 against the Ugandan pathotypes tested.

Out of the 55 pairwise correlations between phenotypic data of the trials, 43 (78%) were significant (Pearson correlation, $P < 0.05$), ranging from 0.12 to 0.73 (Supplementary figure 2). Highest correlations were observed between the replicates of the field experiment in Kawanda, Uganda, and the comparison of field data between years in Darien and Quilichao, Colombia (Supplementary table 3).

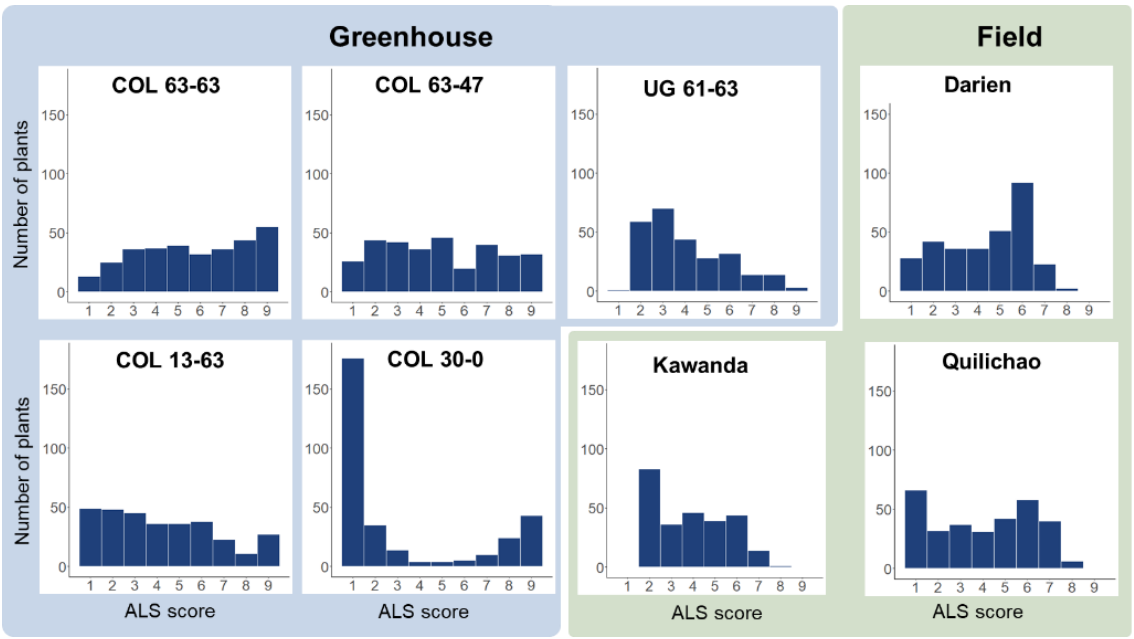

Figure 1: Frequency distributions of disease scores for angular leaf spot (ALS), evaluated in greenhouse (blue) and field trials (green) using the extBALSIT panel containing 316 common bean lines. ALS was scored on a scale from 1 to 9, where 1 is resistant and 9 is highly susceptible. The greenhouse trials were conducted with five different pathotypes, determined by their origin (COL and UG) and race (63–63, 63–47, 61–63, 13–63, and 30–0). Field trials in Colombia (Darien and Quilichao) and Uganda (Kawanda) were inoculated with mixtures of pathotypes previously collected at the corresponding sites. For Darien and Quilichao, the average ALS score from both evaluation years is shown.

Genome-wide association studies confirm ALS resistance loci on chromosomes 4 and 8.

Genotyping by sequencing of the extBALSIT panel revealed 22,765 high-quality SNPs distributed over the eleven choromosomes of common bean (Supplementary figure 3). The population structure of the extBALSIT panel was analyzed with PCA, on the basis of the SNP marker data (Supplementary figure 4). The first PC explained 45% of the genotypic variance and clearly distinguished Andean and Mesoamerican lines, with lines that originated from inter-gene pool crosses clustering between them. The second PC explained 4% and distinguished lines that originated from a cross between G10474 and G5687 (referred to as RAI lines) from the remaining inter-gene pool crosses. The second PC further separated the Mesoamerican lines G10613, G10474, G10909, G18970, G855, Mexico 54, G1805, Flor de Mayo, MAR 2, and G5653. The first six of these accessions were collected in Guatemala or neighboring Oaxaca and likely belong to the highly ALS-resistant subpopulation previously characterized in Guatemala [52-55].

Genotype to phenotype associations were investigated by GWAS. In all but one trial, foliar ALS resistance was significantly associated with a region on Chr 8 (Figure 2). For the field trial in Uganda (Kawanda), a peak is clearly visible in the Manhattan plot, but it is not passing the stringent Bonferroni threshold. Manhattan plots indicate the same resistance locus on Chr 8 to be effective in Colombia as well as in Uganda. The interval where significant associations were found in this study on Chr 8 coincides with the genomic region where molecular markers linked to the *Phg-2* resistance locus in the common bean line Mexico 54 and G10474 were found [24, 56, 57], hence, it will be referred to as the *Phg-2* locus. GWAS analyses of ALS symptoms on pods at one of the field locations in Colombia (Darien) resulted in the same resistance locus on Chr 8. Pod evaluations at the other field locations in Colombia (Quilichao) and Uganda (Kawanda), where phenotypic variability was low, did not result in significant associations to markers in the GWAS analysis (Supplementary figure 5). In addition to the predominant signal on Chr 8, another resistance locus on Chr 4 was effective against the pathotype COL 30–0. This resistance locus coincided with the mapping interval of the *Phg-4* locus [32].

Over all experiments, significantly associated SNPs were found in the interval spanning 61,150,549–62,934,224 bp (total length of 1,784 kbp) on Chr 8 and 46,703,147–46,934,061 bp (total length of 231 kbp) on Chr 4. In the interval on Chr 8, 265 annotated genes were identified, of which two (Phvul.008G284500, Phvul.008G285300) were NB-ARC domain-containing disease resistance genes (PF00931), another two (Phvul.008G267600, Phvul.008G267700) were of the TIR-NBS-LRR class (PF13676, PF01582), and 20 were containing leucine-rich repeats. On Chr 4, 28 annotated genes were found in the interval, but no putative resistance genes were among them. Significant SNPs on Chr 8 explained highest percentages of phenotypic variance, between 8.6–31.4%, in line with the very dominant role of this resistance locus seen in these experiments. Markers associated with the resistance locus on Chr 4 explained 9.3–11.4% of the variance.

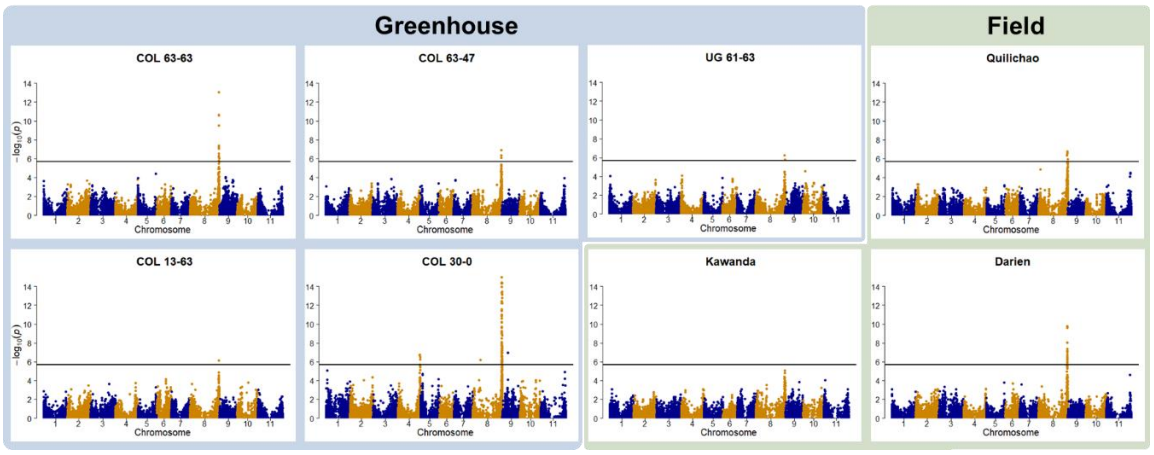

Figure 2: Manhattan plots of the genome-wide association studies (GWAS) for angular leaf spot (ALS) resistance in the extBALSIT panel. The greenhouse trials were conducted with five different pathotypes, determined by their origin (COL and UG) and race (63–63, 63–47, 61–63, 13–63, and 30–0). Field trials in Colombia (Darien and Quilichao) and Uganda (Kawanda) were inoculated with mixtures of pathotypes, previously collected at the corresponding sites. On the x-axis, the genomic position of the markers is given. On the y-axis, the negative logarithm to the base 10 of the *P*-value, representing the significance value, is given. In order to correct for multiple testing, the significance threshold was adjusted through the Bonferroni method, and the new significance threshold is depicted by the black horizontal line.

Haplotypes of the resistance locus on chromosome 8 explain ALS pathotype-specificity

Haplotypes at the *Phg-2* locus, identified through cluster analysis of the SNP data in the *Phg-2* region, were categorized into eleven groups (M1 to M5, M/A, A1 to A5, figure 3) and associated with trial-specific ALS resistance scores (Figure 4, Supplementary figure 6). The haplotype groups M1 to M5, originating from the Mesoamerican gene pool, were resistant against the pathotype COL 30–0, as indicated by its race code. Common bean lines from the Mesoamerican haplotype group M1 were resistant in nearly all experiments but showed intermediate resistance in the trial with the Ugandan pathotype UG 61–63. Lines from the haplotype groups M2 and M3 were resistant against COL 14–63, UG 61–63, and the pathotypes present in the field in Quilichao and Kawanda but were susceptible to pathotypes present in the field in Darien and the most aggressive race COL 63–63. Lines from the haplotype group M4 showed increased resistance against UG 61–63 and COL 13–63 but were less effective compared to M2 and M3. Lines from the haplotype group M5 were largely resistant against pathogen races in Darien and Kawanda, but no clear trend was observed in the other experiments.

Andean haplotype groups at the *Phg-2* locus were mostly associated with susceptibility to ALS. A1 and A2 only displayed effective resistance against COL 30–0, and A1 and A3 appeared resistant against UG 61–63. Lines from the haplotype groups A4, A5, and M/A were mostly susceptible in all experiments. The haplotypes at the *Phg-2* locus were able to explain a much larger fraction of the total phenotypic variability in ALS resistance (R^2 = 0.40 – 0.85, Supplementary table 4) compared to significant single SNP markers.

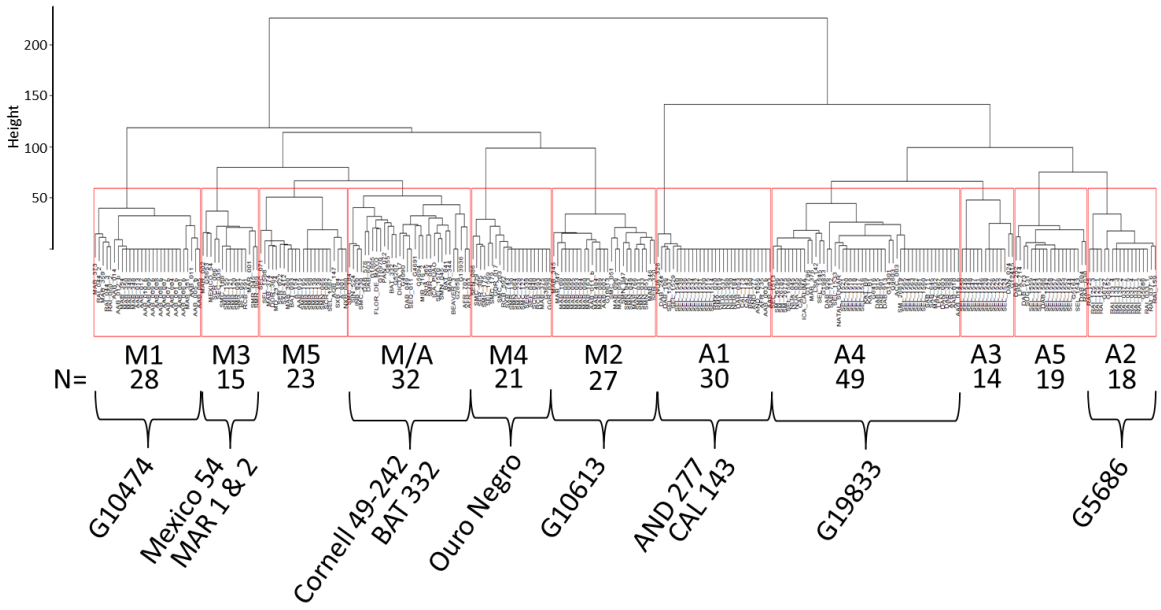

Figure 3: Dendrogram of hierarchical clustering at the *Phg-2* locus. The common bean lines of the extBALSIT panel were clustered according to similarity of their SNP data in the 61.15–62.93 Mbp interval on Chr 8 and divided into eleven haplotype groups. Haplotype groups were named according to the gene pool of the lines (M, Mesoamerican; A, Andean; and M/A = mixed) and numbered. Below the haplotype names, the number of common bean lines in each haplotype group is given and well known ALS resistant common bean lines as well as the reference genome line (G19833) contained in the haplotype groups are indicated. On the y-axis, the Euclidian distance between clusters is shown.

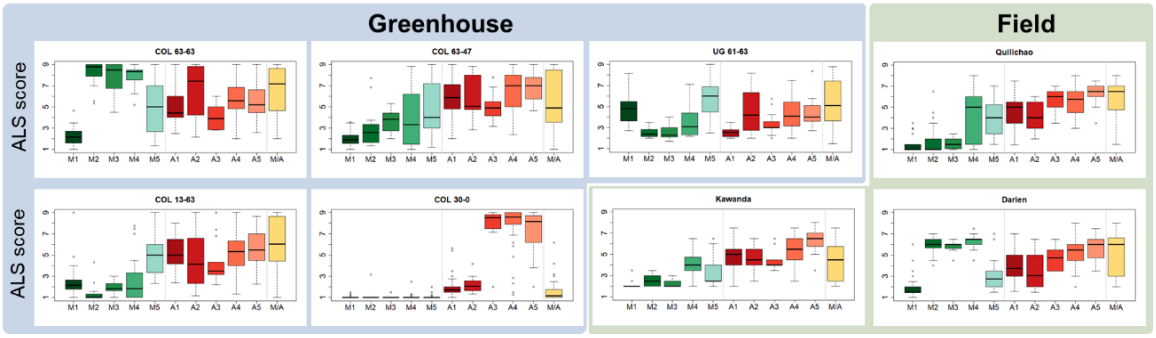

Figure 4: Haplotype groups at the *Phg-2* locus and their ALS response, as evaluated in greenhouse and field trials using the extBALSIT panel. For each trial, the ALS response, scored on a scale from 1 (resistant) to 9 (susceptible), is shown for each of the eleven haplotype groups.

Haplotype-specific SNPs to advance resistance breeding by marker-assisted selection

Seven haplotype groups (M1–3, M5, A1–A3) were identified as potentially interesting for breeding because of the resistance they displayed in multiple experiments. For example, the SNP marker specific for M1, the haplotype group associated with strongest resistance against most pathotypes, offers unique opportunities to trace this effective resistance allele in advanced breeding germplasm (Figure 5A). Similarly, the SNP markers tagging M2

(Figure 5B) and M3 (Supplementary table 5) can be employed for breeding to provide resistance against UG 61–63 (and the region of its occurrence). The SNP markers specific for the Andean haplotype groups A1 and A2 can be used to improve ALS resistance in the Andean gene pool, although their effectiveness is limited to a few pathotypes only. Genomic positions of the SNPs specific for all but one of the seven haplotype groups as well as for the resistance locus on Chr 4 are provided in supplementary table 5.

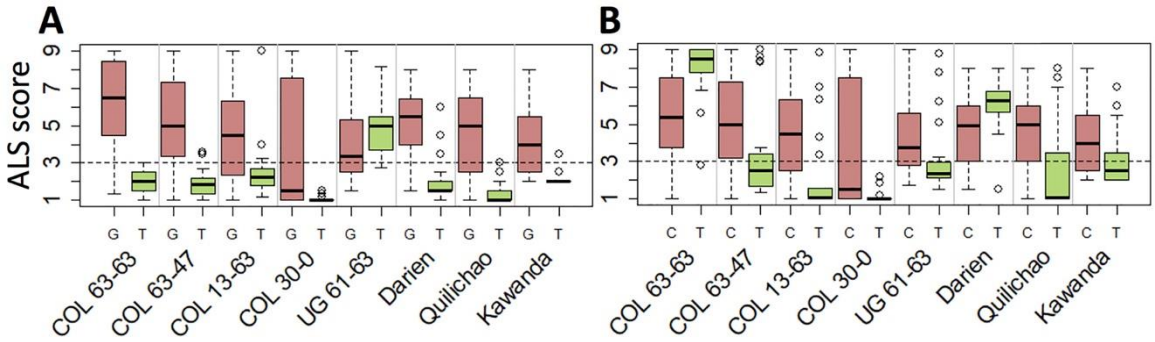

Figure 5: Candidate SNPs for marker-assisted selection of *Phg-2* haplotypes. Shown is the phenotypic distribution of ALS scores of the two alleles at the SNPs, which are specific for the functional haplotypes M1 **(A)** and M2 **(B)**. The SNPs on chromosome 8 at position 61,901,182 bp and 62,188,623 bp of the Pv2.1 reference genome that co-segregated with the haplotype groups M1 and M2, respectively, were used. On the y-axis, ALS response scored on a scale from 1 to 9 is shown, whereas scores below 3 (dashed line) are considered resistant. On the x-axis, greenhouse and field trials are indicated, and for each trial, the ALS-resistance response of the two alleles of the SNP is plotted.

Discussion

This study is the first to thoroughly evaluate the pathotype-specific response of ALS in common bean on the genetic level. Through GWAS in the largest yet assembled diversity panel segregating for ALS resistance, a pathotype-specific resistance locus, likely *Phg-4*, and a broad-spectrum resistance locus coinciding with *Phg-2* were effective against a variety of ALS pathotypes from Colombia and Uganda. For the latter locus, a high haplotype diversity was found, with at least seven different haplotype groups providing resistance in a pathotype-specific manner. Molecular markers specific for resistance loci and haplotype groups will facilitate breeding for pathotype-specific ALS resistance through marker-assisted introgression strategies.

No effect of *Phg-1*, *Phg-3* and *Phg-5* against the ALS pathotypes tested

In common bean, ALS resistance is reportedly controlled by five major resistance loci, named *Phg-1* to *Phg-5* [58]. Our study revealed a preeminent role of *Phg-2*, representing the unmatched source of resistance in effectively all experiments, while *Phg-1*, *Phg-3*, and *Phg-5* did not appear to be relevant. This is unexpected as the resistance loci *Phg-1* and *Phg-5*, originating from the resistance sources AND 277, CAL 143, and G5686 that were extensively used as progenitors in the CIAT breeding program, were present in the extBALSIT panel at frequencies sufficiently high to be detected by GWAS. Our observation may be a consequence of the strong pathotype-specificity of *P. griseola* and the differences in pathotypes prevalent within regions, countries, and continents. Experiments that led to the discovery of *Phg-1* and *Phg-3* were conducted with ALS

evaluation protocols comparable to ours using pathotypes of the races 63–23 and 63–39 from Brazil [23, 59]. *Phg-5* was discovered in CAL143 using a pathotype of race 0–39 and natural field evaluations in Brazil, and in G5686 using a pathotype of race 31–0 from Colombia [32, 33, 60]. Brazilian pathotypes are known to be very aggressive on the current differentials [21, 61-63], and it is possible that specific resistance genes are effective against these pathotypes. Future experiments should involve resistance evaluations of the extBALSIT panel with additional pathotypes, particularly from Brazil, where the resistance loci *Phg-1, Phg-3*, and *Phg-5* were observed to be effective.

In a similar study on resistance to anthracnose in common bean, an Andean bean diversity panel was tested with eight different pathotypes. In contrast to our study that only revealed a small subset of previously reported ALS resistance loci, GWAS for anthracnose resistance found the majority of the known resistance loci to effectively be involved [39]. Our findings undermine the importance of the pathotypes on the efficacy of disease resistance in common bean and call for an increased understanding of the pathogen population structure and virulence to allow prediction of effectiveness of resistance loci. Once the population structure of the pathogen is better known, established GWAS panels can be used to study the pathotype-specificity within and between sub-populations.

Phg-2 is an important ALS resistance locus with functional haplotypes from both, the Mesoamerican and the Andean background

The *Phg-2* locus is one of the most important ALS resistance locus in common bean and originally described in the Mesoamerican cultivar Mexico 54 [24, 57]. In the meantime, several additional Mesoamerican common bean lines were found to contain ALS resistance, either at or in close proximity to *Phg-2* on Chr 8 [25, 28, 29, 64]. This led to the hypothesis that *Phg-2* originated from the Mesoamerican gene pool, and hence, several breeding efforts aimed at its introgression into the Andean gene pool. Our study revealed that ALS resistance at *Phg-2* can also be found in the Andean gene pool: through cluster analysis on the basis of the genotypic data in the *Phg-2* region, we were able to classify eleven haplotype groups, at least seven of which appeared to be functionally different, leading to distinct patterns of resistance against the tested ALS pathotypes. Not only were resistance- and susceptibility- associated haplotypes in both gene pools, but also within each gene pool different haplotype groups of this resistance locus provided resistance to some, but not all evaluated ALS pathotypes.

Genetic determination of pathotype-specificity at *Phg-2*

The different haplotype groups at *Phg-2* largely explained pathotype-specificity for ALS response. For further understanding of the detailed interaction on the molecular level, the underlying genetic determinants need to be identified. To date, the causal genes of any ALS resistance loci, including *Phg-2*, are yet to be determined. Based on our data, it remains difficult to resolve whether the resistance at *Phg-2* is conferred by an allelic series at one resistance gene or by several resistance genes arrayed in clusters within the haplotype region defined by the significantly associated SNP markers.

Both, resistance gene clusters and allelic series are commonly occurring in plants [65]. In common bean, several allelic series have been reported for anthracnose resistance, and there were five alleles described of the *Co-1* and *Co-3* loci, three alleles for the *Co-4* locus,

and two alleles for the *Co-5* locus [66]. In wheat, up to 17 alleles have been found for the powdery mildew resistance gene *Pm3* that showed different pathotype-specific reactions [67]. The *Pm3* gene encoded for a classical nucleotide binding leucine-rich repeat (NR-LRR) receptor and alleles were highly similar, with usually only single amino acid changes differing between the alleles [68]. This pattern is reflecting the evolutionary mechanisms that are promoting the genetic diversification of resistance loci [69, 70].

Although the presence of allelic series may be a plausible explanation for ALS pathotype-specificity, the large extension of the *Phg-2* region, spanning 1.78 Mbp including 265 annotated genes, as well as the pathotype-specific significance peaks at distinct positions within this interval, indicates the involvement of multiple genes. Indeed, the presence of several candidate NB-LRR resistance genes at *Phg-2* in the Andean reference genome and their distinct expression in leaf tissue, as the case with Phvul.008G284500 and Phvul.008G285300 [45], strengthens this hypothesis.

While these are probable candidate genes, it should be noted that the most effective haplotype groups at *Phg-2* originated from the Mesoamerican gene pool, while the reference genome used for SNP discovery and gene identification derived from the Andean gene pool [17]. Resistance gene clusters are repetitive arrays of highly similar gene sequences that are often difficult to correctly assemble [71]. Moreover, they usually differ in the number of repeats between common bean lines and gene pools, and hence, one reference genome might not be fully representative of the structural diversity at resistance loci. In the recent years, novel genome assembly strategies including long-read sequencing technologies have been developed to assemble such regions more accurately. With the increased availability of pan-genomes, it will be possible to take into account even the genetic rearrangements between common bean lines.

Implications for ALS resistance breeding

ALS is one of the most devastating common bean diseases, particularly affecting smallholder farmers in low input agricultural systems. The results are production losses to the poorest, which most depend on the harvest from their fields for food security. Breeding for ALS resistance and other biotic and abiotic stress has been ongoing for a long time in common bean breeding programs of the tropics [72], but in the future, breeding needs to respond quicker than in the past to assure food security and adequate nutrition. Globalization and the increased human mobility have led to a globalization of plant pathogens and will continue to facilitate the exchange of genetic pathogen diversity [73-76]. An additional process that is expected to heavily affect plant pathogen dynamics is climate change. The increased warming and occurrence of extreme weather events will have effects on prevalence and plant-pathogen interactions [73, 77]. In the case of the tropical pathogen ALS, global warming will likely expand its range, and global mobility will lead to a mixing of pathogen populations previously separated by distance. More effective breeding methods are therefore urgently required to develop the varieties that will feed the growing future populations in developing countries. The research presented here will increase breeding efficiency for ALS resistance by providing a screening panel that can be used to find effective resistance loci in different areas. The molecular markers linked to resistance loci and resistance haplotypes will allow development of resistant lines without direct phenotypic screening in the region.

Resistance gene pyramiding is usually the suggested strategy to ensure durable disease resistance for highly virulent pathogens such as *P. griseola* [16]. The fact that ALS resistance in nearly all trials was conferred by the different haplotypes at *Phg-2* is rendering pyramiding difficult or impossible, depending on whether the causal genes are different genes within a resistance gene cluster or allelic series, respectively. Until the causal genes are known, the haplotype groups with very high effectiveness on both continents, M1 for Colombia and M2 and M3 for Uganda, provide the most sustainable strategy to control ALS by marker-assisted selection in one of the globally most important food security crop. However, given the threat of resistance sources to become inefficient, it is crucial to seek new ALS resistant common bean lines and elucidate the genetics of their resistance [13].

Data availability

The datasets analyzed and generated for this study can be found on dataverse at https://doi.org/10.7910/DVN/U2BAWN.

Author contributions

BR and BS conceived the study. MN conducted the experiments and performed data analysis and interpretation. BR, BS, and CM assisted in the experimental setup and data analysis. MN drafted the manuscript, which was improved by BS, BR, and CM. All authors read and approved the final manuscript.

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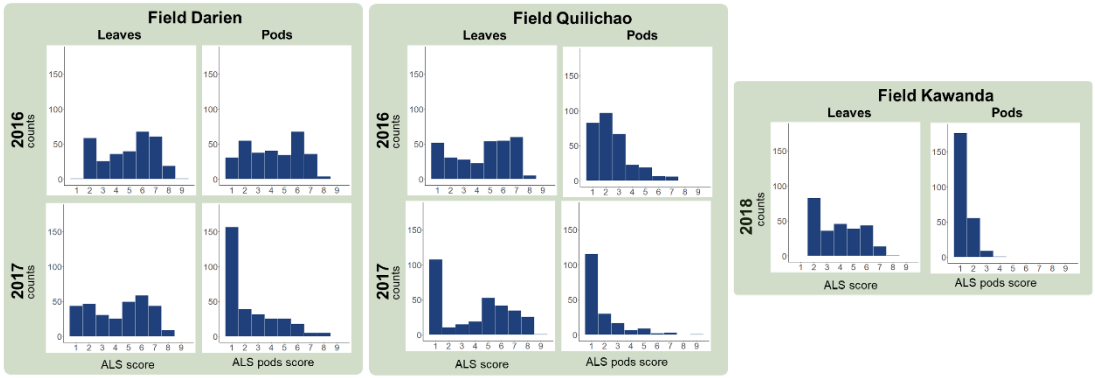
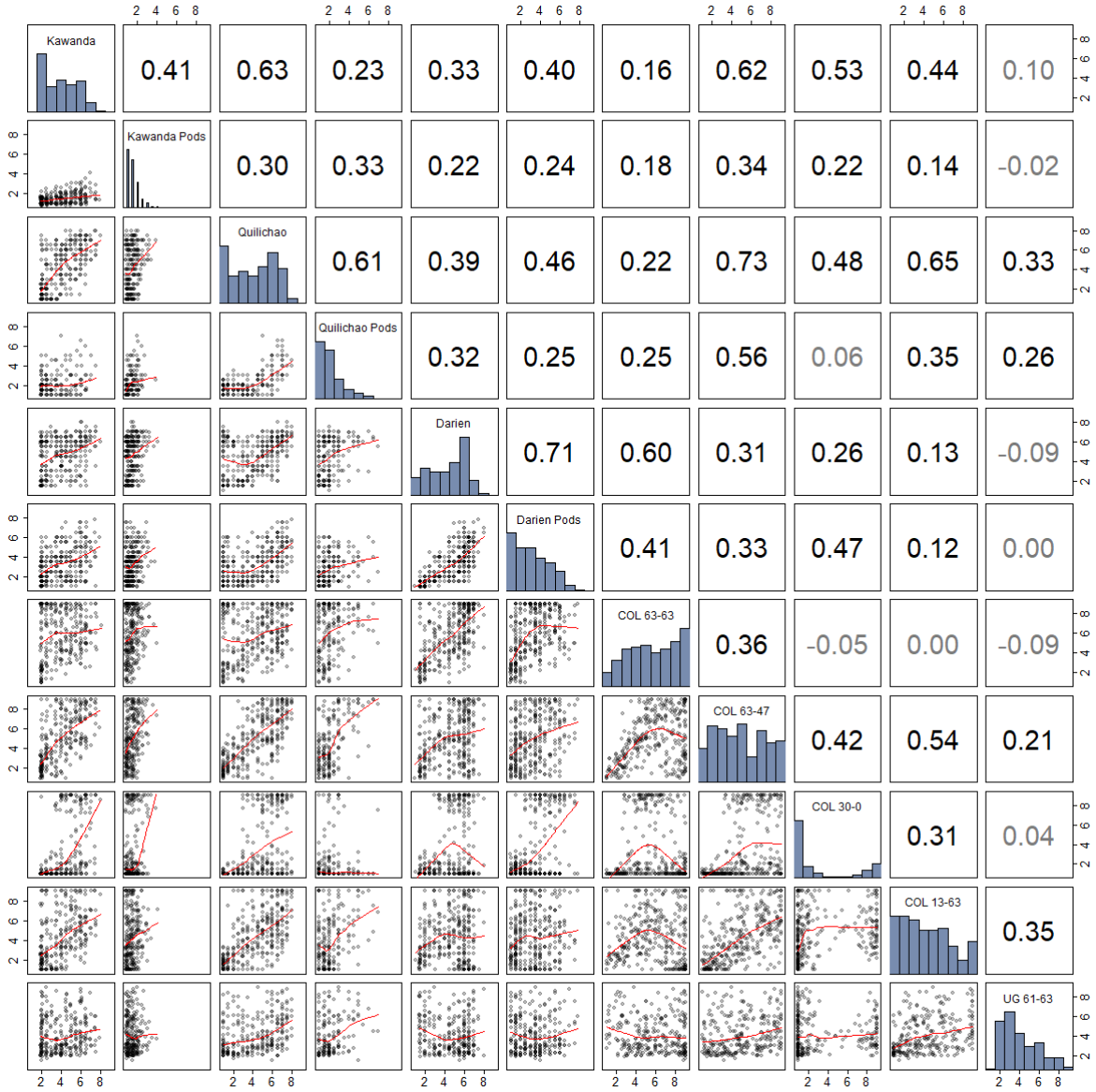
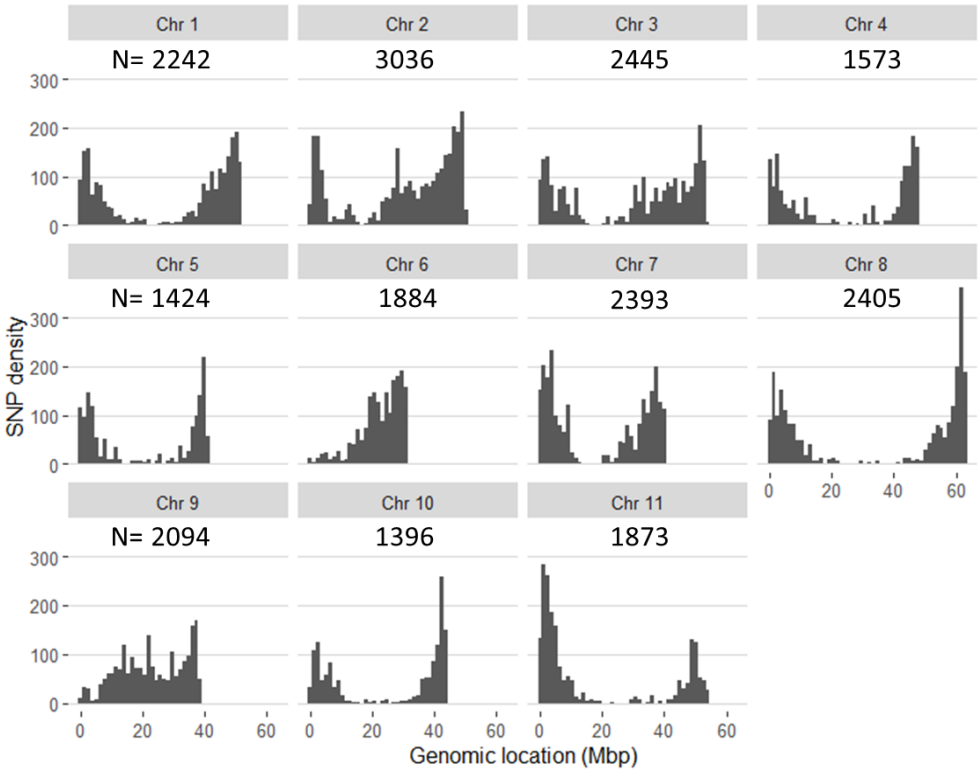
Supplementary material



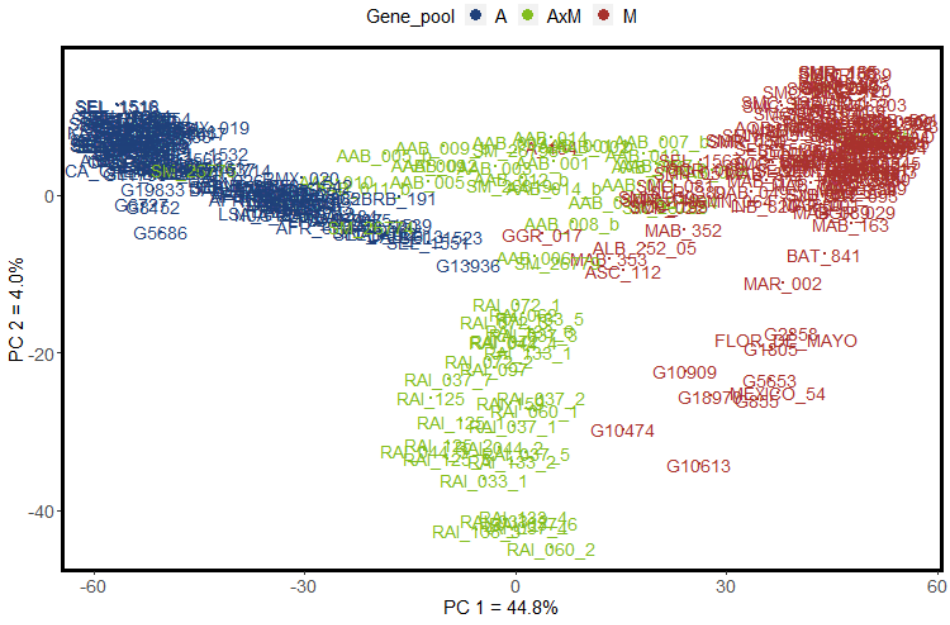
Supplementary figure 1: Frequency distributions of disease scores for angular leaf spot in field experiments using the extBALSIT panel containing 316 common bean lines. Angular leaf spot was scored on leaves and pods on a scale from 1 (resistant) to 9 (highly susceptible). Field trials in Colombia (Darien and Quilichao) and Uganda (Kawanda) were inoculated with mixtures of pathotypes, previously collected at the corresponding sites. Experiments in Colombia were conducted in two different years.



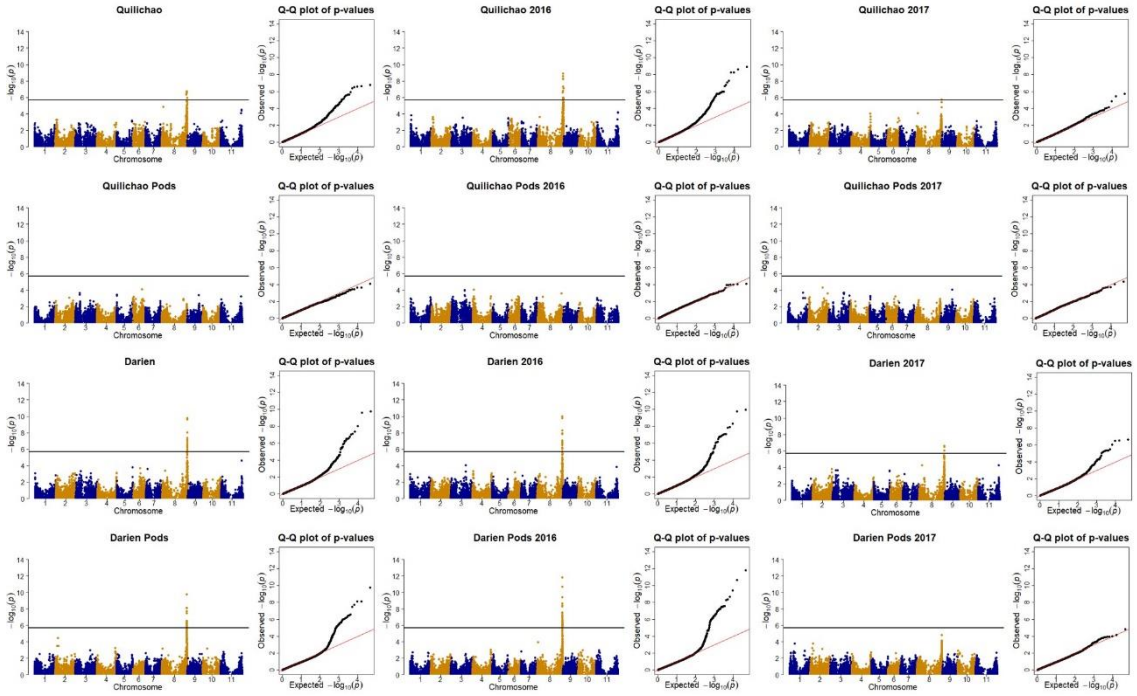
Supplementary figure 2: Correlation matrix of disease scores for angular leaf spot (ALS), evaluated in different greenhouse and field trials using the extBALSIT panel containing 316 common bean lines. Greenhouse trials were conducted with five different pathotypes, determined by their origin (COL and UG) and race (63-63, 63-47, 61-63, 13-63 and 30-0), and ALS scores were taken on leaves. Field trials in Colombia (Darien and Quilichao) and Uganda (Kawanda) were inoculated with mixtures of pathotypes, and ALS scores on leaves and pods were recorded. In the upper diagonal, Pearson correlations between trials are shown as well as their significance. Significant correlations ($P < 0.05$) are shown in black font and non-significant correlations in grey font. In the diagonal, histograms of ALS scores for each trial are given. In the lower diagonal, ALS scores of the two trials are plotted with the red line representing the LOESS (locally estimated scatterplot smoothing) line.



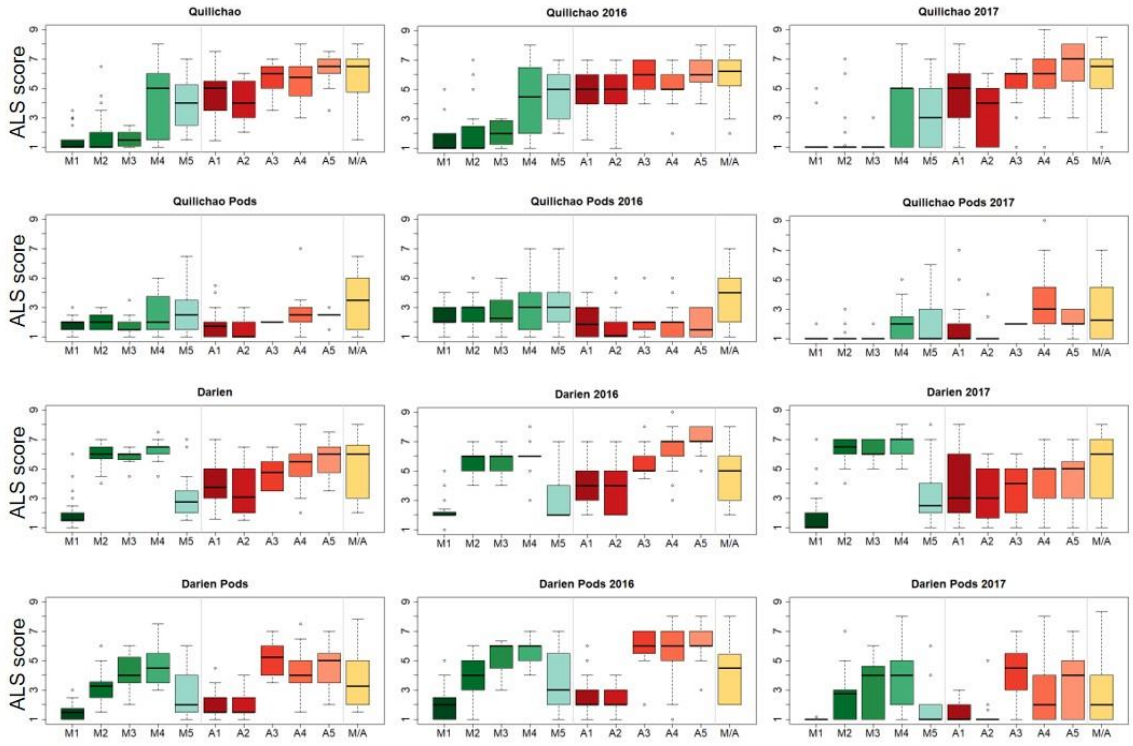
Supplementary figure 3: Distribution of SNP markers in the extBALSIT panel on the eleven chromosomes of the common bean reference genome. Histograms show the SNP density on chromosomes, and the total number of SNPs per chromosome is listed above each histogram. The common bean reference genome v2.1 was used as a reference.



Supplementary figure 4: Population structure of the 316 common bean lines of the extBALSIT panel based on genotypic data. Principal component analysis was conducted using 22,765 SNP markers distributed over the eleven chromosomes. Bean line names are colored according to their gene pool of origin: A (Andean), M (Mesoamerican) and AxM (Inter-gene pool cross).



Supplementary figure 5: Comparison of genome-wide association study results for angular leaf spot resistance of field experiments in two years. Manhattan and quantile-quantile (Q-Q) plot are shown for the years 2016 (central column) and 2017 (right column) separately and for the mean over both years (left column). Field trials in Colombia (Darien and Quilichao) were inoculated with mixtures of pathotypes, previously collected at the corresponding sites. On the x-axis, the genomic position of the SNP markers is given in the Manhattan plot and the negative logarithm to the base 10 of the expected *P*-value is given in the Q-Q plot. On the y-axis, the negative logarithm to the base 10 of the *P*-value, representing the significance value, is given. In order to correct for multiple testing, the significance threshold was adjusted through the Bonferroni method and the new significance threshold is depicted by the black horizontal line.



Supplementary figure 6: Haplotype-specific angular leaf spot responses of the extBALSIT panel in field trials conducted in two years. Common bean lines with genetic similarity at the *Phg-2* locus were grouped and haplotype groups named Mesoamerican (M1-3), Andean (A1-4) and mixed (M/A) based on their gene pool origin. Experiments in Colombia were conducted in two years and haplotype-specific angular leaf spot responses are shown for the years 2016 (center column) and 2017 (right column) separately. In the left column, the mean over the two years is shown. On the x-axis the haplotype group is given. On the y-axis, the angular leaf spot response scored on a scale from 1 (resistant) to 9 (highly susceptible) is shown.

Supplementary table 1: *Pseudocercospora griseola* pathotypes used for angular leaf spot resistance evaluation of the extBALSIT panel in greenhouse and field experiments. For each pathotype, the country of origin (COL = Colombia, UG = Uganda), race, isolate number and the collection site is given**.**

Origin and Race	Isolate	Experiment	Collection site
COL 63-63	Pg 347	Greenhouse	Quilichao
COL 63-47	Pg 431	Greenhouse	Tenerife
COL 30-0	Pg 447	Greenhouse	Palmira
COL 13-63	Pg 61	Greenhouse	Quilichao
COL 31-47	Pg 44	Field Darien	Darien
COL 5-47	Pg 81	Field Darien	Darien
COL 31-47	Pg 261	Field Darien	Darien
COL 15-44	Pg 305	Field Darien	Darien
COL 63-0	Pg 66	Field Darien	Darien
COL 63-0	Pg 3	Field Quilichao	Quilichao
COL 7-35	Pg 15	Field Quilichao	Quilichao
COL 31-55	Pg 32	Field Quilichao	Quilichao
COL 13-63	Pg 61	Field Quilichao	Quilichao
COL 15-39	Pg 318-1	Field Quilichao	Quilichao
COL 31-47	Pg 254	Field Quilichao	Popayan
UG 61-63	KA 060	Greenhouse	Kabale
UG 13-13	KA 045	Field Kawanda	Kabale
UG 0-22*	KA 049A	Field Kawanda	Kabale
UG 1-6*	KA 039A	Field Kawanda	Kabale
UG 17-23	MB 026	Field Kawanda	Mbale
UG 1-22*	KIS 70B	Field Kawanda	Kisoro

* These Ugandan races were evaluated with an incomplete set of differentials. Missing were the Andean common bean lines Poroto (binary value = 2) and Bolon Bayo (4).

Supplementary table 2: Sowing, inoculation and evaluation dates for field experiments conducted to evaluate the resistance of the extBALSIT panel to angular leaf spot in common bean. Given are the dates (and days after sowing in brackets) for each field trial. Plant development was delayed in Uganda (Kawanda) in comparison to Colombia (Darien and Quilichao) because of water limitation during the germination phase. Therefore, inoculation and evaluations were conducted approximately one week later than in Colombia.

Location / Year	Darien 2016	Darien 2017	Quilichao 2016	Quilichao 2017	Kawanda 2018
Sowing date	27.10.16	27.10.17	18.11.16	25.10.17	22.5.18
Inoculations					
1st	18.11.16 (d22)	20.11.17 (d24)	07.12.16 (d19)	17.11.17 (d23)	22.7.18 (d31)
2nd	24.11.16 (d28)	27.11.17 (d30)	14.12.16 (d26)	24.11.17 (d30)	29.7.18 (d38)
3rd	2.12.16 (d36)	4.12.17 (d37)	21.12.16 (d33)	1.12.17 (d37)	6.7.18 (d45)
Evaluations					
1st	2.12.16 (d36)	7.12.17 (d41)	27.12.16 (d39)	7.12.17 (d43)	6.7.18 (d45)
2nd	9.12.16 (d43)	15.12.17 (d49)	4.1.17 (d47)	13.12.17 (d49)	13.7.18 (d52)
3rd	26.12.16 (d60)	21.12.17 (d55)	13.1.17 (d56)	19.12.17 (d55)	20.7.18 (d59)
Evaluation Pods	11.1.17 (d76)	9.1.18 (d74)	31.1.17 (d74)	12.1.18 (d79)	10.8.18 (d80)

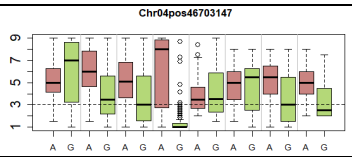
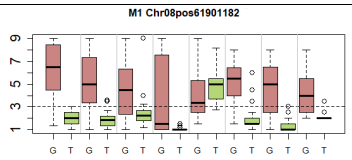
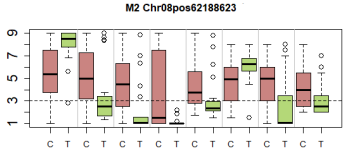
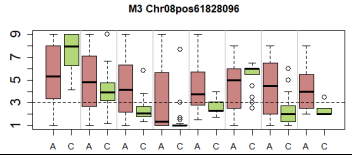
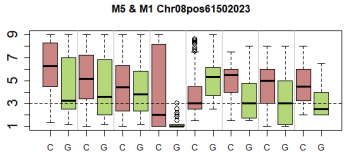
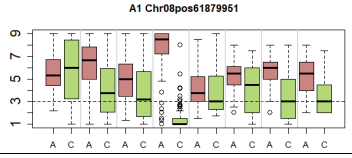
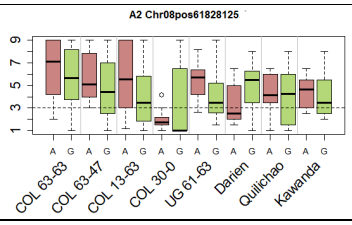
Suplementary table 3: Correlations and mean phenotypic values for angular leaf spot resistance of the extBALSIT panel evaluated in different years or replicates. The field trials in Colombia (Darien and Quilichao) were conducted in different years, while the field trial in Uganda (Kawanda) was conducted with two replicates. Greenhouse trials were conducted with five different pathotypes, determined by their origin (COL and UG,) and race (63-63, 63-47, 13-63 and 30-0). For each trial, the mean values and the standard deviation is given. In the last column, the Pearson correlation (*r*) between years and replicates is given.

Trial	Average	Year / Rep 1	Year / Rep 2	Correlation *r* =
Darien	4.60 ± 1.83	4.94 ± 1.95	4.26 ± 2.15	0.59
Darien Pods	3.31 ± 1.71	4.17 ± 2.00	2.44 ± 1.87	0.56
Quilichao	4.22 ± 2.20	4.39 ± 2.19	3.98 ± 2.55	0.72
Quilichao Pods	2.29 ± 1.27	2.48 ± 1.42	1.86 ± 1.47	0.44
Kawanda	4.10 ± 1.69	3.91 ± 1.85	4.29 ± 1.85	0.67
Kawanda Pods	1.51 ± 0.56	1.57 ± 0.82	1.46 ± 0.68	0.13
COL 63-63	5.74 ± 2.42			
COL 63-47	4.92 ± 2.45			
COL 13-63	4.30 ± 2.44			
COL 30-0	3.25 ± 3.13			
UG 61-63	4.11 ± 1.82			

Supplementary table 4: Phenotypic variance for angular leaf spot (ALS) resistance in the extBALSIT panel explained by the *Phg-2* haplotype groups in greenhouse and field trials. Reported here is the coefficient of determination (R^2) of a linear model with the haplotypes as the predictor and ALS score as target variable.

Isolate	R^2
COL 63-63	0.524
COL 63-47	0.403
COL 13-63	0.421
COL 30-0	0.850
UG 61-63	0.351
Darien	0.511
Quilichao	0.557
Kawanda	0.567
Darien Pods	0.471
Quilichao Pods	0.220
Kawanda Pods	0.161

Supplementary table 5: Locus- and *Phg-2* haplotype-specific SNP markers for breeding applications. Provided is a non-exhaustive list of SNPs that are locus- or haplotype-specific and represent target SNPs for marker-assisted selection in common bean. The SNP position names consist of the chromosome (Chr) number and the genetic position (pos) on the common bean reference genome v2.1. In the SNP column, the resistant allele is given before the dash and the susceptible allele after.

SNP position	SNP	Allele-specific reaction in ALS trials
***Phg-4* on chromosome 4**		
Chr04pos46703147	G/A	
Chr04pos46934061	T/C	
Chr04pos46727398	G/A	
***Phg-2* on chromosome 8**		
<u>M1</u>		
Chr08pos61901182	T/G	
<u>M2</u>		
Chr08pos62188623	T/C	
<u>M3</u>		
Chr08pos61828096	C/A	
Chr08pos61878388	A/C	
Chr08pos61880092	T/C	
<u>M5 and M1*</u>		
Chr08pos61388457	C/T	
Chr08pos61502023	G/C	
Chr08pos61533289	T/C	
<u>A1</u>		
Chr08pos61825787	A/C	
Chr08pos61879951	C/A	
Chr08pos62191492	G/A	
<u>A2</u>		
Chr08pos61828125	G/A	
<u>A3</u>		
Not found		

*No SNPs that specifically tag M5 were found, but one that tags the haplotype group M5 and the highly resistant group M1 was found instead

Chapter 4

Pyramiding of five angular leaf spot resistance loci in common bean

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This chapter will be updated with phenotypic and ALS resistance data from the field season 2019 and submitted for publication after the completion of this thesis.

Abstract

Combining different resistance loci in a single cultivar is a promising strategy for providing broad-spectrum and durable resistance against highly virulent plant pathogens such as *Pseudocercospora griseola*, the causal agent of the angular leaf spot (ALS) disease in common bean (*Phaseolus vulgaris*). Here, we describe a crossing and selection scheme to pyramid five previously characterized ALS resistance loci and combine them with the good agronomic properties of elite cultivars, primarily good grain quality. The crossing scheme involves four ALS resistant common bean cultivars and two elite common bean cultivars. Through marker-assisted selection, seven plants were found in the F_3 generation that combine all resistance loci in a homozygous state. In addition, 84 plants with all resistance loci present, but at least one of them in heterozygous state, were found. Future genotypic and phenotypic selections within the progenies of the 91 plants will be conducted to find lines that combine ALS resistance with good grain and agronomic qualities. The combination of five ALS resistance loci is expected to result in a broad-spectrum resistance to multiple ALS pathotypes and may extend the time it takes for *P. griseola* to overcome resistance.

Introduction

Plant breeding is a lengthy process taking years before cultivars are released and distributed to farmers. In contrast, pathogen evolution is a continuous, rapid process and resistance of new cultivars is usually overcome quickly [1]. Common bean (*Phaseolus vulgaris* L.) with its tropical origin has evolved in conjunction with various pathogens and consequently their domesticated form today is challenged by several fungal, viral and insect pests that attack at various stages of their life-cycle, sometimes with devastating effects on yield [2, 3]. One of these diseases, angular leaf spot, is caused by the fungus *Pseudocercospora griseola* (Sacc.) Crous & U. Braun and is responsible for high yield losses in the tropical common bean producing continents of Africa and Latin America [4]. In the tropics, common beans are mostly produced by poor smallholder farmers, who have limited possibilities to protect their crops from plant diseases and environmental stresses [5, 6]. Disease and stress resistant common beans are therefore the most feasible and sustainable method to stabilize yield on smallholder's farms [7].

Common bean breeding has a long tradition that, because of a complex domestication history with multiple centers of secondary diversification, is based on a high genotypic and phenotypic diversity that can be utilized for crop improvement [8-11]. Common bean has been domesticated at least twice, once in Mesoamerica and once in the Andean zone, which has resulted in the small-seeded Mesoamerican and the large-seeded Andean gene pool [12-14]. Several ALS resistance loci have been found in the two common bean gene pools and a comprehensive review of all quantitative and qualitative ALS resistance loci characterized has recently been published by Nay et al. [15]. Five ALS resistance loci could be repeatedly discovered and were named *Phg-1* to *Phg-5* [16]. *Phg-1* was found on chromosome (Chr) 1 in the Andean cultivar AND 277 [17, 18]. *Phg-2* is one of the most important resistance loci in the Mesoamerican background and resistance loci have been found on the lower end of Chr 8 in the genotypes Mexico 54, Cornell 49-242, BAT 332, G10474 and G10909 [19-24]. While the name *Phg-2* is officially only to be used for the resistance gene found in Mexico 54, we use the term '*Phg-2* locus' here to include all resistance loci found on the lower end of Chr 8 [16]. The resistance locus *Phg-3* was found on the upper end on Chr 4 in the Mesoamercian cultivar Ouro Negro with a potential allelic or nearby locus in G10909 [22, 25, 26]. On the lower end of Chr 4, *Phg-4* was found to confer resistance in the Andean genotype G5686 [27, 28]. *Phg-5* was found on Chr 10 in the Andean genotypes G5686 and CAL 143 [28, 29]. While the initially discovered ALS resistance loci *Phg-1* to *Phg-3* are considered major effect loci, the recently discovered loci *Phg-4* and *Phg-5* as well as several unnamed resistance QTLs suggest an additional quantitative aspect of ALS resistance [15, 28-31]. For all five ALS resistance loci, linked molecular markers are published and their approximate genetic location has been evaluated by mapping these linked markers to the common bean reference genome [15].

The five named ALS resistance loci have been successfully used to breed ALS resistant varieties, but these lines were often only resistant in a few locations and a shift of pathogen population easily rendered them susceptible again [32-34]. ALS is highly pathotype specific, which is probably a consequence of the constant evolutionary arms race of host and pathogen, in which the pathogen tries to avoid host recognition and the host is evolving new mechanisms to recognize the pathogen [35-37]. For ALS, this has resulted in the co-evolution of the pathogen with its Mesoamerican and Andean common bean gene pools [38]. Based on virulence data, *P. griseola* isolates can be categorized as Mesoamerican or Andean, with Andean isolates being mostly pathogenic on Andean beans,

while Mesoamerican pathogens exhibit a broader virulence spectrum and are pathogenic on both Andean and Mesoamerican common beans [32, 33, 38-40]. Based on these observations, strategies to achieve effective resistance were proposed and included; pyramiding of Mesoamerican and Andean resistance genes, planting Mesoamerican common bean cultivars in regions where Andean pathogen races are present or vice versa [32, 38]. Gene pyramiding is expected to make resistance more effective in different environments as well as prolong the time taken by the pathogen to overcome the resistance [41]. Resistance pyramiding in common bean has been difficult because of the limited availability of highly source specific markers that work outside of the experimental population. With the increased availability of next generation sequencing data of non-model species and cost-effective genotyping systems, molecular markers specific for resistance loci have become affordable. In combination with phenotypic selection, gene pyramiding has the potential to develop within a few years highly resistant lines that can be introduced into the main breeding pipeline allowing modern varieties to be one step in advance of pathogen evolution.

The main objective of this study was to pyramid five well-characterized ALS resistance loci into common bean lines that exhibit desired agronomic qualities, mainly good grain types and a bush type growth habit. Specifically, we aimed at i) establishing highly locus-specific molecular markers that allow genotypic selections ii) developing a multi-parental crossing scheme involving ALS resistant cultivars and elite breeding cultivars and iii) conducting phenotypic and genotypic selection at various stages of the crossing scheme to ensure the lines combine all five ALS resistance loci and exhibit good phenotypic and agronomic characteristics.

Materials and methods

Plant material, molecular markers and genotyping

The ALS resistance sources G10474, G5686, AND 277, 10909 and the well-accepted grain genotypes CAL 143 and KAT B1 were obtained from the CIAT common bean breeding program. The following ALS resistance loci were selected for pyramiding; *Phg-1* from AND 277 [17, 18], *Phg-2* from G10474 [24], *Phg-3* from G10909 [22] and *Phg-4* and *Phg-5* from G5686 [28]. Two lines named RAI 97 and RAI 62 with the pedigree G10474 x G5686 containing all resistance loci known in G10474 and G5686 were available at CIAT from a previous project.

Molecular single nucleotide polymorphism (SNP) markers specifically targeting resistance loci within the six parental genotypes were used for genotyping. Two published markers, ALSChr08_CT_57798588 and MAS_ALS10c, from Lobaton et al. [42] were used. In addition, SNP markers in proximity to the published approximate location of resistance loci (Supplementary table 1) were selected based on genotypic data of all six parental genotypes on the BARC Bean SNP Chip and whole genome sequencing data of G5686 [42, 43]. Conversion of SNPs to KASP markers was done at LGC (LGC Ltd, Teddington, UK) or Intertek (Intertek Group plc, London UK), based on a reference sequence 50bp before and after the SNP. For genotyping at LGC, DNA was extracted with an SDS protocol from leaf tissue and with a CTAB protocol from seeds [44, 45]. Genotyping at Intertek was done according to their internal standard protocol [46].

Sanger sequencing was conducted for a fragment that contained the SNP targeted by the Phg1Chr1_AG_51617802 marker, which was not successfully established at LGC. PCR was conducted with the marker named 3_Phg1 _51617802, with forward primer sequence 5'-TGCAC-CAAAATCCATTCCATGA-3' and reverse primer sequence 5'-GCCGGTTTTATGGGGT-TGAG-3' (Microsynth, Balgach, Switzerland). DNA was extracted according to Afanador et al. [45] and PCR was performed in a volume of 10 µl with 1x Green GoTaq reaction buffer (Promega, Maddison WI, USA), 0.2 mM dNTP (Promega), 0.2 µM forward primer, 0.2 µM reverse primer, 0.08 µl GoTaq polymerase (Promega, Maddison WI, USA) and 1 µl DNA template (5 ng/µl). PCR was amplified on a Bio Rad C1000 thermal cycler (Bio Rad Laboratories Inc., Hercules CA, USA) for 15 min at 95°C followed by 35 cycles of 30 s at 95°C, 30 s at 60°C and 90 s at 72°C, and to finish 10 min at 72°C. Sequencing was conducted at Microsynth using the 3_Phg1 _51617802 reverse primer. Sanger sequences were compared using the BLAST megablast algorithm [47, 48].

Based on the Sanger sequencing data obtained, an allele-specific PCR marker named 1_Phg1 _51617802 was established to target the T/C SNP on the forward DNA strand on Chr 1 at 50,901,342 bp with the forward primer 5'-TGCAATTGAGTTAGGGT-TAAAGT-3' and the reverse primer 5'-ATATTATGGATAGCCCGATAATGA-3' (Microsynth). DNA extraction and PCR were conducted the same as for Sanger sequencing with a modified PCR protocol that involved 15 min at 95°C followed by 35 cycles of 30 s at 95°C, 30 s at 62.2°C and 90 s at 72°C, and to finish 10 min at 72°C. The PCR products were visualized through gel electrophoresis with a 2.5% agarose gel where 3 µl of PCR product was loaded and run for 50 min at 50 V.

Pyramiding scheme

Resistance locus pyramiding was initiated using four ALS resistance sources and two elite common bean lines. The crossing scheme is summarized in figure 1. In the following descriptions, good grain type lines (KAT B1 and CAL 143) and the RAI lines (RAI 62 and RAI 97) were used alternately, which is designated by a '/' that signifies 'or'. Initially 4-way crosses of (AND 277 x G10909) x (RAI 62/RAI 97 x KAT B1/CAL 143) and 3-way crosses of (G10909xAND277) x KAT B1/CAL 143 were established (see supplementary table 2 for the pedigrees of crosses used, number of seeds genotyped and selected, and planting dates of the trials). Molecular markers used in each round of genotyping are given in the last four columns of table 1. Where available, plants that were homozygous at the resistance locus were preferred.

For the 4-way crosses, half of the F_1 seeds were planted in Darien (N3°53'31" W76°31'0", 1469 m a.s.l) and the other half in Popayan (N2°31'02" W76°38'05", 1753 m a.s.l). In the first round of genotyping, DNA from leaf tissue of F_1 plants was genotyped at LGC. Plants containing *Phg-2*, *Phg-4* and *Phg-5* were selected and their F_2 seeds planted in Darien. Plant tissue was harvested from these plants shortly after emergence, DNA extracted and sent to LGC for a second round of genotyping. The F_2 4-way plants containing *Phg-2*, *Phg-4* and *Phg-5* were selected and crossed to 3-way plants in the same generation.

For the 3-way crosses, F_2 seeds were planted in Quilichao (N3°04'22" W76°29'55", 995 m a.s.l) and plants with a bush-type growth habit and a good grain type were harvested and genotyped. To reconstruct the genotype of the harvested F_2 plants, seed tissue of five

F_3 offspring per plant were pooled and genotyped. Families that contained the *Phg-1* and *Phg-3* loci were selected and their seeds planted in Darien along with the 4-way crosses. Plant tissue was harvested from plants shortly after emergence for a second round of genotyping. The F_3 3-way plants containing *Phg-1* and *Phg-3* were used for crossing to the 4-way plants.

Selected 3- and 4-way plants were crossed to each other (Supplementary table 2). For successful crossing, the genotypes needed to flower or be ready for pollination at the same time, therefore only a subset of the plants with good genotypes could be used. Successful crosses were advanced to the F_2 generation and genotyped at Intertek in a third round of genotyping with the markers listed in table 1. Plants containing all resistance loci in at least a heterozygous state were selected and their F_3 seeds sown in Palmira. These plants were again genotyped at Intertek in a fourth round with the markers listed in table 1.

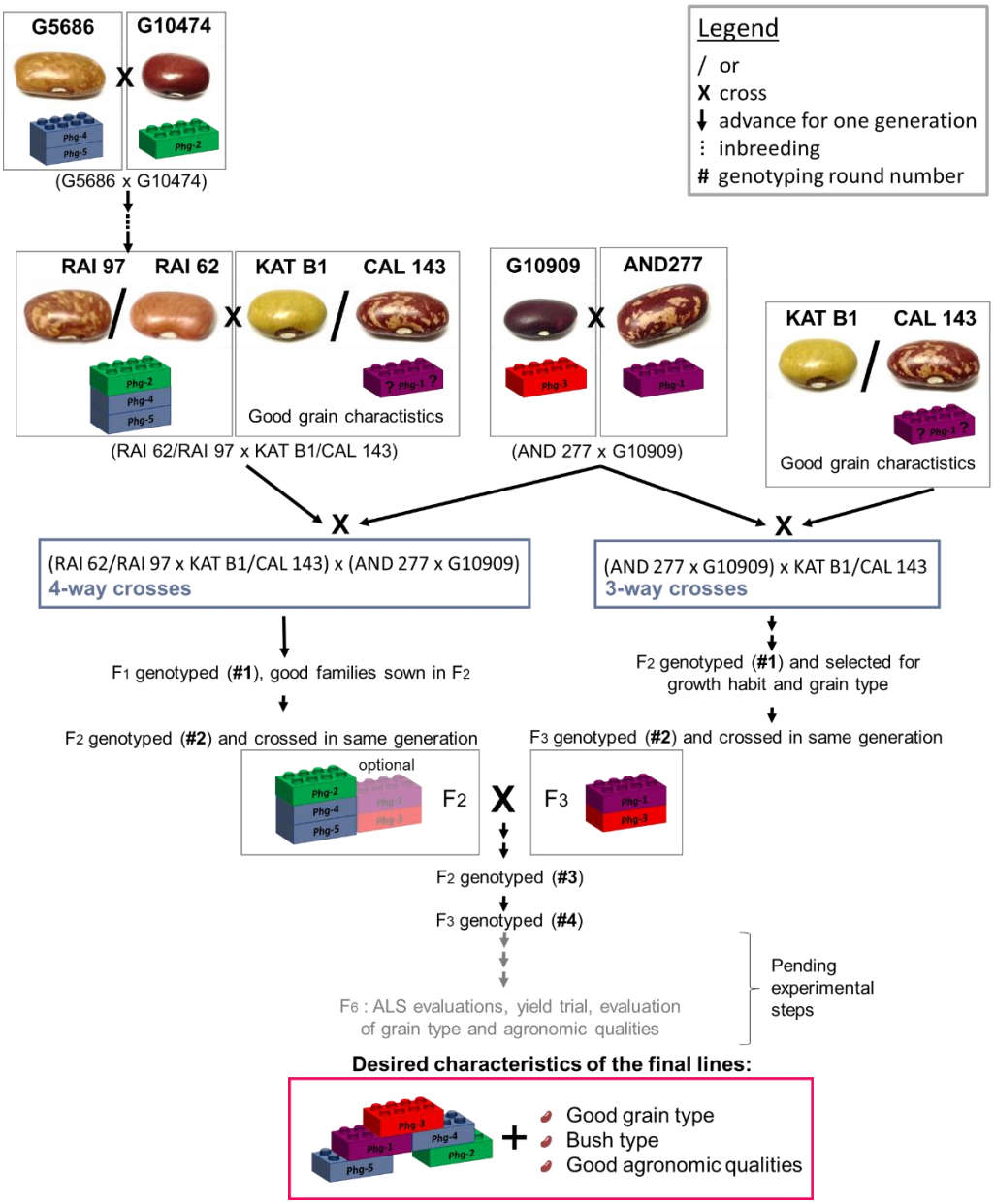

Figure 1: Crossing and genotyping scheme for angular leaf spot resistance (ALS) pyramiding in common bean. Four ALS resistant common bean lines with known resistance loci were crossed to two elite grain lines according to the scheme drawn above. For each starting cultivar (name given in bold) a photo of the grain type is shown and the ALS resistance locus contained is given as a brick. The *Phg-1* locus is probably contained in CAL 143 in addition to the known source AND 277, which is indicated with the question mark next to the locus name. Crosses are indicated with an 'x', advancing for one generation is indicated by an arrow, inbreeding (advancing several generations) is indicated by '...' and if lines were crossed to multiple starting materials it is indicated by a '/'. In short, initial crosses involving four parents (4-way crosses) and three parents (3-way crosses) were established. The 4-way crosses were advanced and selected to contain the *Phg-3*, *Phg-4* and *Phg-5* loci and because of their pedigree may also contained *Phg-1* and *Phg-3*. The 3-way crosses were phenotypically selected and genotyped to contain the *Phg-1* and *Phg*-3 locus. The best genotypes originating from 4-way crosses were crossed to the best genotypes originating from 3-way crosses and the resulting offspring genotyped to contain all five resistance loci.

Table 1: Molecular SNP markers to pyramid five angular leaf spot resistance loci. Molecular markers established at LGC, Intertek and in-house are listed with their genetic position on the *Phaseolus vulgaris* reference genome v2.1 [49]. The first nucleotide mentioned in the SNP column is tagging the resistant allele contained in the genotypes listed in the source column. Four rounds of genotyping (#1-#4) were conducted and markers used in the different rounds are given in the last four columns, where 'OK' signifies the marker produced the expected results in the parental genotypes, 'NW' signifies the marker was not working, and a gap signifies the marker was not tested in the genotyping round. Markers were tested at LGC (#1-2) and/or Intertek (#3-4). Abbreviations: Chr: chromosome; WGS: Whole genome sequence.

Marker name LGC	Intertek	Position	SNP	Chr	Source	Origin	#1	#2	#3	#4
Phg-1										
Phg1Chr1_AG_51617802	snpPV0051	50,901,342	A/G	1	AND 277 & CAL143	BARC chip	NW		OK	OK
Phg1new_TC_51617802		50,901,342	T/C	1	AND 277 & CAL143	BARC chip		OK		
1_Phg1_51617802		50,901,342	T/C	1	AND 277 & CAL143	BARC chip		Only in-house use		
ALS_Phg1_01_TC_51653736*	snpPV0080	50,937,212	C/T	1	AND 277 & CAL143	BARC chip			OK	OK
ALS_Phg1_01_CT_5183712*	snpPV0081	51,167,602	C/T	1		BARC chip			OK	OK
sc00003ln2130026_318965_G_A_46§		50,840,540	G/C	1	AND 277 & CAL143	BARC chip		OK		
sc00618ln186586_180008_C_T_254§		51,167,602	C/T	1		BARC chip		OK		
Phg-2										
ALSChr08_CT_57798588	snpPV0033	61,230,352	T/C	8	G10474	[42]				OK
Phg2Chr8_GA_57941925		61,375,044	G/A	8	G10474	BARC chip	OK	OK		
ALS_Phg2_08_GT_61901182	snpPV0071	61,901,182	T/G	8	G10474 & G10909	[50]			OK	OK
Phg2Chr8_GA_58703798	snpPV0052	62,170,581	G/A	8	G10474	BARC chip	OK	OK	NW	
Phg-3										
Phg3Chr4_AG_6241077		6,576,995	A/G	4	G10909	BARC chip		OK		
Phg3Chr4_GT_6493282	snpPV0053	6,847,471	G/T	4	G10909	BARC chip	NW		OK	OK
Phg3Chr4_GA_4915296	snpPV0078	5,174,371	G/A	4	G10909	BARC chip	OK	OK	OK	OK
Phg-4										
Phg4Chr4_CA_43340341		45,465,414	C/A	4	G5686	BARC chip	OK	OK		
sc00716ln161188_139140_T_G_271	snpPV0054	45,500,982	T/G	4	G5686	BARC chip	OK	OK	OK	OK
Phg-5										
MAS_ALS10c	snpPV0027	39,052,977	T/C	10	G5686	[42]	OK	OK	OK	OK
ALSChr10_AG_4390652	snpPV0079	4,352,041	A/G	10	G5686	WGS	OK	OK	OK	OK

*,§ These two marker pairs are haplotype markers and were always tested in combination.

Results

Establishment of molecular markers for marker-assisted selection

Five previously described ALS resistance loci, *Phg-1* to *Phg-5*, from four different common bean cultivars were chosen for pyramiding. For genotypic selection, source-specific molecular SNP markers were successfully established for all but the *Phg-1* locus at the genotyping service providers LGC and Intertek (Table 1). The only SNP that, based on the SNP chip data [43] distinguished AND 277 from the remaining parental lines in proximity of the *Phg-1* region was successfully converted at Intertek but not at LGC. Sanger sequencing of the Phg1Chr1_AG_51617802 target SNP region revealed several polymorphisms up- and downstream of the targeted polymorphism (Figure 3), which may have interfered with the primer design at LGC. Based on Sanger sequencing data, an in-house allele-specific PCR marker named 1_Phg1_51617802 and a LGC marker named Phg1new_TC_51617802 were designed that target the SNP from the opposite DNA strand. The allele-specific PCR marker amplified an approximately 150 bp long fragment in the genotypes AND 277 and CAL 143 simultaneously (Figure 3).

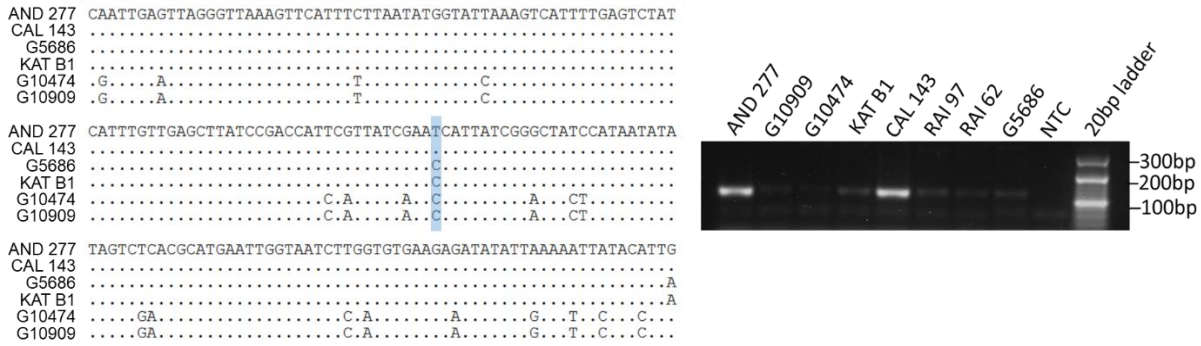

Figure 3: Characterization and marker conversion of a SNP in proximity to the *Phg-1* locus. Left, a local alignment of DNA sequences of all five parental lines that were intercrossed in this study around the target T/C SNP at 50,901,342 bp on chromosome 1 (marked in blue) is shown. The sequence shown corresponds to the range of 50,901,247 to 50,901,426 bp on chromosome 1 on the forward strand of the common bean reference genome v2.1. The AND 277 sequence was used as reference and the parental lines were compared to it: no deviations are indicated by a dot and deviations by stating the observed nucleotide below the reference. Right, DNA amplification products obtained with the allele-specific PCR marker 1_Phg1_51617802 tested on all parental lines and a 'no plant DNA template' control (NTC) is shown. The PCR products were visualized through agarose gel electrophoresis.

Sanger sequencing of the Phg1Chr1_AG_51617802 target SNP and the PCR results in figure 3 revealed that the genotyping information of the SNP chip was erroneous, since both, AND 277 and CAL 143 had a thymine nucleotide at position 50,901,342 on Chr 1 on the forward DNA strand. Based on the available SNP chip and Sanger sequencing data, no polymorphisms that distinguished between AND 277 and CAL 143 could be found within the large region of 39.0 to 50.6 Mbp on Chr 1, overlapping with the expected region of the *Phg-1* locus (49.8Mbp to 50.0Mbp, Supplementary table 1). Because of the shared ancestry between AND 277 and CAL 143, we assumed that the *Phg-1* resistance locus is contained

in both genotypes and for subsequent rounds of genotyping, markers distinguishing *Phg-1* in AND 277 and CAL 143 from the remaining parental lines were established and used for selection (Table 1).

Initiation of resistance gene pyramiding

Resistant common bean lines were intercrossed and crossed to elite common bean breeding lines as shown in figure 1. The starting material for pyramiding of the five resistance loci were 4-way crosses obtained by crossing AND 277 x G10909 to RAI 97/62 x KAT B1/CAL 143 and 3-way crosses by crossing AND 277 x G10909 to KAT B1/CAL 143 (for the exact pedigree of crosses see supplementary table 2). Based on their pedigree, 4-way crosses can contain all five resistance loci, while the 3-way crosses can only contain *Phg-1* and *Phg-3*.

In the first round of genotyping, out of 110 F_1 4-way cross plants, eight plants that contained the resistance loci *Phg-2*, *Phg-4* and *Phg-5* were selected (Table 2). For the F_2 3-way crosses, 71 plants with a bush type growth habit and an acceptable grain type were harvested and genotyped, of these, ten plants that contained the *Phg-1* and *Phg-3* loci were retained (Table 2).

Table 2: Genotypic results of selected F_1 4-way and F_2 3-way plants, whose families were used in subsequent crosses. Plant identification codes (ID) for 4-way and 3-way crosses are given and their genotyping results with the markers listed in the first column are shown. For 3-way crosses, which based on their pedigree can only contain *Phg-1* and *Phg-3*, the markers for the remaining resistance loci are not shown. For each marker, the angular leaf spot resistance locus (ALS Locus) it targets and the two alleles at each SNP are given, whereas the resistant allele is mentioned first.

Marker	ALS Locus	SNP	Plant ID 4-way crosses								Plant ID 3-way crosses									
			P84	P110	D39	P107	D26	D19	D14	D23	Q8	Q14	Q17	Q19	Q31	Q33	Q57	Q59	Q69	Q71
1_Phg1 _51617802 *	*Phg-1*	T/C	T	T	T	–	T	T	–	T	T	T	T	T	T	T	T	T	T	T
Phg2Chr8_GA_ 58703798	*Phg-2*	G/A	G:A	G:A	G:A	G:A	G:A	G:A	G:A	G:A										
Phg2Chr8_GA_ 57941925	*Phg-2*	G/A	G:A	G:A	G:A	G:A	G:A	G:A	G:A	G:A										
Phg3Chr4_GA_ 4915296	*Phg-3*	G/A	G:A	A:A	A:A	G:A	G:A	A:A	A:A	G:A	G:A	G:G	G:G	G:A	G:A	G:A	G:A	G:A	G:G	G:A
sc00716ln161188_ 139140_T_G_271	*Phg-4*	T/G	G:T	G:T	G:T	G:T	G:T	G:T	G:T	G:T										
Phg4Chr4_CA_ 43340341	*Phg-4*	C/A	C:A	C:A	C:A	C:A	C:A	C:A	C:A	C:A										
ALSChr10_AG_ 4390652	*Phg-5*	A/G	A:G	A:G	A:G	G:G	G:G	G:G	A:G	A:G										
MAS_ALS10c	*Phg-5*	T/C	T:C	T:C	T:C	T:C	T:C	T:C	C:C	C:C										

*The allele specific PCR marker 1_Phg1_51617802 was dominant, therefore only absence (–) and presence (T) could be scored.

Generation of crosses and genotypic selection of plants combining all five resistance loci

Seeds derived from selected single plants in the first genotyping round were planted in Darien. Leaf material of 375 plants (145 4-way crosses, 198 3-way crosses and 32 parental lines) was sampled shortly after germination and genotyped in a second round. Of these, 21 F_2 4-way plants containing *Phg-2, Phg-4* and *Phg-5*, and 84 F_3 3-way plants containing *Phg-1* and *Phg-3* were selected and deemed suitable for the final crossing round in the same generation. Because of differences in flowering time, successful crosses were made between nine F_2 4-way and 24 F_3 3-way plants. Of the 81 crosses, 49 were successful and resulted in 355 F_1 seeds that were advanced to F_2. The large F_2 population of 2,668 plants was sown and genotyped with ten SNPs targeting all five *Phg* resistance loci in the third round. The markers used for genotyping the *Phg-2* locus in this round were not fully informative, as the Phg2Chr8_GA_58703798 marker did not show the expected pattern in the parental lines and the ALS_Phg2_08_GT_61901182 marker tagged *Phg-2* of G10909 and G10474 simultaneously.

Using the ALS_Phg2_08_GT_61901182 marker data for the *Phg-2* locus, 32 plants were selected and their F_3 seeds were sown and genotyped again. In the F_3 generation 309 plants were genotyped and this time no difficulties in interpretation of genotyping data occurred. We found seven plants that contained all resistance loci in a homozygous state and 84 plants that had all five resistance loci, but at least one was in a heterozygous state. These 91 plants were harvested and their progenies will be used for future experiments.

Discussion

Resistance pyramiding to enhance angular leaf spot resistance in farmer-desired grain types

This is the first time that all five well-characterized ALS resistance loci have been pyramided in common bean lines. Within three years, we have designed and implemented a strategy to pyramid five resistance loci and combine them with good grain types of donor common bean lines. Our efforts resulted in seven genotypes that have all resistance loci in a homozygous state already in the F_3 generation, and 84 genotypes which will undergo another generation of inbreeding and from which we will be able to select progenies with all resistance loci in a homozygous state in the F_4 generation. After finalization of this thesis, the grain types of the selected genotypes will be examined and yield trials in the F_6 generation will give us an indication of their performance in the field.

The resulting lines will be distributed among CIAT's African and Latin American partners to confirm resistance with the ALS pathotypes present in their respective field locations and with aggressive pathotypes in the greenhouse. Besides serving as a test for intercontinental ALS resistance, the lines will allow different breeding programs to introgress resistance genes into elite germplasm. The established molecular markers in this study (Table 1) were specific enough to target resistance loci within the used parental lines. In order to make them useful for breeding, molecular markers are needed that work in a diverse set of breeding material. In addition, for some loci pyramided in this study, more research to delimit their genetic position and determine their allelic series is necessary to allow confident introgression.

Phg-1 resistance locus may be present in AND 277 and CAL 143

Finding multiple highly source-specific molecular markers was achieved in the first attempt for four out of five resistance loci. However, for the *Phg-1* locus, an extensive search for a marker within the approximate genetic location of *Phg-1* that specifically targets AND 277 was not successful. This could have a biological reason as AND 277 and CAL 143 may both harbor the *Phg-1* locus, because CAL 143 derived from a G12229 x AND 277 cross and both, CAL 143 and AND 277, are highly ALS resistant [51]. In contrast, QTL mapping with CAL 143 as a parent using a single pathotype has revealed several QTL on different chromosomes, but none of these in proximity to *Phg-1* [29]. Despite the missing evidence from QTL mapping, which was conducted with only a single pathotype and hence may not have revealed all resistance loci contained in CAL 143, there is a 50% chance that CAL 143 has inherited the *Phg-1* locus from its parent AND 277. Furthermore, the fact that it was not possible to distinguish the genomes of AND 277 and CAL 143 in the *Phg-1* region is indicative that they share the *Phg-1* locus. For this study we therefore assumed that AND 277 and the CAL 143 share the *Phg-1* locus, however future studies that compare the resistance of the two parental lines are needed, to prove or disprove our assumptions.

Need for flanking markers to confidently assess resistance locus presence

In this study, we have incorporated the best available information to design molecular markers with shortest possible physical distances to the approximate location of the resistance loci (Supplementary table 1). However, in some cases these markers were not tightly linked and in order to assess the resistance locus more confidently, flanking markers are desirable [52]. To find flanking markers for MAS, the resistance loci need to be delimited to an interval where recombination occurs rarely, which can be achieved through fine- or high-resolution QTL mapping with high-density SNP markers [53, 54]. Flanking marker data is available for the ALS resistance loci *Phg-1*, *Phg-2* and *Phg-4* [18, 28, 55], but not for *Phg-3* and *Phg-5* [22, 28]. In addition, to find flanking molecular markers that specifically target the desired locus within a diverse parental population, high-density genotypic data is required [56]. Because of the limited SNP density of the available data from the SNP chip, this was not possible in this study. With the increasing availability of WGS data of common bean it should be possible to find source-specific SNPs, but until then, genotyping by sequencing data available at CIAT for most breeding lines may offer the possibility to find useful flanking markers within the population of intended crossing partners.

For two of the five resistance loci, further research is needed to delimitate their genetic position to an interval that allows reliable selection by MAS. The genetic position of the QTL *Phg-5* in G5686 is not delimitated as only a single molecular marker was reported to be associated with disease resistance [28]. Additional positional information is available for the *Phg-5* resistance locus in CAL 143 [29, 57], however besides a rough positional analysis, there is no evidence that the *Phg-5* loci in CAL 143 and G5686 represent the same gene, even though both loci were officially assigned the locus name '*Phg-5*' by the Bean Improvement Genetics Committee [15, 16]. Similarly, for the *Phg-3* locus of G10909, the only published marker is reported at a distance of 13cM [22]. A tightly linked molecular marker is available for the *Phg-3* locus in Ouro Negro [26], but again, no studies have assessed whether the two loci are the same or distinct in Ouro Negro and G10909.

Angular leaf spot pathotype-specificity and its implication for the established gene pyramid

The highly pathotype-specific reaction of common bean to the ALS pathogen has been known for a long time [32, 33, 38, 51, 58], but these interactions have only been systematically studied on the gene level for pathogen populations in Colombia and Uganda after the initiation of this study. Surprisingly, only two of the five resistance loci known and pyramided in this study were found to confer resistance against a broad selection of pathotypes in field and greenhouse studies [50]. The *Phg-2* resistance locus and its haplotypes were found to be the predominant locus, next to the *Phg-4* locus that was conferring resistance against a single pathotype [50]. This finding likely affects the durability of the resistance loci pyramid described in this study.

The theory suggests that by pyramiding several resistance genes, the pathogen is less likely to overcome the resistance of several genes at the same time, than it is to overcome the resistance of one gene [59, 60]. The boom and bust cycle frequently observed when only single resistance genes are deployed is therefore prevented or at least delayed [61]. During field and greenhouse trials in Colombia and Uganda it was observed that the resistance loci *Phg-1*, *Phg-3* and *Phg-5* were not effective [50]. Because these are three of the five resistance loci pyramided in this study, it signifies that the pathotypes in these field locations only need to overcome one or two resistance loci to become virulent again on our lines containing all five resistance loci. While the actual durability of a resistance pyramid can only be judged in retrospect because of the mostly unknown barrier function of resistance loci and the dynamics of pathogen populations, pathogen-specificity is an important aspect to consider when designing the gene pyramid.

In comparison to the durability of resistance, which can only be assessed after multiple years of field experience of a new cultivar, the resistance spectrum of pyramided loci is comparatively easy to assess. The lines resulting from this study will be tested with multiple ALS pathotypes in different continents and, because of the wide variety of qualitative and quantitative resistance genes incorporated, we expect a broad spectrum of resistance. In similar experiments in common bean, a superior ALS resistance to race 61-63 of plants containing five ALS resistance loci from four resistance donors was observed in comparison to the original resistance donors [62]. Two resistance loci selected by Ddamulira et al. [62], *Phg-1* from AND 277 and *Phg-4* from G5686, are the same as in the experiment described here. In another study, a pyramid of three rust resistance genes resulted in a high resistance to the three pathotypes that were specific for each resistance gene, without interfering with agronomic and grain characteristics [63]. However, none of the above-mentioned gene pyramids were systematically evaluated with a broad variety of pathotypes from different continents because of their recent availability. For the two available resistance gene pyramids in common bean, no epistatic effects that interfered with the effectiveness of resistance or affected agronomic qualities were described [62, 63].

In conclusion, forecasting the field performance of the common bean lines established in this study is difficult because it depends on the selection of resistance genes and the pathogen populations present in the fields [41, 60]. We have included two quantitative and three qualitative ALS resistance loci and the causal agent of ALS is thought to reproduce asexually, hence it will not frequently reshuffle different effector genes [64]. Pyramiding genes for such an asexual pathogen should be more or less stable. However, predicting the interaction of the *Phg-1* to *Phg-5* loci and the time it takes until they are overcome by new pathotypes is impossible, hence it remains to be seen how long their resistance can persist in the field.

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Supplementary material

Supplementary table 1: Position of molecular markers linked to angular leaf spot (ALS) resistance loci selected for pyramiding in common bean. Molecular markers reported to be linked to ALS resistance loci (named *Phg-1* to *Phg-5*) are listed with the common bean line they were discovered in. The genetic linkage of the marker to the resistance locus in centi Morgan (cM) is given where available and the physical position of the marker is given with chromosome (Chr) and position in base pairs (bp) on the common bean reference genome v2.1.

Marker name	Resistance locus and source	Genetic linkage	Chr	Position (bp)	Publication
CV542014	*Phg-1* in AND 277	0.7 cM	1	49,795,296	[18]
TGA1.1	*Phg-1* in AND 277	1.3 cM	1	50,022,788	[18]
ALS_08_61730261	*Phg-2* in G10474		8	61,730,261	[55]
ALS_08_62139256	*Phg-2* in G10474		8	62,139,256	[55]
Pv-gaat001	*Phg-3* in G10909	13.0 cM	4	9,432,363	[22]
Marker63	*Phg-4* in G5686		4	45,670,275	[28]
4M439	*Phg-4* in G5686		4	46,152,467	[28]
Marker 17	*Phg-5* in G5686		10	39,108,247	[28]

Supplementary table 2: Overview and number of common bean genotypes used in the selection process aiming at pyramiding five angular leaf spot resistance loci. This table describes for every generation the number of plants sown, genotyped, selected and where applicable crossed. The plant ID of the selected plants genotyped in #1 corresponds to the plant ID in the #2 section, where plots with seeds of the selected plants were sown. Plants selected in #2 were crossed in the same generation. In the #2 section, in addition to the plants that were selected from the plot it is indicated how many times they produced successful crosses. As an example, plant P84 was selected as promising in #1, the progenies of P84 were planted on the plot with ID 17ADB00246.000 and 35 plants on this plot were genotyped. Of the genotyped plants, four plants were selected for subsequent crosses and these four plants were involved in 27 successful crosses together.

Plant material sown	Genotyped plants	Selected plants
Genotyping #1		
F_1 4-way sown in Darien (6.5.2016) and Popayan (12.5.2016)		
43 seeds (AND277 x G10909) x (RAI62 x KATB1)	34	2 (Plant ID = P84, D14)
26 seeds (AND277 x G10909) x (RAI62 x CAL143)	14	2 (P107, P110)
54 seeds (AND277 x G10909) x (CAL143 x RAI62)	17	4 (D19, D23,D26, D39)
10 seeds (AND277 x G10909) x (RAI97 x CAL143)	5	0
64 seeds (AND277 x G10909) x (RAI97 x KATB1)	40	0
F_2 3-way sown in Quilichao on the 6.4.2016		
5 rows (G10909 x AND277) x KATB1	3	0
25 rows (G10909 x AND277) x CAL143	27	4 (Q14, Q31, Q33, Q59)
50 rows CAL143 x (G10909 x AND277)	41	6 (Q8, Q17, Q19, Q57, Q69, Q71)
Genotyping #2		
F_2 4-way sown in Darien on the 11.4.2017		
35 seeds from P84 (Plot ID = 17ADB00246.000)	35	4, crossed 27x
20 seeds from P110 (17ADB00248.000)	20	4, crossed 17x
11 seeds from D39 (17ADB00249.000)	11	1, crossed 1x
39 seeds from P107 (17ADB00250.000)	39	0
22 seeds from D19 (17ADB00252.000)	22	0
36 seeds from D23 (17ADB00254.000)	30	0
6 seeds from D14 (17ADB00256.000)	6	0
1 seed from D26 (17ADB00257.000)	1	0
F_3 3-way sown in Darien on the 11.4.2017		
28 seeds from Q8 (17ADB00223.000)	20	1, crossed 2x
16 seeds from Q14 (17ADB00225.000)	16	0
25 seeds from Q17 (17ADB00226.000)	25	13, crossed 21x
36 seeds from Q19 (17ADB00228.000)	26	0
10 seeds from Q31 (17ADB00230.000)	10	0
21 seeds from Q33 (17ADB00231.000)	19	0
36 seeds from Q57 (17ADB00232.000)	20	4, crossed 17x
21 seeds from Q59 (17ADB00234.000)	21	0
36 seeds from Q69 (17ADB00235.000)	33	6, crossed 13x
18 seeds from Q71 (17ADB00237.000)	18	0
Generation advance		
Intercrossing of selected F_2 4-way and F_3 3-way plants resulted in 49 successful crosses, which yielded 355 F_1 seeds. These seeds were planted in Darien on the 27.10.2017 and F_2 seeds were harvested		
Genotyping #3		
F_2 seeds of intercrossed 4-way and 3-way plants were planted in Palmira on the 12.2.2018	2668	32
Genotyping #4		
F_3 seeds from 32 plants selected in the third genotyping round were sown in Palmira on the 17.08.2018	309	91

Chapter 5

Introgression of Ascochyta resistance from *Phaseolus dumosus* to common bean (*Phaseolus vulgaris*) – insights from resistance evaluation in field and greenhouse

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Abstract

Ascochyta, caused by the fungus *Boeremia diversispora*, is an emerging common bean (*Phaseolus vulgaris*) disease in the tropical highlands and in temperate regions. Improving Ascochyta resistance by breeding is challenged by the lack of strong resistance in the common bean gene pool. Resistance however has been found in the extended gene pool of common bean and its sister species *Phaseolus dumosus* is considered immune to Ascochyta. Interspecific crosses of common bean and *P. dumosus* conducted at CIAT have recently resulted in lines combining enhanced levels of Ascochyta resistance with a common bean type grain.

Here, progenies of these putative Ascochyta-resistant, interspecific lines crossed to elite climbing common bean cultivars were evaluated in greenhouse and field trials. Greenhouse evaluations revealed a resistant reaction of the interspecific lines and F_2 families with a clear segregation for resistance were observed. Two of these families were advanced and partial inbred lines were tested for Ascochyta resistance in the field. The interspecific lines did not show a superior resistance over the elite cultivar in the field. In addition, the progenies exhibited a narrow phenotypic variation, as 82.1% of the experimental plots showed intermediate scores. In spite of that, four partial inbred lines with a repeatedly low disease score were identified that can be used for further breeding activities.

Our results emphasize the need for greenhouse evaluation methods that more accurately mimic field conditions to confidently select promising plant material. Furthermore, to tackle this emerging disease, a better understanding of the Ascochyta resistance mechanism in *P. dumosus* and its genetics is needed to transfer it to common bean.

Introduction

Ascochyta blights are the most important foliar diseases of legumes worldwide and responsible for high yield losses [1]. Breeding for Ascochyta resistance is a high priority in common bean (*Phaseolus vulgaris* L.), pea (*Pisum sativum* L.), chickpea (*Cicer arietinum* L.), lentil (*Lens culinaris* Medikus) and faba bean (*Vicia faba* L.) [2]. Although the symptoms are similar across crops and collectively referred to as Ascochyta, the causal agent, is specific for each crop species [1]. Because of the weak genetic resistance found in several grain legumes against Ascochyta, control is currently relying heavily on fungicides [3]. Chemical control is expensive and can lead to pesticide residues on the produce [4]. A more sustainable and feasible option is therefore to exploit the genetic resistances found in closely related species to improve the inherent genetic resistance of cultivars [5].

Ascochyta in common bean is caused by the ascomycete fungus *Boeremia diversispora* (Bubák) Aveskamp, Gruyter & Verkley, previously known as *Phoma exigua* var. *diversispora* or *Ascochyta phaseolorum* [6-8]. Ascochyta has been reported to be a locally important disease in the middle- and high-altitude common bean growing regions of Latin America and the Great Lakes region of Eastern Africa [9]. The causal agent of the disease, *B. diversispora*, thrives in the tropical highland's cool and humid conditions, where mostly climbing common bean cultivars are grown [9, 10].

Breeding for Ascochyta resistance is challenging because, despite extensive testing, no complete and only partial resistance could be detected in the common bean gene pool [9, 11]. Strong Ascochyta resistance has however been found in the secondary gene pool of common bean and all of the tested gene bank accessions of the sister species *Phaseolus dumosus* Macfady were resistant against Colombian pathotypes [12]. Crosses of *P. dumosus* with common bean have been achieved, but combining Ascochyta resistance with marketable grain types and acceptable agronomic properties in yield and growth habit have been very hard to obtain [13].

After some attempts with limited success in the 1990s, the increasing importance of the disease in tropical and temperate regions has sparked new investigations. By now, Ascochyta has reached southern Europe and has been observed in Greece and Spain [14, 15]. In Spain and Rwanda, several locally resistant common bean varieties were reported [15, 16]. Also in Latin America, Ascochyta disease incidences have increased and breeding has been taken up again (H. Buendia, personal observation). At CIAT, recent efforts involving a cross of *P. dumosus* to common bean now seem to have resulted in successful combination of Ascochyta resistance and acceptable grain types (S. Beebe, personal communication).

The main objective of this study was to investigate the inheritance of Ascochyta resistance of the interspecific, CIAT-bred lines. Specifically, we aim at i) evaluating F_2 families derived from crosses of interspecific lines with elite climbing common bean lines in the greenhouse and ii) evaluating partially-inbred lines of the two best families for Ascochyta resistance under field condition.

Materials and methods

Plant material

Interspecific lines in the F_{15} generation, originating from a cross of *P. dumosus* G35575 to the Andean common bean cultivar CAL 96, were selected for grain type and Ascochyta resistance in Popayan (C. Cajiao, unpublished data). Crosses of common bean with *P. dumosus* usually result in undesired grain types, hence lines combining acceptable grain type and increased resistance for Ascochyta were chosen and coded ASC at CIAT (C. Cajiao, personal communication). Three ASC lines (ASC 144, ASC 145 and ASC SCO 1331D) were crossed to elite climbing common bean lines (Cargamanto Blanco, ENF 25, ENF 26) and eight F_2 families were evaluated in the greenhouse. The ASC 144 line was segregating for flower color, hence the crosses were labelled either as flor blanca (white flower) or flor morada (purple flower), depending on the flower color of the ASC 144 parent involved. Families with pedigree ENF 26 x ASC 145 and ENF 26 x ASC 144 flor blanca were advanced by single seed descent to F_3 and their progenies (referred to as $F_{3:4}$) were used for field testing.

Greenhouse evaluation

In order to evaluate Ascochyta resistance in the greenhouse, trials were conducted at CIAT headquarters in Cali, Colombia (3°30'10.6"N 76°21'18.2"W, 967 m a.s.l.) under natural light regime at ambient temperature. The average temperature in Cali in May, when the greenhouse trial was conducted, is 23.9°C (avg. max. and min. temperatures are 29.3 – 19.2°C, [17]). Ten pots per treatment per F_2 family were planted with three seeds per pot and grown for 17 days before inoculation. For each parental line, one pot containing three seeds was planted. Inoculations were conducted with *B. diversispora* isolate ASC 1 at a concentration of 10^6 conidia per milliliter according to CIAT guidelines [18]. Two treatments were applied: 1) spraying of trifoliate leaves with inoculum and 2) spraying trifoliate leaves with inoculum and mechanical damage (MD) by punching each leaf twice with a cork that has four nails sticking out. After inoculation, the plants were put in a humidity chamber for 10 days to ensure good development of the disease. Disease scores were evaluated 13 days after inoculation on a 1 to 9 scale, whereas scores of 1-3 were considered resistant, 4-6 intermediate and 7-9 susceptible [19].

Field evaluation

Field-testing of partially inbred lines with two different pedigrees was conducted at CIAT field station in Popayan, Colombia (2°31'02.1"N 76°38'05.4"W 1753 m a.s.l). Average temperatures in Popayan from November to January, when the field trial was conducted, were 18.7°C (avg. max. and min. temperatures are 13.6 – 24.0°C, [17]). Onehundred single seed descended lines in the $F_{3:4}$ generation were used per family with the pedigrees ENF 26 x ASC 145 and ENF 26 x ASC 144 flor blanca. Lines were sown on November 16, 2017 in a complete randomized block trial with three replicates. A replicate consisted of one row with ten seeds sown, which were thinned to eight plants after emergence. Row lengths were 1 m and the spacing between rows was 0.6 m. Inoculations with *B. diversipora* isolate ASC 1 were conducted according to CIAT guidelines at 34, 49 and 61 days after sowing [18]. Disease scores were evaluated 75 and 85 days after sowing

on a 1 (resistant) to 9 (susceptible) scale established by van Schoonhoven and Pastor-Corrales [19].

Data analysis

Phenotypic results were analyzed and visualized with R version 3.4.4 [20] using the packages lme4, psych and ggplot2. To calculate heritability, the genotypic and residual variances were estimated by fitting a linear mixed model in R with the genotypic effect as random effect. Broad sense heritability was calculated by dividing genetic variance (Vg) by the phenotypic variance (Vp): h^2=Vg/Vp. The phenotypic variance is calculated as the sum of the genetic variance and the residual variance (Vres) divided by the number of replicates (n): Vp=Vg+ Vres/n according to Piepho & Möhring [21].

Results

Segregation of Ascochyta resistance in greenhouse trials

Crosses of interspecific, putative Ascochyta-resistant ASC lines with elite climbing common bean lines were evaluated in the F_2 generation in a greenhouse trial (Table 1). Plants subjected to the 'spray' treatment generally appeared resistant and only few plants showed intermediate or susceptible reactions. In the 'spray + MD' treatment, the plants were more affected by the disease and different segregation ratios were observed. The ASC parental lines and the *P. dumosus* resistance donor (G35575) were highly resistant. The elite climbing common bean lines showed different levels of resistance: In the 'spray + MD' treatment, Cargamanto Blanco was susceptible, ENF 26 intermediate and ENF 25 resistant, while in the 'spray' treatment, most parental lines were resistant. The F_2 families with pedigree ENF 26 x ASC 145 and ENF 26 x ASC 144 flor blanca were selected because of the difference in resistance of their parental lines and the clear segregation into resistant and susceptible plants in the F_2 generation, when subjected to the 'spray + MD' treatment. These two families were advanced for future resistance evaluations.

Table 1: Distribution of resistance phenotypes of interspecific ASC lines, elite climbing common bean lines and the progeny of crosses between them. In a greenhouse trial, F_2 families were evaluated for their resistance to Ascochyta. Plants were subjected to two different treatments: 1) Spray inoculation and 2) spray inoculation and mechanical damage (MD). Ascochyta disease symptoms were evaluated on a 1 to 9 scale, with disease scores 1-3 considered resistant (Res), 4-6 considered intermediate (Int) and 7-9 considered susceptible (Sus). The two families marked in bold were advanced for field testing.

Plant material	Spray Res:Int:Sus	Spray + MD Res:Int:Sus
Pedigree of F_2 plants		
Cargamanto blanco x ASC 144	9:15:0	0:0:28
ASC 144 x Cargamanto blanco	14:10:0	1:9:14
ENF 25 x ASC 144	8:18:0	11:10:1
ENF 25 x ASC 145	1:20:7	22:3:4
ENF 26 x ASC 145	**30:0:0**	**10:7:10**
ENF 26 x ASC 144 flor blanca	**24:0:0**	**13:4:6**
ENF 26 x ASC 144 flor morada	29:0:0	19:2:1
ENF 26 x ASC SCO 1331D	28:0:0	21:1:2
Parental lines		
ENF 25	0:2:0	3:0:0
ENF 26	3:0:0	0:3:0
Cargamanto blanco	3:0:0	0:0:3
ASC 144	3:0:0	3:0:0
ASC 145	3:0:0	2:0:0
ASC Sco 1331D	2:0:0	2:0:0
G35575	2:1:0	3:0:0

No effective Ascochyta resistance observed in field evaluation

Replicated field testing of partially inbred lines with two different pedigrees was conducted under artificial Ascochyta inoculation in Popayan, Colombia. Environmental conditions were conductive for disease development and because of the high correlation of the resistance evaluations 75 and 85 days after planting (Pearson correlation, $r = 0.75$), only the results of the evaluation on day 85 are shown in the subsequent analyses unless mentioned otherwise (analyses for the evaluation on day 75 are given as supplementary figures 1 and 2). The range of observed disease scores in individual plots ranged from 3 to 8 and the correlations between the replicated blocks were between 0.38 - 0.54 (Pearson correlation, Figure 2). Analysis of variance analysis revealed a significant effect ($p < 0.05$) of pedigree, line and block on the Ascochyta score ble 1). The calculated broad sense heritability for Ascochyta resistance in the trial was $h^2 = 0.71$.

The parental lines did not show the expected contrasting Ascochyta resistance pattern (Figure 1): The putative Ascochyta resistant parents ASC144 and ASC145 had mean disease scores of 5.3 ± 1.2 and 6 ± 1.0, respectively, while the susceptible parent ENF 26 had a mean disease score of 6 ± 1.0. The ASC 144 and ASC 145 parental lines were therefore not significantly more resistant under field conditions than the elite cultivar ENF 26 (Student's t-test**,** $p = 0.49$ and $p = 1.00$, respectively). Still, there are four lines (13, 108, 165 and 174) with a consistently low disease score of 4 over the three replicates (Figure 1).

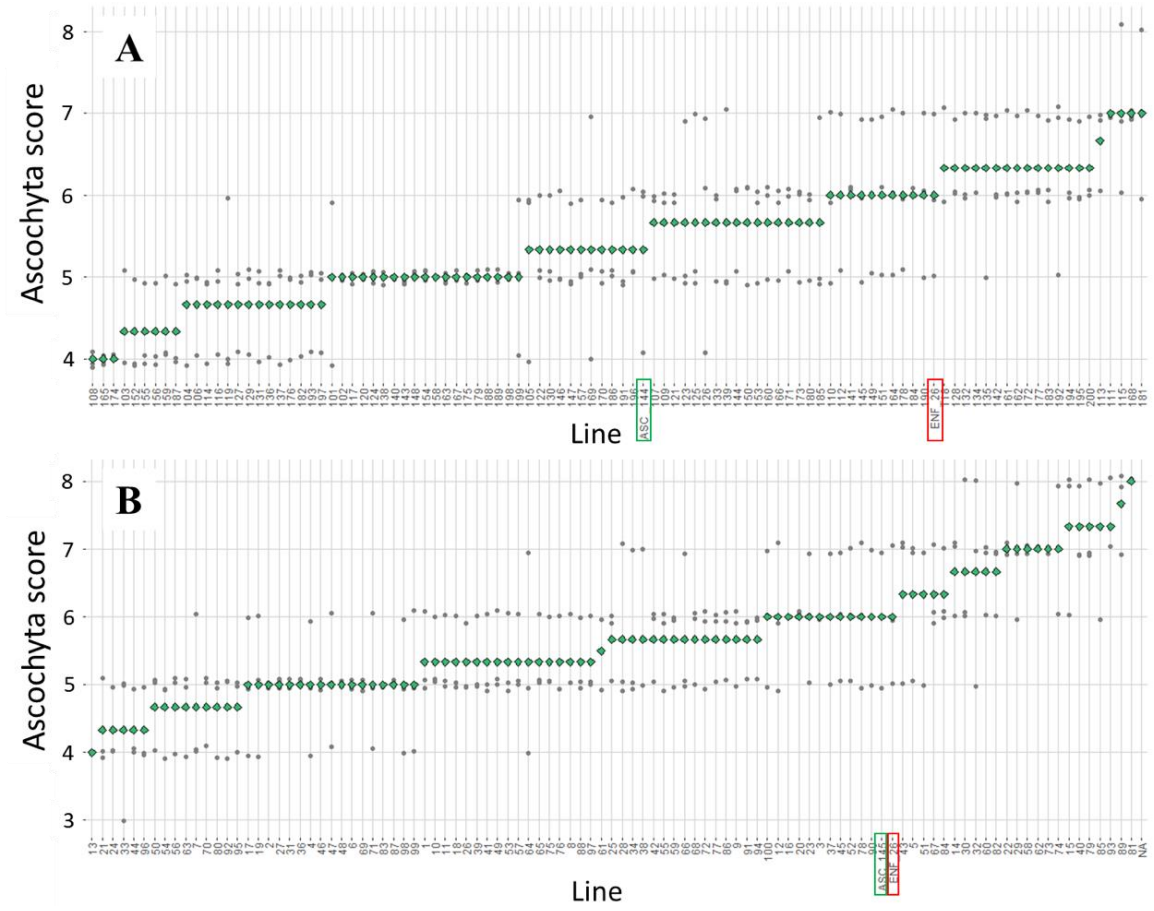

Figure 1: Ascochyta field resistance of partially inbred lines with a pedigree of ENF 26 x ASC 144 (A) and ENF 26 x ASC 145 (B). Experiments were conducted under artificial Ascochyta inoculation in Popayan, Colombia. Disease scores were obtained 85 days after planting on a 1 to 9 scale, with 1 being resistant and 9 susceptible (y-axis). For each partially inbred line (x-axis), the Ascochyta score of each replicate is shown as a grey dot and the mean score over the replicates as a green diamond. The parents ASC 144 (A) and ASC 145 (B), originating from interspecific crosses of common bean to *Phaseolus dumosus*, are marked with a green box and the elite climbing common bean parent ENF 26 with a red box.

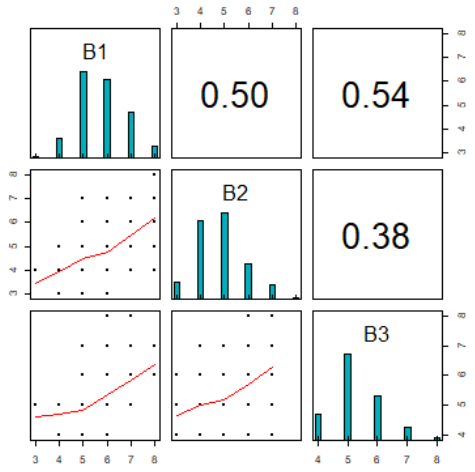

Figure 2: Correlation and distribution of Ascochyta disease scores of partially inbred lines between the three replicate blocks (B1 - B3) in the field experiment. Experiments were conducted under artificial Ascochyta inoculation in Popayan, Colombia. Disease scores were obtained 85 days after planting on a 1 to 9 scale, with 1 being resistant and 9 susceptible. In the upper diagonal, Pearson correlations between blocks are shown, in the diagonal, histograms of Ascochyta scores in each block are given, and in the lower diagonal, Ascochyta scores of two blocks are plotted against each other with the red line representing the LOESS (locally estimated scatterplot smoothing) line.

Discussion

Ascochyta resistance in interspecific lines shows similarity to Ascochyta resistance in other legumes

This study revealed a complex inheritance of Ascochyta resistance in CIAT-bred lines that originated from a cross of a resistant *P. dumosus* to a susceptible common bean. The two interspecific lines used in this study were the result of extensive testing at CIAT and were thought to be the first lines combining both Ascochyta resistance and acceptable grain types. Our research, however, has shown that their resistance is highly dependent on the evaluation conditions as they exhibited strong resistance in greenhouse trials but only intermediate resistance in field trials. In addition, evaluation of the progenies of a cross with elite climbing common bean lines revealed a highly quantitative pattern of resistance inheritance.

The host-pathogen interaction and genetic control of the Ascochyta resistance in interspecific lines are not well understood, but exhibit some striking parallels to the Ascochyta diseases in other legumes, where resistance is difficult to find in breeding germplasm and highly quantitative. In pea and chickpea, only low to intermediate levels of resistance have been observed and resistance is highly quantitative, with more than 30 QTL identified spread across all chromosomes in both crops [22-26]. In lentil and faba bean, resistance is conferred by a combination of major and quantitative resistance genes and although resistant varieties are available, their resistance is frequently overcome by new pathotypes [27-29]. Comparisons of the mode of resistance, phenotyping strategies and integrated pest management strategies against the Ascochyta disease in other legumes may offer solutions that can be transferred to the common bean crop.

Disparity of Ascochyta resistance in greenhouse and field evaluation

Greenhouse screening protocols that accurately predict resistance under field conditions are crucial to justify their use. Through carful development of such screening techniques and frequent reassessment of these techniques, accurate prediction of field performance can be achieved [30]. In our study, however, large disparities in the effectiveness of Ascochyta resistance in common bean were found when experiments were conducted in greenhouse and field conditions according to the CIAT standard evaluation method. While the difference of the generation in which the material was tested, F_2 in greenhouse and F_4 in the field, may has influenced the resistance of the population, the parental lines were included in both tests and also showed an inconsistent resistance pattern under the two testing schemes.

In the greenhouse trial, the ‘spray’ treatment was generally not sufficient to induce disease symptoms, but in combination with MD the fungus was able to colonize plants and cause characteristic disease symptoms. The latter treatment is more comparable to field conditions, where insects and wind cause small wounds that help leaf colonization of the pathogen [12]. Another reason that may have contributed to the disparity in Ascochyta resistance, is the difference in ambient temperature of the field and greenhouse site located at 1753 m and 967 m a.s.l., respectively. The causal agent of Ascochyta, *B. diversispora*, prefers a cold and humid climate [31] and because of the limited possibility to control temperature and humidity in the greenhouse used in Cali, plants had to be placed in a

humidity chamber for ten days to ensure pathogen infection (V. Arredondo Domínguez, personal communication). This may have created an environment favorable enough for the fungus to colonize damaged leaves, but the disease pressure in the greenhouse experiment was probably was much lower compared to field conditions.

The contrasting observation of Ascochyta resistance in greenhouse and field experiments requires more attention and further research is needed to establish a reliable screening technique. Screening for Ascochyta resistance in other legumes has previously emphasized the importance of the environmental conditions, inoculum concentration and age of plants used on the ability of these screens to predict field resistance [32, 33]. In chickpea an efficient screening technique based on seedling or cut-twigs has been established that allowed the prediction of field performance with a correlation of $r = 0.88$, but required the setup of a special screening chamber with controllable conditions [34]. For Ascochyta resistance determination in common bean, screening techniques involving either cut-twig, leaf material or seedlings [15, 34] may be more feasible as they require less space and can be conducted in growth chambers that better allow to control climate variables than the greenhouse used in this study.

Interspecies transfer of Ascochyta resistance to common bean resulted in intermediate resistance levels under field conditions

Opposite to our expectations, the ASC lines were not highly resistant and only showed intermediate resistance in field trials. Furthermore, they were not significantly more resistant than the elite breeding line ENF 26. A possible explanation for our observation may be, that even though the greenhouse and field trials were inoculated with the same pathotype, aggressive pathotypes naturally occurring in Popayan may have co-inoculated the trial. Another reason could be that the usually favorable conditions for disease development in Popayan in combination with the heavy Ascochyta inoculations were too strong for the resistance of the ASC lines. To have a reference point of the maximal disease pressure that could possibly be withstood, the resistance donor G35375 should have been included as a control line.

Similar to the narrow range of observed disease scores in field experiment in the parental lines, the Ascochyta scores of the progenies showed a narrow range and 82.1% of the experimental plots had a disease score between 4 and 6. This range of intermediate scores is the most difficult to evaluate, because the leaves are severely diseased and are scored on a qualitative scale, while the disease progression is continuous. Hence, the differences between an early 5 and a highly diseased 4 are very small and even though the scoring was done by scientists with experience in scoring common bean diseases, slight deviations of the scores are common [35]. To overcome this limitation of inaccurate visual phenotyping, digital photography and hyperspectral imaging in combination with image analysis pipelines implementing recent advances of machine learning will be useful [36, 37]. Once implemented, these techniques can give a more accurate and quantitative assessment of disease symptoms, including percentage of leaf covered by lesions, lesion size and lesion number, that are possibly under different genetic control [38].

Despite the narrow range of observed disease scores and the difficulty in scoring intermediate-resistant plants, the high heritability of $h^2 = 0.71$ indicates a surprisingly high genetic component of resistance. This suggests that multiple resistance loci with small effects are responsible for the observed differences of resistance. Through the crosses of

P. dumosus to the common bean lines CAL 96 and subsequently to ENF 26, several quantitative resistance loci may have been lost and hence, the resulting lines did not exhibit strong resistance. A similar observation of dilution of Ascochyta resistance by subsequent crosses has been observed in previous studies that resulted in only intermediate Ascochyta resistance [39].

Non-host resistance might be conferring Ascochyta resistance in *Phaseolus dumosus*

Ascochyta is an important common bean pathogen, but our understanding of the causal agent *B. diversispora* and the mode of resistance in *P. dumosus* are limited. The pattern of Ascochyta resistance in common bean has been described as highly quantitative [39], which is in accordance with our observations. The observed quantitative resistance pattern does not point to a gene for gene interaction that is characteristic for qualitative, effector-triggered immunity but rather a pathogen-triggered immunity that is often exhibiting incomplete resistance [40, 41]. Because of the observation that all *P. dumosus* lines are resistant while all common bean are susceptible [12], resistance may be conferred through non-host resistance (NHR). NHR is the most common, broad-spectrum and durable resistance, as most plant species are non-hosts to most pathogens [42]. The mechanism of NHR is however not as well understood as host resistance and usually involves several layers of defense [42, 43]. To determine non-host status of *P. dumosus* to Ascochyta, a larger collection of *P. dumosus* lines should be tested, preferably in combination with histological studies that may allow the observation of the stage of the colonization process where the resistance is effective [44].

Studying NHR is usually hampered by the crossing incompatibility between species to produce families segregating for the trait of interest [45]. Common bean and its cross-compatible secondary and tertiary gene pool domesticated species are hence a very interesting system to study the inter-species transfer of disease resistance and potentially NHR. In the case of Ascochyta resistance, several previous studies have attempted resistance introgression through crosses that involved at least two common bean genotypes and selection for Ascochyta in later generation. So far these have not been successful and only led to intermediate resistant lines [39, 46, 47]. A more promising solution may be to conduct QTL mapping directly with the cross of *P. dumosus* and common bean, instead of trying first to introduce the trait of interest in the common bean background. This would give a first indication on the extent of quantitative resistance that is involved [48]. In a next step, the most important QTLs could then be introgressed by marker-assisted selection in combination with a backcrossing scheme. This strategy would allow the selection of individuals containing only the desired interspecific introgression and may also be better suited to overcome the often observed hitch-hiking of undesired characteristics by linkage drag of interspecific introgressions [49].

The immense genetic diversity that potentially could be transferred from sister species to common bean is frequently highlighted when the limits of common bean improvement are discussed [50, 51]. In practice, despite some successes in the incorporation of abiotic stress tolerance [52], the transfer of disease resistance from the secondary gene pool to common bean has only resulted in incomplete resistance for bacterial blight, fusarium root rot and white mold [50]. The rate of successful trait transfer from the extended gene pool to common bean may be increased through systematic studies of the genetic causes of the trait before trying to combine it with agronomic qualities.

The quest for Ascochyta resistant common bean is to be continued

Conclusively, the search for stable and efficient Ascochyta resistance in common bean needs to be continued. Because of the described findings that ASC lines were not more resistant than the ENF26 parental lines and the narrow range of observed phenotypes, genotyping and subsequent QTL mapping, which was the initial goal of this study, was not promising and hence not conducted. Instead, we focused on identifying suitable lines with partial resistance for the breeding program.

The true value of the Acochyta resistance in ASC lines could not be determined in this study because of the observed large difference in efficiency in field and greenhouse experiments. Therefore the ASC lines should be further tested under different conditions to get a more accurate assessment of resistance. In addition to that, other sources of Ascochyta should be explored. Recently, Ascochyta resistant common bean lines have been reported from Spain and Rwanda [15, 16] and the usefulness of their resistance against Colombian strains should be investigated. Further efforts should also focus on improving and testing the screening protocols to give comparable results in field and greenhouse conditions. These protocols should then be used to screen diverse germplasm from the primary and secondary genepool as well as interspecific crosses for Ascochyta resistance. Because of the durability and efficiency of resistance conferred by NHR, systematic investigation of the extended common bean gene pool for NHR and its genetic causes should be continued.

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Supplementary material

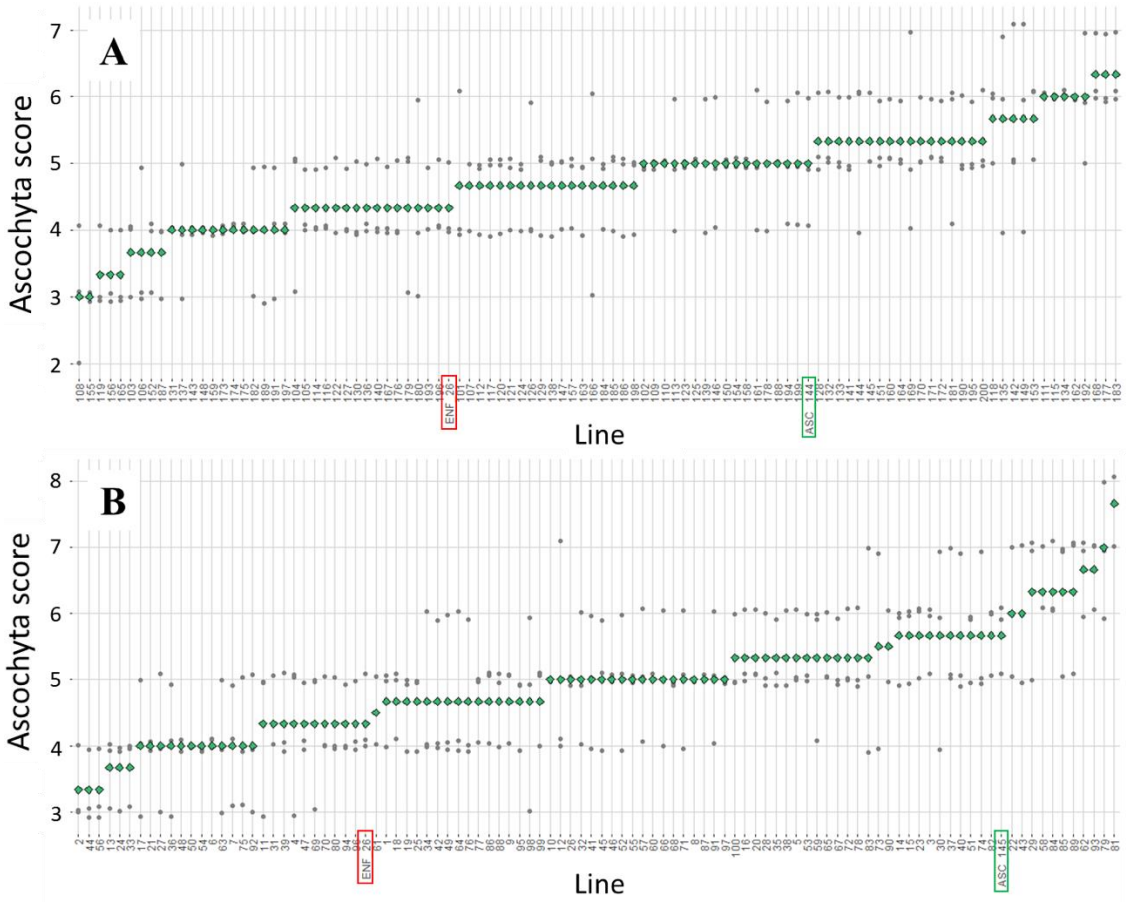
Supplementary table 1: Analysis of variance (ANOVA) of the effect of pedigree, block and line on the Ascochyta resistance score. The ANOVA analysis were conducted for the evaluation on day 75 (upper panel) and day 85 (lower panel) separately. In the ANOVA tables, the partitioning of variance according to their source is listed.

Source of variation	df	Sum of Squares	F-value	*p*-value
Pedigree	1	5.7	9.921	0.001759
Line	198	355.1	3.097	< 2e-16
Block	2	8.8	7.563	0.000598
Residuals	395	228.7		

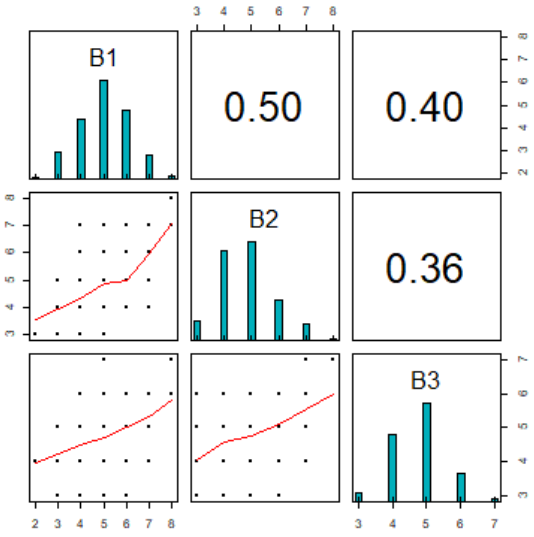
12 observation deleted due to missingness

Source of variation	df	Sum of Squares	F-value	*p*-value
Pedigree	1	5.3	11.084	0.000956
Line	197	350.8	3.725	< 2e-16
Block	2	17.3	18.130	3.02e-08
Residuals	379	181.2		

29 observation deleted due to missingness



Supplementary figure 1: Ascochyta field resistance of partially inbred lines with a pedigree of ENF 26 x ASC 144 (A) and ENF 26 x ASC 145 (B). Experiments were conducted under artificial Ascochyta inoculation in Popayan, Colombia. Disease scores were obtained 75 days after planting on a 1 to 9 scale, with 1 being resistant and 9 susceptible (y-axis). For each partially inbred line (x-axis), the Ascochyta score of each replicate is shown as a grey dot and the mean score over the replicates as a green diamond. The parents ASC 144 (A) and ASC 145 (B), originating from interspecific crosses of common bean to *Phaseolus dumosus*, are marked with a green box and the elite climbing common bean parent ENF 26 with a red box.



Supplementary figure 2: Correlation and distribution of Ascochyta disease scores of partially inbred lines between the three replicate blocks (B1 - B3) in the field experiment. Experiments were conducted under artificial Ascochyta inoculation in Popayan, Colombia. Disease scores were obtained 75 days after planting on a 1 to 9 scale, with 1 being resistant and 9 susceptible. In the upper diagonal, Pearson correlations between blocks are shown, in the diagonal, histograms of Ascochyta scores in each block are given, and in the lower diagonal, Ascochyta scores of two blocks are plotted against each other with the red line representing the LOESS (locally estimated scatterplot smoothing) line.

Chapter 6

General Discussion

The role of common bean as a nutritious food crop for the future

Common beans (*Phaseolus vulgaris* L.) are the predominant source of protein and micronutrients for the poor in many African and Latin American countries [1, 2]. In Africa, although the percentage of people qualifying as poor has decreased, the absolute numbers have increased and 43% of the population was considered to live in poverty in 2012 [3]. Due to the continuing high population growth and the unstable economic situation in many African countries, common beans will continue to be an important food security crop [2, 4]. Additionally, in Latin America, poverty is on the rise and around 31% of the population is affected [5], hence the demand for common beans will likely increase. Furthermore, common beans are the central ingredient for many traditional dishes in most Latin American countries.

On both continents, Africa and America, common beans have special importance in conflict stricken regions to ensure food security. In South Sudan, refugees were handed out common bean seeds to reduce reliance on food aid and in Rwanda they were an important crop to recover after the genocide because of their short cycle of return in contrast to cassava [6-9]. In Nicaragua, a small survey revealed that 60% of farmers planted common beans before the conflict in the 1970s and that 100% of them planted common beans during and after the conflict [10]. In Colombia, efforts are underway to make best seed genetics available to help farmers in rural areas recover from the over 50-year long conflict between guerilla groups and the government [11].

While the focus of this thesis is mostly on the importance of common bean as a food security crop in the tropics, common beans are becoming increasingly popular in the northern hemisphere. The change in lifestyle towards a flexitarian, vegetarian or vegan diet has brought attention to the pulses as a protein source in an amino acid balanced diet [12]. While common bean has suffered acceptance problems in the Global North because of the long cooking time and the flatulence it can cause for some people, with the rise of 'super-foods' consumers are willing to change from fast-food to more traditional foods. Dry beans, with their high-fiber, high-protein, low-fat and nutrient-dense grains are destined to become the new 'super-food' and several studies have shown their beneficial nutritional effects [13-15]. Breeders, food scientists and geneticists are currently working towards an increased acceptance of common beans within the broader public. Breeding for a short cooking time is underway and will be a welcomed trait in rural Africa as well as in the fast-paced, developed world [16] (B. Raatz, personal communication). In addition to the traditional canning of common beans, processing technologies like extrusion cooking allow the conversion of common beans and other legumes into a wide array of products with distinct textural, nutritional and sensory characteristics. Examples of such processed products, where pulses can improve the nutritional quality, are snacks (chips), textured vegetable protein and pasta [17]. Furthermore, geneticists are aiming at modifying the biosynthetic pathways to make common beans more digestible and reduce flatulence [18].

With the increasing importance of common bean in the Global North and the levels of poverty not expected to decrease in Africa and Latin America in the coming years [3, 5], the demand for common bean as a nutritious grain legume will continue. Common bean breeding for the temperate and tropical region will be crucial to ensure a stable and healthy supply of common beans. Because of the lengthy process required to produce varieties ready for distribution to farmers, common bean breeders have to anticipate future developments to have suitable varieties ready.

Plant diseases – a major challenge for common bean production in the future

An important future development to consider for common bean breeding are the projections that forecast increasing importance of plant pathogens [19]. Plant pathogen populations are highly dynamic and in a constant evolutionary arms race with prevalent cultivars to overcome resistance [20]. The evolutionary potential of pathogens to overcome resistance in plant cultivars is affected by many factors that, among others, include their reproduction system, population sizes and their efficiency of dispersal [21]. The latter two factors will be affected by the projected changes in climate and crop production, that are expected to affect common bean producing countries, and may result in a higher pathogen pressure [22].

The causal agent of the ALS disease, *P. griseola*, is a tropical pathogen with no known sexual reproduction that requires a temperature of 16 to 28°C to infect and cause the characteristic disease symptoms on common bean plants [23]. Due to global warming, average temperatures will increase and more extreme weather patterns are expected [24, 25]. Hence, *P. griseola*'s geographic range of suitable habitat will shift and likely expand polewards. The fungus *P. griseola* has shown high survival rates in soil and on plant debris [23], which will be a useful feature to survive unfavorable conditions and thrive again in good years. An additional factor affecting the spread of the pathogen is globalization. The increased mobility of humans, agricultural produce and seeds will lead to a globalization of pathogens and pathogen races that were previously separated by oceans or mountain ranges [22, 26, 27].

While climate and globalization affect the spread and geographic range of the pathogen, changing cultivation practices influence the abundance of suitable host plants. Today, smallholder farming in Africa and Latin America is very heterogeneous and small patches of land, often considerably smaller than two hectares, are planted with a variety of crops [28]. An estimated 90% of seeds used by farmers in Sub-Saharan Africa come from the informal market with most of the seeds being landraces obtained on the local market or saved from last year's harvest [29]. The combination of small cropping plots and the high genetic diversity of landraces planted in the field has probably been a limiting factor of virulence evolution so far. With the increased market demand for common beans, cultivation as a cash crop will increase [30, 31]. This shift is often associated with an intensified production that includes the adoption of high-yielding varieties and increased production as monoculture [32]. Because of the projected loss of traditional cultivars and the focus on a few high yielding cultivars, the directional selection on pathogens will be enhanced and once the resistance of commonly used cultivars is overcome, pathogens can spread unhindered [33]. This process has already been observed in Brazil, which pioneered the large-scale common bean production in the tropics. Brazil currently has a high disease

pressure and the most aggressive races of *P. griseola* [34-36]. Another example of ALS becoming an important disease due to the intensification of common bean production comes from Central America. ALS had been a minor disease in Central America, however with the introduction of new common bean varieties from CIAT combining high yields with resistance to bean common mosaic virus and rust, but lacking ALS resistance, this disease progressively became a major constraint in Central America (M. Pastor-Corrales, personal communication). The two examples from Brazil and Central America are just a glimpse of the pathogenicity the ALS pathogen has evolved with the shift from small-scale to intensive, large-scale production. While the causes of this are unknown, investigations into the genetic population structure of *P. griseola* would probably give some hints that could explain the dynamics that led to the outbreaks.

While the above described processes focused on the ALS disease, which was the main subject of this thesis, many of the processes involved apply conceptually to other tropical common bean and crop diseases. Given the favorable prospects for tropical the pathogens, a thought-through concept to manage the ALS disease and other common bean diseases in the future is urgently needed to ensure resistant cultivars are available and knowledge bases are established before the disease becomes a major threat.

Disease management strategies for durable resistance

Disease management strategies take a holistic approach and include on-field management as well as genetic tools to minimize the damage of plant pathogens. Management options include adjusting planting time and location to avoid pathogen-conductive environments, practicing field hygiene and planting of disease-free seeds to minimize inoculum levels, and the application of agrochemicals to prevent infection or cure infected plants [37]. While these methods are knowledge- or capital-intensive, genetic tools, specifically the planting of disease resistant cultivars, does not rely on special training or recurrent acquisition of agrochemicals for disease control and is therefore an important pillar in managing crop diseases on smallholder farms.

Breeding for fungal diseases, and biotic stresses in general, is thwarted by the virulence diversity and evolution of pathogen populations. Single-gene resistance cultivars are usually succumbing to new virulent pathotypes within a few years after establishment [38]. More promising are strategies based on several resistance genes that are applied dynamically to avoid directional selection of the pathogen [33]. This can be achieved by combining (pyramiding), rotating or mixing (multiline cultivars) different resistance genes [39]. Rotations of cultivars and multiline cultivars aim at reducing the directional selection in pathogen populations while pyramiding resistance genes aims at challenging the pathogen with several barriers (resistance loci) at the same time instead of single barriers that it can easily overcome [39]. In order to select the most efficient resistance loci, a sound understanding of the available resistance sources in the host gene pool, the resistance loci contained and their efficacy in target areas is crucial.

For the common bean disease ALS, several resistance sources and loci have been identified, but the studies described in chapter 3 of this thesis are the first to describe the pathotype-specific interaction on the genetic level. We discovered that two ALS resistance loci and several haplotypes within one resistance locus are conferring resistance against a diverse collection of single pathotypes and pathotype populations in two countries, Colombia and Uganda. To aid implementation of these findings, a toolbox was established,

which involves a collection of the most ALS-resistant lines, information about their resistance against different pathotypes on two continents and molecular markers linked to resistance loci and haplotypes (Appendix 1). This toolbox will greatly help breeders in Colombia and Uganda to select appropriate resistance sources. Furthermore, the collection of ALS resistant common bean germplasm is available on request from CIAT and can be used to provide an overview on effective resistances in new target regions. A detailed description on how to use these resources including phenotypic information of the grain types of common bean lines contained in the collection and scripts to analyze the data is given in the appendix 1.

Because of the finding that haplotypes at the same resistance locus are mainly conferring resistance, pyramiding of these loci is either impossible or very difficult, depending on whether the causal genes are allelic series or different genes located closely together, respectively. Currently, the only feasible strategy to increase durability of resistance are multiline cultivars or frequent rotations of cultivars. Future studies should therefore urgently aim at the discovery of new sources of resistance, since the resistance of all haplotypes is overcome in at least one trial in chapter 3. In the future, knowledge of the causal genes conferring resistance and the increasing availability of genetic engineering techniques may allow pyramiding resistance loci cis-genetically as has been successfully demonstrated in potato and wheat [40, 41].

Specifically for common bean, which is reportedly co-evolving with its diseases, evolutionary-based disease management strategies have been proposed. Guzmán et al. [42] suggested to plant Andean common bean cultivars where Mesoamerican pathogens prevail and vice versa. In addition to that, Pastor-Corrales et al. [43] suggest to pyramid Mesoamerican and Andean resistance loci. While the first strategy may not be very promising based on the increased exchange of pathotypes due to globalization [26], the latter strategy was applied and described in chapter 4. We pyramided five resistance loci, three of the Andean and two of the Mesoamerican gene pool and found 91 common bean genotypes that contained all five resistance loci. The common bean lines that will be selected from the progenies of these genotypes are expected to confer broad-spectrum resistance through the five resistance loci incorporated. However their resistance may not be more durable than single gene resistance, because of the findings in chapter 3, which showed that only one or maximal two resistance loci are involved in resistance against the pathotypes tested on two continents. Nonetheless, whether such a pyramid confers durable resistance can only confidently be assessed in retrospect after several years of field-testing. Meanwhile, an indication on the durability is given by the resistance mechanism involved, as different resistance mechanisms generally differ in their durability.

Resistance mechanisms affect durability of resistance

Different disease resistance mechanisms have been discovered in plants with distinct modes of action. Non-host resistance (NHR) is considered the most durable and effective resistance, since plants are non-hosts to most pathogens [44, 45]. Pattern-triggered immunity (PTI) is generally exhibiting broad spectrum resistance, but can be overcome by pathotypes especially if they have evolved together [46]. Effector-triggered immunity (ETI) is considered the least durable and is the product of specialized interaction between a specific host genotype and a specific pathotype [47].

For the Ascochyta disease resistance studied in chapter 5, we proposed a possible involvement of NHR in the secondary gene pool species *P. dumosus*. Transferring this immunity to common bean was not entirely successful and only intermediate resistance was achieved. Because of the quantitative nature of Ascochyta resistance in *P. dumosus*, subsequent crosses to two different common bean lines may have diluted resistance in our study similarly to previous observations [48]. Because of its enormous potential to confer durable and broad-spectrum resistance, NHR studies for Ascochyta should be continued. NHR is not as well understood as host resistance and does not follow certain patterns as the latter, but rather is mediated through multiple pathways involving PTI and ETI among others [44, 45, 49]. So far, only in a few cases NHR was exploited for crop improvement [50-54]. According to the most accepted theory, the evolutionary distance between host and non-host plant species determines the involvement of either ETI or PTI, with closer related species predominantly protected by ETI and more distant species by PTI [55]. Because of the close evolutionary distance of the Ascochyta-susceptible *P. vulgaris* and the Ascochyta-resistant *P. dumosus*, the theory would postulate ETI conferring resistance. In the case of Ascochyta resistance, the observed quantitative pattern does not fit well with the ETI explanation, that generally confers qualitative resistance [56]. Hence, resistance may be conferred by other mechanisms potentially involving different plant pattern recognition receptors of the PTI or structural barriers such as different epidermal waxes or incompatibilities in the nutrient acquisition that hinder pathogen colonization [46, 57, 58]. These are however only speculations on the mode of action of Ascochyta resistance based on our observations and the limited knowledge about the Ascochyta disease in common bean. Further studies on interspecific Ascochyta resistance should be conducted using the interspecific crosses directly, without diluting the resistance through additional crosses to common bean. These studies should be complemented with microscopy approaches that will elucidate at which point pathogen colonization is hindered. Such studies may also help with the inter-gene pool transfer of resistance for other diseases including ALS, of which the secondary common bean gene pool is known to be a good source [59]. Besides the potential application in crop improvement, the proposed studies are also interesting for fundamental research, as investigations into NHR are frequently hampered by the inter-species reproductive incompatibility [60]. Common bean with its cross-compatible secondary and tertiary gene pool species is therefore an interesting system to study NHR.

In contrast to Ascochyta, ALS resistant common bean lines have been identified. However, the efficacy of ALS resistance has been reported to be largely dependent on the pathotype it is challenged with [43, 61-65]. Such pathotype-specific resistance effects are usually observed for resistance loci that are part of the ETI defense. Another indication, that the resistance system depends on ETI, is the reported co-evolution of host and pathogen, as the pathogen isolates are usually more aggressive to either the Mesoamerican or Andean gene pool [42]. The ETI defense system relies on effective pathogen perception in order to initiate defense pathways [66]. The largest and most studied family of resistance genes belong to the NB-LRR gene family that contain a nucleotide binding (NB) domain and a leucine-rich repeat (LRR) domain that mediates pathogen recognition [67, 68]. Hence, NB-LRR are usually candidate genes for host-pathogen interaction that point to an involvement of ETI [67, 69]. The finding that NB-LRR genes where often present between flanking markers of ALS resistance loci is therefore another indication on the possible involvement of ETI in ALS resistance [70-72].

Because of the rapid generation time of plant pathogens, the NB-LRR genes in plants are under constant selection to diversify and recognize the mutated pathogen individuals [73, 74]. The NB-LRR genes are often clustered on the genome and frequently differences in the number of repeats and coding sequences are observed among genotypes [73, 75]. The haplotypes at the *Phg-2* locus, the main ALS resistance locus found effective against the tested pathotypes in chapter 3, likely represents such resistance gene clusters that differ in gene content and coding sequence between haplotypes. Resistance gene clusters have been found on most of the eleven common bean chromosomes and are harboring resistance genes against multiple common bean diseases [76]. In common bean, these resistance gene clusters are often located in the highly dynamic subtelomeric regions that represent hotspots of recombination and contribute to the rapid evolution of resistance genes [77, 78].

Classical NB-LRR resistance genes, which we expect to be responsible for the pathotype-specific resistance as explained above, mainly encode for qualitative or complete resistance [47]. Assuming that qualitative resistance is the only mechanism of resistance in the panel used for GWAS, this would have led to bimodal distributions of resistance scores and a clear separation of resistant and susceptible lines. In chapter 3, however, a continuous distribution of ALS scores was observed for all but one trial, suggesting an additional quantitative control of resistance for most pathotypes. Possibly, the GWAS approach taken was not successful in catching the quantitative nature of ALS resistance that may consist of PTI. Genome-wide association studies reportedly are a good method to detect common alleles with large effects on the phenotype of interest, but its power is limited when alleles are rare in the panel or their effect is small [79]. To improve the resolution of the GWAS analysis, more lines could be included or measures to decrease the population structure could be taken, as markers are often confounded with population structure and may not be specific to the trait, but the population carrying the trait. In common bean, where two gene pools are present, it would be beneficial to have separate panels for the Andean and the Mesoamerican gene pool to increase the detection power of the GWAS analysis.

Future investigations

As mentioned above, the GWAS approach was successful in finding resistance loci with large effects. To find resistance loci with smaller effects, a more powerful approach than GWAS is required such as QTL mapping within bi-parental or multi-parental mapping populations [80]. Although establishing mapping populations is more laborious and the populations represent a lower diversity than GWAS panels, they ideally have no population structure and balanced allele frequencies and, hence, are more suitable to reveal effects of small sizes. Bi-parental mapping populations have successfully been used to find qualitative and quantitative ALS resistance loci, but previous experiments were conducted with a single or maximal two pathotypes, and hence, failed to describe the pathotype-specific interaction of resistance loci (reviewed in chapter 2). To find quantitative resistance loci, more QTL mapping populations should be developed, preferably recombinant inbred line (RIL) populations that allow precise phenotyping in replicates and with different pathotypes. Several RIL mapping populations with an ALS resistant common bean line as parent have already been established and genotyped in previous projects [72, 81-85]. Until further mapping population are established, QTL mapping with multiple pathotypes should be conducted using the existing RIL mapping populations. In addition, QTL mapping populations should be developed using as parents the highly resistant common bean lines from the extBALSIT panel, whose resistance could not be explained by the *Phg-2*

haplotypes or the *Phg-4* locus. This may identify previously undiscovered resistance loci. Such a QTL mapping approach should represent a promising method to study the quantitative nature of ALS and at the same time describe the pathotype specific effect of resistance.

Determining the causal gene conferring disease resistance is the ultimate goal of molecular plant breeders and has many advantages. Knowing the resistance gene sequence and gene family can give an indication of the barrier function and, for breeding application, allows the identification of completely linked molecular markers and the comparison of resistance genes between cultivars. In the conducted GWAS analysis, the limited representation of genetic polymorphisms in genotyping by sequencing (GBS) data means that the causal polymorphism is likely not genotyped and GWAS therefore does not pinpoint to the causal gene. However, the interval where significantly associated SNPs were found may be used to narrow down the genetic region to a few candidate genes. Although significantly associated SNPs in the *Phg-2* region have been found in a large region spanning 1.78 Mbp on chromosome 8, different SNPs were associated to resistance in the different trials. These intervals, where significant associations were discovered for single trials, may give an indication of the genetic region within the haplotype where we can expect the causal genes to lie.

A complicating factor for gene identification within the *Phg-2* haplotype, which we expect to be a resistance gene cluster, is that sequencing reads of different common bean lines were mapped to the reference genome and therefore only represent the diversity and gene content of the reference genotype. This is problematic because structural variants or large genetic rearrangements between the reference genome and the genotype of interest, which in common bean may even come from a different genepool, can hinder the detection of the causal gene(s). In addition, such highly repetitive regions may not be accurately represented in reference genome sequences produced over the last years, since they were difficult to assemble using short reads [86]. Recent new technological developments that enable long-read sequencing, mainly PacBio and Oxford Nanopore Technology, have been successful in producing continuous sequences of 10-100 kbp [87, 88]. Such long-read sequencing data from common bean lines in the GWAS panel would accurately assemble repetitive regions to be able to compare the gene content and genomic diversity between genotypes and haplotypes.

Another technique that could be used to characterize the NB-LRR gene repertoire of common bean lines independently of a reference genome sequence is resistance gene enrichment sequencing (RenSeq) [69, 89]. RenSeq is a targeted resequencing approach, where interesting regions are enriched through sequence capture. While this technique is very useful to find NB-LRR resistance genes, the disadvantage is that only the previously prioritized gene families for which the baits were designed will be discovered [69]. The verification of gene function by gene cloning requires the availability genetic engineering techniques for common bean. Because of the recalcitrant reaction of common bean in tissue culture, genetic engineering in common bean is not routinely conducted. However, recent achievements in gene editing and virus resistance engineering in common bean give hope [18, 90] that these protocols will become available in the next years.

With the advances in understanding the genetic determinants of ALS resistance, their effect on pathogen interaction should be investigated. ALS resistance is known to work on different levels, ranging from the inhibition of spore germination, colonization, sporulation or a combination of the three [23]. Pathogen genetic resources of *P. griseola* are not

available, but investigations that will produce the first genome assemblies are underway and will reveal a first insight into effector diversity (T. Wood, personal communication). Linking pathogen and host genetic data will be crucial for a better understanding of the pathotype-specificity of ALS resistance. In fact, knowledge of the pathogen population structure would have allowed to make an informed choice on pathogen isolates to be used in chapter 3 to systematically compare the reaction of the GWAS panel against different pathotypes between and within populations. Genetic information about *P. griseola* populations worldwide and their inter- and intra-population genetic diversity will be crucially important to inform resistance management strategies and may allow to predict the efficacy of ALS resistance loci in different regions.

Most plant pathology studies, including the ones conducted in this thesis, determine the 'resistance' of a plant by visual evaluation of the severity of the symptoms. While in our studies we observed that diseased plants lost many leaves and were generally not healthy looking, only a few studies have actually quantified the yield loss in relation to ALS. In field experiments conducted in Brazil, ALS disease score or area under disease progression curve were not correlated to yield, while healthy and effective leaf area did correlate with yield [91-93]. In another study from Brazil, an average yield reduction of 7.88% for each 10% increase in leaf area affected across nine common bean varieties was found [94]. The remaining publications on ALS resistance in common bean only assess disease severity as a measure of ALS resistance, despite the fact that the effect of disease severity on yield is largely unknown. In addition, some genotypes may not halt pathogen reproduction and therefore appear susceptible, but are tolerating pathogen infection and still produce high yields [95]. An indication that this mechanisms plays a role in ALS resistance in common bean is given by Rava Seijas & Sartorato [94], who found that two common bean varieties with comparable yield losses of 43.7% and 45.5%, had a highly different percentage of the leaf area affected, with 24.7% and 44.3% affected, respectively. Future investigations should therefore also consider the genotype-specific yield loss effect when assessing ALS resistance. An interesting study would be to measure ALS tolerance, the percentage of yield lost under strong ALS infection, of each extBALSIT common bean line and conduct GWAS to find the genetic determinants of the trait. Comparing ALS tolerance loci to classical resistance loci that hinder pathogen reproduction would give an indication on the agronomical relevance of the currently used visual disease resistance measurements.

Implications of this work for resistance breeding at CIAT

Common bean breeding at CIAT is centered at its headquarter in Cali, Colombia, where two large breeding programs develop cultivars for several grain types (market classes) within the Mesoamerican and Andean common bean gene pools separately. Two smaller CIAT bean breeding programs are active in Uganda and Malawi. From Colombia, improved common bean lines and germplasm are distributed among partners in Latin America and Africa (Figure 1), which use this germplasm to either register CIAT cultivars as varieties or further use them in their breeding activities. A major focus of common bean breeding at CIAT is breeding for Africa and based on its initiative, the Pan African Bean Alliance (PABRA) was established. PABRA maintains several breeding stations in Africa and works together with national agricultural research systems (NARS), non-governmental organizations (NGO), universities and private companies in 30 countries to ensure CIAT bred cultivars are distributed, used for further improvement and released as varieties. Testing for biotic and abiotic stress responses is conducted in the first instance during the

breeding process in Colombia. Common bean lines selected for distribution are frequently tested again in the receiving countries against local diseases and challenges, but feedback of these tests rarely reaches back to CIAT Uganda and CIAT Colombia, hence it is not commonly incorporated in the breeding process in Colombia (B. Raatz, personal communication). To improve the cooperation and data exchange between partners, a new project termed AVISA (for Accelerated Varietal Improvement and Seed Delivery of Legumes and Cereals in Africa) was recently launched and will hopefully improve communication between common bean breeding programs.

The CIAT approach of breeding at one geographic location and the subsequent distribution and use in a different location seems reasonable for abiotic stresses, which are comparable in the same climate zones of different continents, but not necessarily for biotic stresses, which are highly affected by the prevalent pathogen populations as our research on intercontinental pathotype-specific ALS resistance has shown (Chapter 3). Angular leaf spot resistance at CIAT Colombia is traditionally pre-screened at the field locations in Quilichao and Darien. Experience has shown that similar common bean lines were resistant at the field location in Quilichao and in Eastern Africa, hence evaluations for this target area were predominantly conducted in Quilichao (C. Jara, personal communication). We could, in chapter 3, indeed show that the resistance at the locations Quilichao (Colombia) and Kawanda (Uganda) correlate well (Pearson correlation r = 0.63). A similarly high correlation was achieved, when the panel was tested in the greenhouse with the pathotype COL 63-47 (r = 0.62). For evaluations in Colombia aiming at resistance in the target region Eastern Africa, the pathotype COL 63-47 could therefore be used in the future to determine resistance instead of field-testing in Quilichao. This would result in approximately the same accuracy within a third of the time.

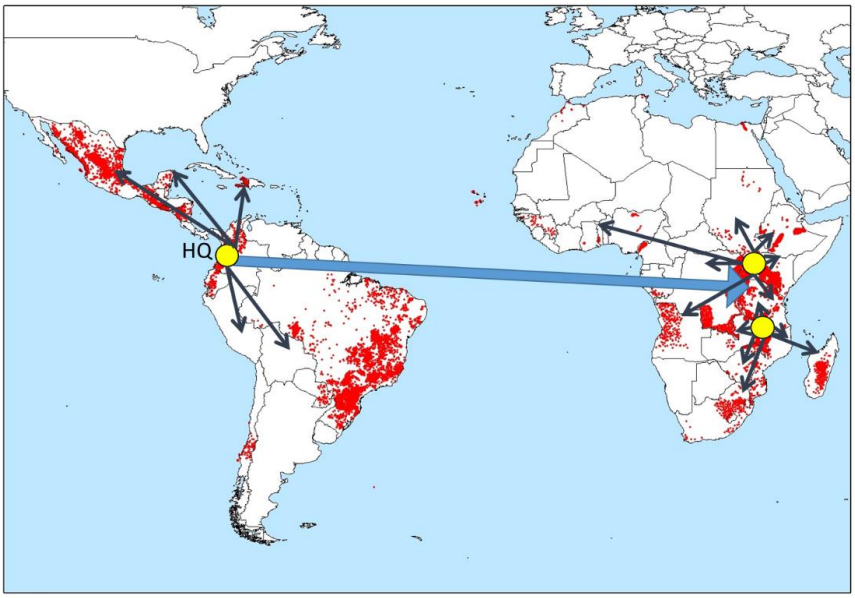


Figure 1: Common bean production and CIAT germplasm exchange routes. Common bean production areas in Latin America and Africa are shown as red dots on the map and common bean breeding stations in Colombia, Uganda and Malawi are shown as yellow dots. The general distribution of common bean germplasm is symbolized by arrows. From CIAT headquarters (HQ) in Colombia, germplasm is provided across Latin America and to the African breeding stations. From the breeding stations in Uganda and Malawi, common bean germplasms is distributed to Pan African Bean Alliance (PABRA) partners across Sub-Saharan Africa. Figure courtesy of B. Raatz.

An even more efficient way to ensure resistance is effective in the target area is to determine its genetic cause and use marker-assisted selection (MAS) for resistance incorporation. In chapter 3, we have found such pathotype- and location-specific ALS resistance and molecular markers co-segregating with resistance. Using this information, common bean lines containing the haplotype that conferred resistance in Uganda can now be bred in Colombia. By testing the established GWAS panel in additional locations, the effectiveness of loci in these locations can be determined and more accurate breeding can occur. Even further accuracy in determining the effective resistance sources in a target location would be achieved by establishing a set of near isogenic lines from a susceptible common bean background that contain introgressions from known resistance genes for all relevant common bean diseases. Based on the results of this artificial differential-like set, effective resistance loci could be selected and incorporated in the resistance breeding strategy.

The SNPs co-segregating with ALS resistance loci or haplotypes found in chapter 3 will increase efficiency of resistance breeding. By converting these SNPs to molecular markers, they can be used to screen germplasm for the presence of desired resistance loci in early generations. Hence, unsuitable genotypes can be discarded very early in the breeding process in the seedling or even seed stage and available resources can be invested in promising genotypes. This reduces labor and area requirements for field experiments. Molecular markers can be developed targeting SNPs for in-house or outsourced genotyping. Dependent on the availability of laboratory equipment, different marker systems can be used such as competitive allele specific PCR (KASP) markers, allele-specific PCR markers, high-resolution melting (HRM) markers or melting temperature shift (Tms) markers [96, 97]. Molecular marker testing can also be outsourced cost-effectively, as DNA extraction and testing of ten SNPs cost approximately 1.50 CHF per sample, which also allows breeding programs without access to lab facilities to benefit from MAS [98] (B. Raatz, personal communication). At this price level, genotyping is affordable to breeding programs in developing countries with tight research budgets. Despite the tremendous potential of MAS to increase efficiency, it should be noted that field trials will remain the last step of the process, as the final resistance may not be the sum of the incorporated genes as epistatic interaction with resistance or agricultural traits may be involved.

Finally, resistance breeding for ALS currently relies on very few resistance loci: *Phg-2* from Mexico 54 in Africa, *Phg-2* from G10474 and *Phg-4* from G5685 in Latin America and *Phg-3* from Ouro Negro in Brazil (Chapter 2). Despite these resistance loci being the strongest known, pathotypes overcoming resistance in all these sources are known [62, 65, 99, 100]. To ensure resistance in the future, new resistance sources should urgently be sort out and the application of known resistance loci should be reviewed. Resistance loci ought to be considered valuable resources that need to be applied strategically, preferably in pyramids of several resistance loci effective in the target location. Only then will individual resistances not be broken down one by one by new virulent pathotypes and thus offering disease protection in the field for a long time.

Conclusion

Common beans, either consumed as dry beans, snap beans or in processed from, will continue to be an important part of a healthy diet in the developed and developing world. Disease resistance is a crucial trait to ensure stable yields, reduce pesticide residues on produce and make common bean production more sustainable. The findings of this thesis will be directly incorporated at the largest common bean breeding program in the tropics and are available to interested breeders. The tools established, will allow breeders to select efficient ALS resistance loci in their target areas and equip them to react faster to future threats and developments. In addition, the established germplasm will be of use in breeding programs aiming at producing ALS and Ascochyta resistant varieties. Improved varieties that incorporate findings and germplasm described in this thesis will make a difference in farmer's field by stabilizing yields and ensuring a healthy produce of common beans. In the tropics, improved common bean varieties will contribute to an appropriate nutrition quantity and quality, hence contributing to food security and alleviating malnutrition.

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Appendix

Appendix 1: Breeding report

Manual to facilitate incorporation of angular leaf spot resistance in breeding programs

This short report describes the different angular leaf spot (ALS) resistance breeding tools established throughout this thesis and how they can be incorporated in a breeding program. While the main scientific results are described in chapter 3 of this thesis, this report is designed as an easy to understand user manual that helps breeders with the implementations of the findings.

In short, chapter 3 revealed that two resistance loci were effective in the extBALSIT common bean diversity panel against a broad variety of pathotypes in greenhouse and field experiments in Colombia and Uganda. The resistance locus on chromosome 8 was found to be effective in all trials and locations, while the resistance locus on chromosome 4 was detected when the panel was tested against the Andean ALS pathotype COL 30-0 (Figure 1, 2). For the resistance locus on chromosome 8, different functional haplotypes were found responsible for the pathotype-specific ALS reaction observed.

Surprisingly, the previously characterized resistance loci *Phg-1*, *Phg-3* and *Phg-5* were not found to be effective against pathotypes tested in chapter 3. This suggests a highly pathotype-specific ALS response, with only a subset of the known resistance loci effective in Colombia and Uganda. In order to breed for effective resistance, it is hence crucially important to know which resistance loci are functional in the target region. The resources presented here serve to assist breeders with the implementation of the findings in chapter 3 by providing phenotypic data of the common bean lines contained in the extBALSIT and molecular markers linked to haplotypes. In addition, we describe how the extBALSIT panel can be used in future experiments and give the TASSEL and R scripts to analyze future experiments.

Materials and methods available

ExtBALSIT GWAS panel: The panel was assembled especially for ALS genome-wide association studies (GWAS) studies. It includes the Bean ALS International Trial (BALSIT) panel of 55 lines, assembled by the CIAT bean pathology unit to be able to identify germplasm resistant to ALS in different locations in the world for breeding applications. For the extBALSIT panel used in the studies in chapter 3, the BALSIT was complemented with previously characterized resistance sources, breeding material with phenotypic variability for ALS response and susceptible checks. The panel consists of 124 large-seeded Andean common bean lines, 129 small-seeded Mesoamerican common bean lines and 63 lines from inter-gene pool crosses. A description of the grain type of these lines, their pedigree and haplotype at the *Phg-2* locus is given as supplementary material 1.

Genotyping and phenotyping data: Genotypic and phenotypic data of the extBALSIT panel is available on dataverse (https://doi.org/10.7910/DVN/U2BAWN). The phenotypic data is also listed as supplementary table 1.

Tassel GWAS script: The script that was used to run GWAS through the command line is given in supplementary material 2. Furthermore, a step by step explanation how this is implemented in the TASSEL 5 graphical user interface is given.

Haplotyping script in R: The R script, which is implementing haplotype clustering at a desired interval, is given as supplementary material 3.

SNP testing script in R: The R script that produces a boxplot to compare the ALS resistance of common bean lines with different alleles at a SNP is given as supplementary material 4.

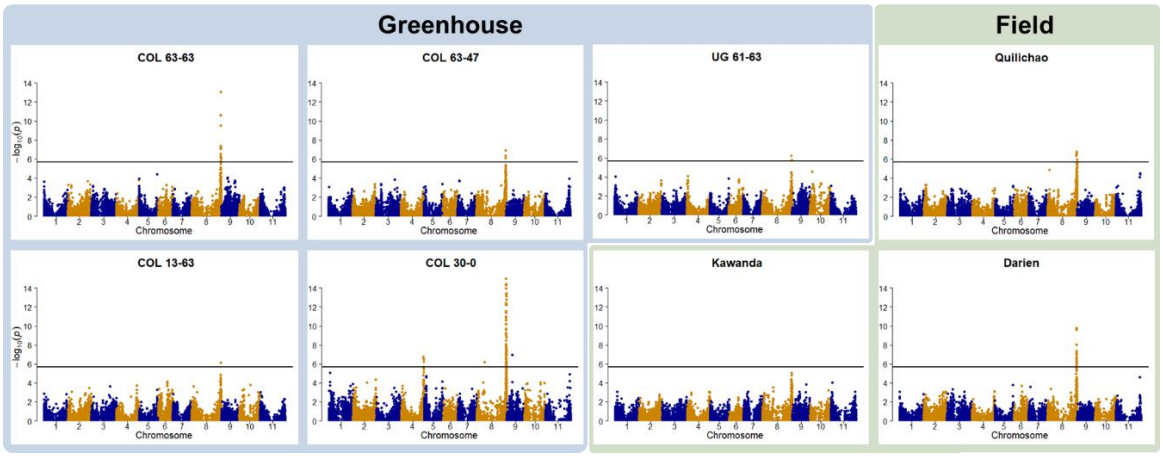


Figure 1: Manhattan plots of the genome-wide association studies for angular leaf spot (ALS) resistance in the extBALSIT panel. The greenhouse trials were conducted with five different angular leaf spot pathotypes, determined by their origin (COL and UG,) and race (63-63, 63-47, 61-63, 13-63 and 30-0). Field trials in Colombia (Darien and Quilichao) and Uganda (Kawanda) were inoculated with mixtures of pathotypes, previously collected at the corresponding sites. On the x-axis, the genomic position of the markers are given. On the y-axis, the negative logarithm to the base 10 of the *P*-value, representing the significance value, is given. In order to correct for multiple testing, the significance threshold was adjusted through the Bonferroni method and the new significance threshold is depicted by the black horizontal line.

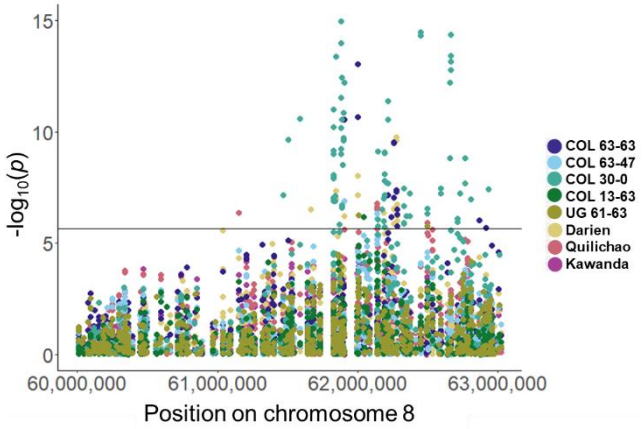


Figure 2: Magnification of the region on chromosome 8, where most significant associations were found. The significance of the marker-trait association of the genome-wide association studies were plotted on the y-axis for markers in the 60-63Mbp interval (x-axis). The different trials are depicted by differing colors.

Step by step explication of how to use the resources for breeding

● Step 1: Find out which resistance loci or haplotypes are useful in the target breeding region

Based on figure 1, decide which resistance loci are useful in the target breeding region. Because of the predominance of the locus on chromosome 8, this locus was further dissected: Common bean lines were clustered based on their genotypic data at the interval where significant associations were found (Figure 3). For each of the eleven haplotype groups, the haplotype-specific ALS score was calculated and plotted in figures 4 and 5. Select the haplotype that performed best against the pathotypes in the target area. Generally, in Colombia haplotype M1 performed best, while in Uganda the haplotypes M2 and M3 performed best.

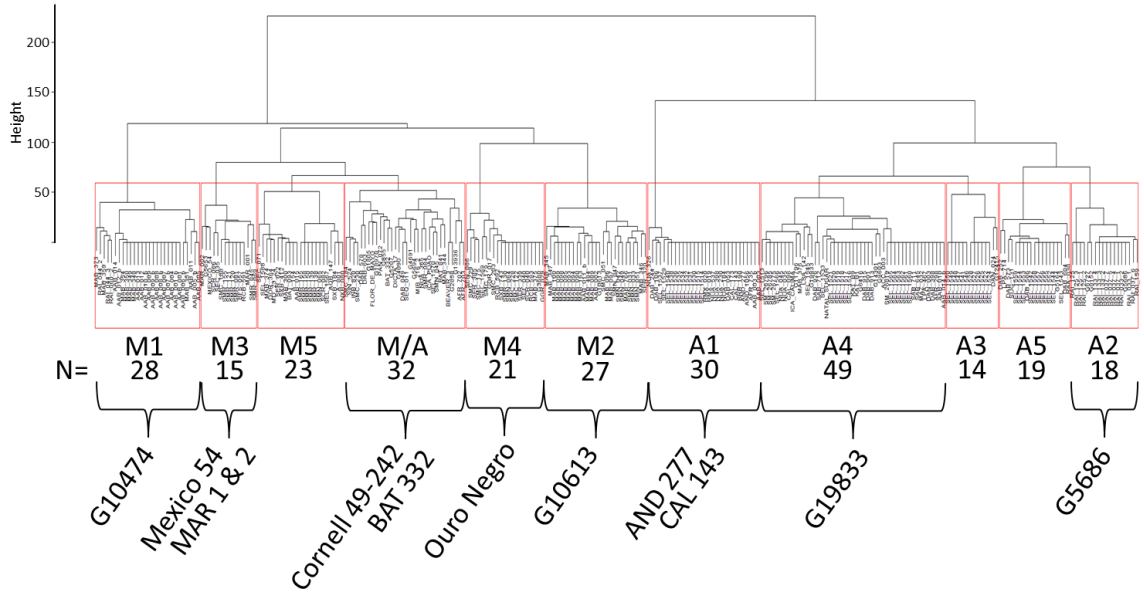


Figure 3: Dendrogram of hierarchical clustering at the *Phg-2* locus. Common bean lines were clustered according to their SNP data similarity in the 61.15-62.93 Mbp interval on chromosome 8 and divided into eleven haplotype groups. Haplotype groups were named according to the common bean gene pool origin of the contained lines (M = Mesoamerican, A = Andean and M/A = mixed) and numbered. Below the haplotype names, the number of common bean lines in each haplotype group is given and famous ALS resistant common bean lines are listed.

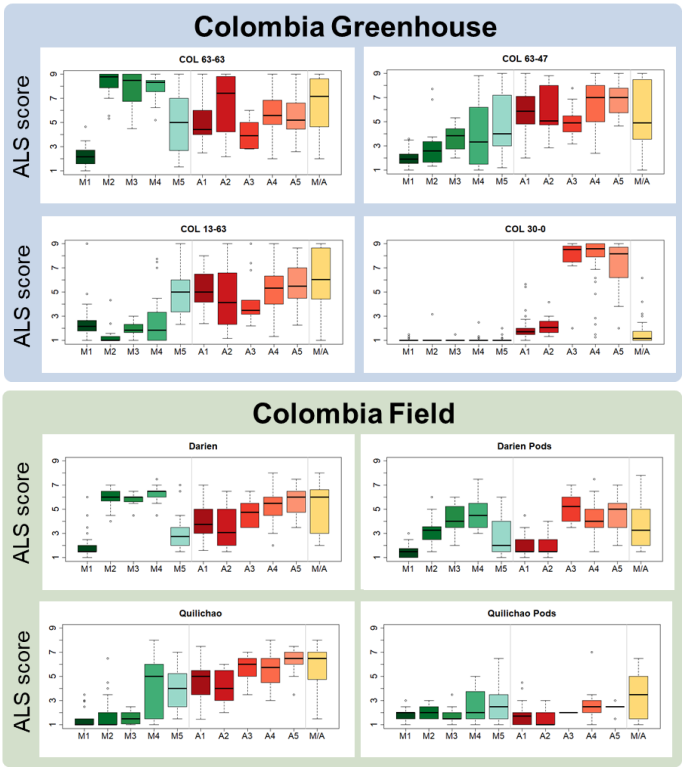


Figure 4: Haplotype-specific angular leaf spot (ALS) response of the *Phg-2* locus in greenhouse and field trials in Colombia. The extBALSIT panel was tested with five different pathotypes of race 63-63, 63-47, 13-63 and 30-0 in the greenhouse and with mixtures of pathotypes previously collected at the corresponding site at the field locations Darien and Quilichao. For each trial, the response of the eleven haplotype groups is plotted. On the x-axis the haplotype group is given and on the y-axis, the ALS response scored on a scale from from 1 (resistant) to 9 (highly susceptible).

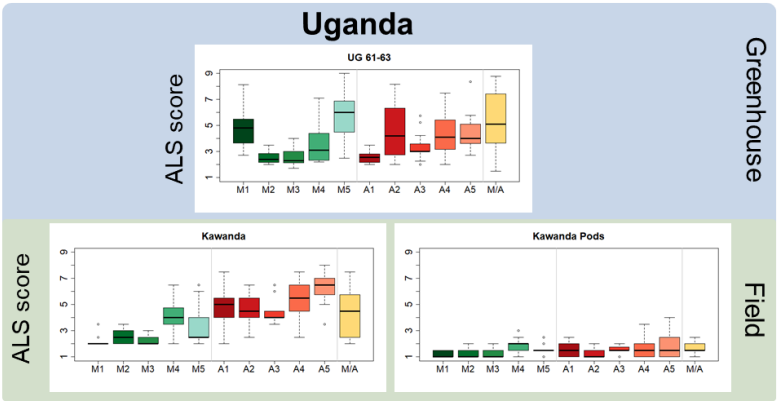


Figure 5: Haplotype-specific angular leaf spot (ALS) response of the *Phg-2* locus in greenhouse and field trials in Uganda. The extBALSIT panel was tested with a pathotype of race 63-47, in the greenhouse and with mixtures of pathotypes previously collected at different locations in Uganda at the field location Kwanda. For each trial, the response of the eleven haplotype groups is plotted. On the x-axis the haplotype group is given and on the y-axis, the ALS response scored on a scale from from 1 (resistant) to 9 (highly susceptible).

● Step 2: Find common bean lines within haplotype that resemble the desired grain type.

Once you have determined the haplotype you would like to use for ALS resistance breeding in the target area, check which common bean lines contain this haplotype (Supplementary material 1). Within these, find the genotype that most resembles your desired grain type and make sure, based on previous ALS evaluations, it shows the desired resistance.

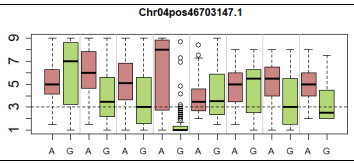
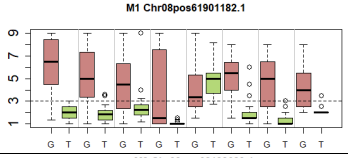
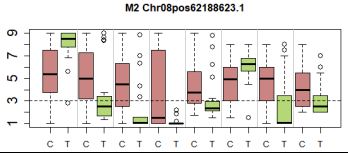
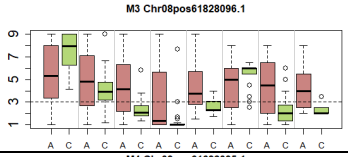
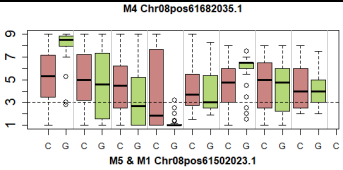
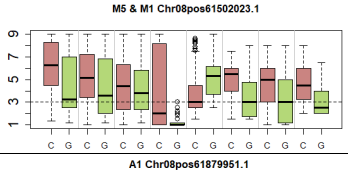
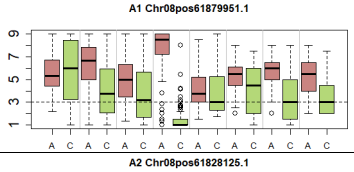
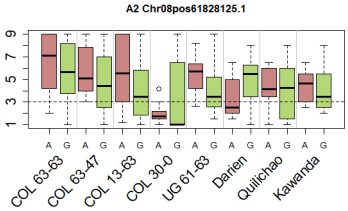
● Step 3: Choose a marker specific to the haplotype

Locus- and haplotype-specific SNP markers were found for the resistance locus on chromosome 4 and most haplotypes on chromosome 8 (Table 1). To facilitate the establishment of molecular markers, DNA sequence of 50bp before and after the SNPs are reported in supplementary material 5.

● Step 4: Cross resistance sources to elite common bean breeding lines

The chosen marker should allow to distinguish the resistant offspring containing the selected haplotype from the remaining offspring.

Table 1: Selection of SNPs for molecular marker development. Non-exhaustive list of SNPs that are locus- or haplotype-specific and represent target SNPs for marker assisted selection. In the SNP column, the resistant allele is given before the dash and the susceptible allele after. For each haplotype, a representative SNP was selected and the allele-specific reaction in the ALS trials is plotted. DNA sequence for molecular marker development of these SNPs is given in supplementary material 5:

SNP position	SNP	Allele-specific reaction in ALS trials
	***Phg-4* on chromosome 4**	
Chr04pos46703147 Chr04pos46934061 Chr04pos46727398	G/A T/C G/A	
	***Phg-2* on chromosome 8**	
<u>M1</u> Chr08pos61901182	T/G	
<u>M2</u> Chr08pos62188623	T/C	
<u>M3</u> Chr08pos61828096 Chr08pos61878388 Chr08pos61880092	 C/A A/C T/C	
<u>M4</u> Chr08pos61681984 Chr08pos61682023 Chr08pos61682035	 C/T A/G G/C	
<u>M5 and M1*</u> Chr08pos61388457 Chr08pos61502023 Chr08pos61533289	 C/T G/C T/C	
<u>A1</u> Chr08pos61825787 Chr08pos61879951 Chr08pos62191492	 A/C C/A G/A	
<u>A2</u> Chr08pos61828125	G/A	
<u>A3</u> Not found		

Using the extBALSIT GWAS panel for further studies

ALS resistance

The extBALSIT panel has been extensively tested in field and greenhouse trials in Colombia. In addition, a field and a greenhouse trial have been conducted in Uganda. These two locations are however only a small subset of the many countries and location that cultivate common beans. To gain a better understanding of location specific resistance, the extBALSIT panel should be distributed to several countries for ALS testing. For example, the resistance loci *Phg-1*, *Phg-3* and *Phg-5* have been reported in studies conducted with Brazilian pathotypes, but were not found in the Ugandan and Colombian trials. Testing the panel in Brazil would probably result in different resistance loci significantly associated to ALS resistance.

The information gained through GWAS with the extBALSIT panel will also be useful when breeding is conducted at one geographic location, but should be useful in other target areas. As an example, breeding in Uganda is conducted for several different African countries. By testing the extBALSIT panel in target locations, the effective resistance loci in these regions are revealed. By knowing which ALS resistance loci are effective in the target regions, breeders in Uganda can select the appropriate resistance loci when initiating the crosses.

Additional traits

Any trait that shows phenotypic variability in the extBALSIT panel, which can be scored or measured, can be used for association testing. This has successfully been demonstrated in the Kawanda (Uganda) field trial, where in addition to ALS score, growth habit and black root disease symptoms were assessed. The GWAS analysis for growth habit revealed a significant association on chromosome 1 (Figure 6). This locus has been associated with growth habit in previous studies and is in proximity to the *PvTFL1y* gene, which is responsible for a determinate growth [1, 2].

In the field trial in Kawanda, a high natural occurrence of the black root disease was observed. The severity of the disease was assessed on a score ranging from 1 to 4 on a plot basis (1 = resistant, 2 = 1-2 plants affected, 3 = heavy infection, 4 = all plants affected) and the marker-trait associations were calculated. The strongest associations were observed for markers on chromosome 2, with other significantly associated markers on chromosomes 7 and 9 (Figure 6). Black root disease symptoms are caused under certain conditions by the infection of the plant with the bean common mosaic necrotic virus (BCMNV) on plants that contain the *I* virus resistance gene [3, 4]. The *I* gene was characterized on chromosome 2 [5], coinciding with the significant associations in our study.

Although the extBALSIT panel was specifically assembled to contain variability for ALS resistance, it is sufficient diverse to be used to study other traits. Interesting would be to use it for studies of other common bean diseases such as anthracnose, bacterial blights, rust and root rots.

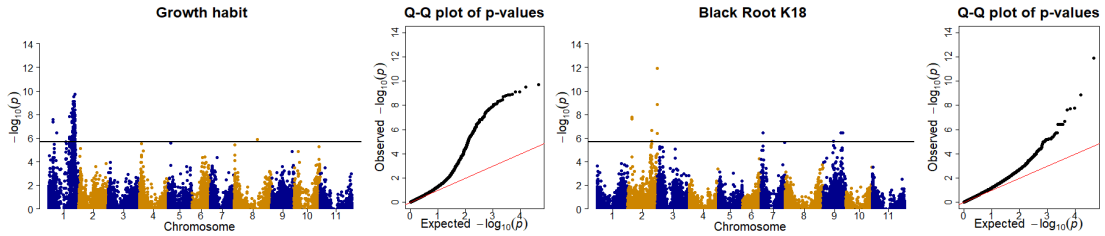


Figure 6: Manhattan and quantile-quantile (Q-Q) plots of the genome-wide association studies for growth habit (left) and black root disease (right). The extBALSIT panel of 316 lines was evaluated for growth habit and black root disease symptoms in the field in Kawanda, Uganda. On the x-axis, the genomic position of the markers are shown. On the y-axis, the negative logarithm to the base 10 of the *P*-value, representing the significance value, is given. The significance threshold was adjusted through the Bonferroni method and the new significance threshold is depicted by the black horizontal line.

How?

To receive the extBALSIT panel, CIAT bean breeding program should be contacted well in advance of the planned experiments since the common bean lines in the panel may need to be multiplied and phytosanitary certificates for seed shipments need to be obtained. Once the lines are received, they can be tested in field and greenhouse trials. Genotypic information for the panel is provided on dataverse and genome-wide association testing can be conducted using the script provided in supplementary material 2. Using the script in supplementary material 3, haplotypes at any locus of interest can be clustered and tested for their haplotype-specific effect on the trait. Furthermore, candidate SNPs for molecular marker development can be tested in silico using the R script in supplementary material 4.

Literature

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Supplementary Material

Supplementary material 1: Common bean lines of the extBALSIT panel. Common bean lines are listed with their corresponding gene pool (A, Andean; M, Mesoamerican; AxM, inter-gene pool cross), haplotype at the *Phg-2* locus, angular leaf spot resistance (ALS) phenotypes, pedigree and grain phenotypic characteristics. Resistance to ALS was scored in greenhouse trials with five different pathotypes, determined by their origin (COL and UG) and race (63-63, 63-47, 61-63, 13-63 and 30-0). Field trials were conducted in Colombia (Darien and Quilichao) and Uganda (Kawanda) in different years (if year is not specified, mean values are given) and ALS symptoms were scored on leaves and pods. ALS was scored on a 1 (resistant) to 9 (susceptible) scale. Black root disease symptoms were only observed in Kawanda (Uganda) and were scored on a 1 to 4 scale (1= no symptoms, 2 = 1 – 2 plants affected in plot, 3 = heavy infestation in plot, 4 = all plants in plot showed disease symptoms). Growth habit was scored on a 1 to 3 scale in the field in Kawanda, where 1 corresponds to bush type, 2 to intermediate and 3 to climbing type. Abbreviations: COL, Colombia; int, intermediate; UG, Uganda; var, variable.

Line name	Meso or Andean	Haplotype	COL 63-63	COL 63-47	COL 30-0	COL 13-63	UG 61-63	Quilichao	Darien	Kawanda	Darien Pods	Quilichao Pods	Kawanda Pods	Darien 2016	Darien 2016 Pods	Darien 2017	Darien 2017 Pods	Quilichao 2016	Quilichao 2016 Pods	Quilichao 2017	Quilichao Pods 2017	BlackRoot Kawanda	Growth_habit	Grain size	Grain Color	opaque/ shiny	Pedigree
AAB_003	AxM	A1	5	5	1	2.5		1	4		1.5			2	1	6	2			1							NUA184xMAB353
AAB_003_b	AxM	A1	7.2	8.8	1	3.8	3	6	7	5.5	3.5	3	2	6	4	8	3	5	3	7	3	1	2	int./large	dark-red	s	NUA184xMAB353
AFR_735	A	A1	3	2.3	1	4.2	2	3.5	2.5	5	1.5	1	1	4	2	1	1	4	1	3	1	1	1	large	red with white mottles	s	T23xAND924
AND_1056	A	A1	4.5	6.7	1.4	7.2	2	4	2	5	1		1.5	3	1	1	1	5	1	3		1	1	large	light-brown - dark striped	s	
AND_277	A	A1	5.2	8	2	5	3.5	3.5	2	4.5	1.5	1	2	2	2	2	1	4	1	3	1	1	1	large	dark-red	s	G21720xBAT1386
BRB_190	A	A1	6.4	4.4	1.5	5	2.3	4	3.5	6	2	1	2	4	3	3	1	3	1	5	1	1	1	large	red with white mottles/calima	s	CAL143x(CAL122xMCR2511)
CAL_143	A	A1	6.3	4.3	1.7	3.4	2.3	3.4	2	6.5	1.4	1.7	2.5	2.5	1.7	1.4	1	3.8	1.7	1	2	1	1	int./large	red, with white spots or mottles	s	BOLAxAND277
CAL_173	A	A1	4.2	3.2	1.5	3.6	2.2	2.5	2	4	1	1	1	3	1	1	1	4	1	1	1	1	1	large	red with white mottles	s	
DAB_053	A	A1	4.2	4.5	2.2	3.3	3.1	2	3	4.5	1.5		1.5	4	2	2	1	3	1	1		1	1	large	red with brown mottles		SEQ11xR.C.WONDER
DAB_106	A	A1	9	8.7	1.8	5	2.1	5	3.5	4	2		1.5	5	3	2	1	5	1	5		1	1	small	red	s	RAA21xCAL143
DAB_244	A	A1	4	4.8	5.7	4.9		5	5		3.5			7	4	3	3	5	3	5							(SAB628xCAL143)XSAB659
NUA_184	A	A1	4.2	2	1.8	2.4	2.3	1.5	1.6	2	1	1.2	1	2	1	1.3	1	1.6	1.3	1	1	1	1	large	beide with light red/violet stripes	s	SUG131x(SUG131xG21242)
NUA_230	A	A1	7.7	8	2	5.7	2.3	5.5	3.5	7.5	1	4	2	4	1	3	1	5	1	6	7	1	2.5	int./large	red with white mottles/calima	s	CAL144x(CAL144xG21242)
NUA_326	A	A1	6	5.3	5.4	7	2.8	3	4.5	5.5	4.5	1.5	2	7	6	2	3	5	2	1	1	1.5	1	large	red with white mottles/calima	s	NUA56x(BID29xSUG131)
NUA_398	A	A1	6	7	2.8	8	2.4	4	3	5	1.5	2	1	4	2	2	1	3	3	5	1	1.5	1.5	large	red with white mottles/calima	s	NUA35x(SUG131xG23823E)
NUA_537	A	A1	4.3	6.8	2	6.5	2.8	4	2.5	6	3		2.5	3	5	2	1	5	1	3		1	1	large	dark-red	s	AFR298xBID29
RMX_019	A	A1	5.8	6.7	1.3	5	2.1	5	3	5.5	1.5	2	2	4	2	2	1	6	3	4	1	1	1.5	large	light-brown with brown mottles	s	CAL143x((A483xG6416)x(VAX3xAFR298))
RMX_020	A	A1	5.4	6.2	1.5	4	2.1	2.5	4.5	4.5	1.5		1	4	2	5	1	4	1	1		1	2	large	light-brown with brown mottles	s	CAL143x((A483xG6416)x(VAX3xAFR298))
SEL_1514	A	A1	3.4	4.8	3	6.2	2.5	5.5	5.5	6	2.5		1.5	5	3	6	2	6	3	5		1	1	large	dark-red	s	L.CATRACHITAI-17AX(L.CATRACHITAI-17AXBRB153)
SEL_1515	A	A1	4	5.3	2	6.7		6	3.5		2			5	2	2	2	6	2	6							L.CATRACHITAI-17AX(L.CATRACHITAI-17AXBRB181)
SEL_1529	A	A1	4.3	4.8	1.3	4.8	3.1	5	5	4.5	1.5		1	4	2	6	1	5	1	5		1	1	large	brown-red	s	G76X(G76XBRB186)
SEL_1530	A	A1	5.2	9	1.5	8	2.7	7.5	5	5.5	2.5		1.5	5	4	5	1	7	3	8		1	1	large	brown	s	G76X(G76XBRB186)
SEL_1531	A	A1	6	8	3.5	5	3	6.5	6	4	2.5		1.5	5	3	7	2	6	2	7		1	1	large	brown	s	G76X(G76XBRB186)
SEL_1532	A	A1	3.5	8.8	1.5	5.5	2.8	7	5.5	3.5	2.5	4.5	1	5	3	6	2	7	4	7	5	1	1	large	light-brown	s	G76X(G76XBRB186)

SEL_1534	A	A1	2.5	5	2	6.3	2.7	5.5	4	6.5	2.5			4	3	4	2	6	2	5		1	1	large	dark dark-red	s	G76X(G76XBRB186)
SEL_1535	A	A1	3.8	5.6	1.8	7.4	2.7	4	4.5	5.5	1.5		1	4	2	5	1	3	2	5		1	2.5	large	dark-red	s	MONTCALMxBRB182
SEL_1536	A	A1	4	6.6	1	6.5	2	6.5	5	4	2		1	4	2	6	2	6	1	7		1.5	1	int./ large	dark-red	s	MONTCALMxBRB183
SEL_1537	A	A1	3.8	5	1.8	6.3		3	5.5		1.5			4	2	7	1			3							MONTCALMxBRB181
SEL_1538	A	A1	5	6.6	1.5	7.6	2.6	5	4.5	3.5	2.5		1	4	2	5	3	4	2	6		1	2.5	large	dark-red	s	MONTCALMxBRB184
SEL_1540	A	A1	4.3	7.1	1.5	4.7	2.7	5	3	4	1.5	2	1.5	3	2	3	1	4	3	6	1	1	1.5	large	beige	s	MONTCALMxBRB182
G5686	A	A2	3.3	5	2.4	3.8	2.8	4.3	3.1	4.8	2.1	1.3		4.5	2.5	1.7	1.7	4.2	1.1	5	2.5	1.1	1	large	brown mottled, barely visible	s	
G6727	A	A2	2.2	3	1.3	2.3	2	3	3	3	4	1	1	4	3	2	5	2	1	4	1	1	1	large	brown with dark stripes		
G8152	A	A2	2.2	2.8	1.5	2.4	2.2	2	2.5	3.5	1.5	1	1	4	2	1	1	3	1	1	1	2.5	1	large	light brown with dark stripes		
RAI_033_1	AxM	A2	6.7	8	2.6	1.5	2.5	3	2	4	1	2	1.5	2	1	2	1	5	3	1	1	3.5	3	small	brown with lighter, brown spots	s	G5686xG10474
RAI_033_2	AxM	A2	7	8.8	1.8	1.7	3.4	2.5	1.5	6.5	1.5	1.5	2	2	2	1	1	4	2	1	1	2.5	3	small	dark-red	s	G5686xG10474
RAI_037_1	AxM	A2	8	7.8	2	6.6	7.7	6	6.5	4.5	2	2.5		7	3	6	1	7	4	5	1	3.5	1	very small	dark-red	s	G5686xG10474
RAI_037_4	AxM	A2	8.8	4.8	3	4.5	3	6	4	4	2.5			3	4	5	1	7	3	5		2.5	1	very small	var. brown red-black	s	G5686xG10474
RAI_037_5	AxM	A2	9	3.5	1.5	9	8.2	6	5	4.5	2	1	1	6	3	4	1	6	1	6	1	3	1	very small	dark-red	s	G5686xG10474
RAI_037_6	AxM	A2	9	8.7	1.8	6.2	5.4	6	5	6.5	1.5	3	1	5	2	5	1	6	2	6	4	4		small	var. brown and dark red-brown	s	G5686xG10474
RAI_037_7	AxM	A2	7.7	5.5	1.5	3		4	1.5		1	1		2	1	1	1	7	1	1	1						G5686xG10474
RAI_125	AxM	A2	8	4.8	4.2	9	4.2	4	5	5.5	3	1	1	5	4	5	2	3	1	5	1	1.5	1	int./small	brown	s	G5686xG10474
RAI_125_1	AxM	A2	4.3	3.6	2	9	2.6	3	4.5	4	2.5	1	1	4	4	5	1	1	1	5	1	2	1	small/int.	dark-red	s	G5686xG10474
RAI_125_2	AxM	A2	3.3	4.8	3	2.3	3.6	3	2	5.5	1	1	1	2	1	2	1	5	1	1	1	2.5		small/int.	brown	s	G5686xG10474
RAI_125_3	AxM	A2	6.7	5.3	2.2	3.2	5.7	4.5	3.5	2.5	1.5	3	1.5	4	2	3	1	5	5	4	1	2	1	small	red-brown with brown spots	s	G5686xG10474
RAI_133_2	AxM	A2	9	5.2	2.2	5.8	7.4	5.5	5.5	4.5	2.5	1	1	5	4	6	1	5	1	6	1	2	3	small	brown with lighter, brown spots	s	G5686xG10474
RAI_133_3	AxM	A2	7.2	8	1.7	5.4	7.9	3.5	2.5	6	1.5	1	1.5	2	2	3	1	4	1	3	1	2.5	3	int. small	brown with lighter, brown spots	s	G5686xG10474
RAI_133_4	AxM	A2	8.8	4.8	2.7	9	6.3	5		5.5		2	1					5	2			1	4	small	brown with lighter, brown spots	s	G5686xG10474
RAI_159	AxM	A2	9	8.3	2.5	1.2	5	4	1.5	3	1.5	1.5	1	2	2	1	1	7	2	1	1	3.5	1	small	brown	s	G5686xG10474
DAN_024	A	A3	5	5.4	2	4.2	5.8	6.5	5	3.5	3.5		1.5	5	2	5	5	7	2	6		1	1	int./large	dark red	s	(SAB645xKATB9)xNUA430
G12415	A	A3	6	7.8	8	6.8	5.2	6.5	4.8	3.5				4.5		5		7		6		1	4	int./large bola	brown	o	
SEL_1522	A	A3	3.3	4.5	8.8	3.2	3	6	6	4	7		1.5	8	7	4	7	6	1	6		1	1	large	light-brown	s	G76X(G76XBRB211)
SEL_1524	A	A3	2.8	3.3	8.7	3.3	2.8	6	4.5	4.5	5.5		2	5	7	4	4	6	2	6		1	1	large	beige	s	G76X(G76XBRB186)
SEL_1525	A	A3	2.8	3.2	7.2	3.5	3.4	6	6	6	5		1.5	7	6	5	4	6	5	6		1	1	large /int.	beige	s	G76X(G76XBRB186)
SEL_1526	A	A3	2.8	3.4	9																						
SEL_1527	A	A3	5	5	8.2	9	4.3	7	6.5	6.5	6		1	7	6	6	6	7	2	7		1	1	large	brown-red	s	G76X(G76XBRB186)
SEL_1528	A	A3	5.5	6.9	7.3	7.4	3.6	5	5.5	4.5	6			6	6	5	6	5	2	5		1	1	large but thin	brown-red	s	G76X(G76XBRB186)
SEL_1541	A	A3	3.3	4.8	8.8	3.8	3	5.5	4.5	4	5.5		2	6	6	3	5	6	2	5		1	1	large	dark-red	s	MONTCALMxBRB181
SEL_1546	A	A3	4.5	5.2	8.7	2.5	3	4	3.5	4	4		1.5	6	7	1	1	4	1	4		1	1	large	red	s	MONTCALMxBRB189
SEL_1547	A	A3	3.2	4.2	8.4	2.4	2	6.5	3.5	4	4		1	5	5	2	3	7	1	6		1	1	large	red	s	DRK57xBRB211
SEL_1548	A	A3	2.8	5.8	9	2.2	3	4	3.5	4	4		1.5	5	5	2	3	5	2	3		1	1				DRK57xBRB213
SEL_1549	A	A3	4.6	4.8	7.5	4.3	3.6	5.5	5	3.5	6		1.5	5	7	5	5	5	2	6		1	1	large	dark-red	s	DRK57xBRB214
SEL_1550	A	A3	4.8	5.5	9	3.3	2.3	3.5	3.5	4	4.5	2	2	5	7	2	2	6	2	1	2	1	1	large	red with light-brown mottles	s	DRK57xBRB215
AAB_010	AxM	A4	2	2.4	1.3	1.4		5.5	5.5		1.5			6	1	5	2	6	2	5							MAB352xNUA45
AAB_014_b	AxM	A4	4.7	4.2	8.7	3.3		3	4		3	2		6	5	2	1	5	3	1	1						MAB349xNUA184
ACC_003	A	A4	5	7	9	4	5.2	7	6	6.5	7.5		1	7	7	5	8	7	2	7		1	1	large	dark-red	s	AFR298x(RMX2xBRB266)
AFR_298	A	A4	6	6.7	8	5.7	2.6	6	5.5	6	4		3	6	6	5	2	6	1	6		1	1	large	dark-red	s	G6592xA487

CAL_096	A	A4	6.6	7	8.3	6.2	2.9	5.6	4.8	5.2	4.5	1.9		6.7	6.8	2.6	1.8	5.6	1.6	6	7	1.1	1	large	red with white mottles	s	CALIMA-2xARGENTINO1
DAB_398	A	A4	8.6	7.7	8.3	7.3	5.5	7	6	5.5	5		2.5	8	7	4	3	6	3	8		1	1	large	light-brown with red spots or stripes	s	(CAL143xSAB620)XSAB626
DAB_901	A	A4	7.2	3.7	8	3.3	3.5	4	5.5		6.5			6	7	5	6	2	2	6		1		large	red with white mottles /calima	s	SAB645xKATB9
DAB_913	A	A4	5	6.9	8	2.8		5	6		5			7	6	5	4	5	2	5							SAB645xKATB9
DAB_915	A	A4	9	8.7	9	4.4	4.8	6.5	6.5	7.5	4.5		1.5	8	7	5	2	6	1	7		1	1	large	light-brown with red spots or stripes	s	SAB645xKATB9
DAB_926	A	A4	4	4.6	7.3	3.4	5		4.5	5	3.5		1	6	6	3	1			6		1	1	int./large	white	s	SAB712xKATB1
DAN_003	A	A4	6.5	8.4	9	5.8	5.3	6.5	6.5	6.5	6			7	7	6	5	7	2	6		1	1	large	red with light-brown mottles	s	(SAB645xKATB1)xNUA424
G11796	A	A4	6	5	8.3	4.6		6	4.5					4		5		6		6							
G14301	A	A4	3.3	5.6	1.5	1.3		3	2		2			3	3	1	1	5	5	1					brown with dark stripes	s	
G19833	A	A4	4.2	3.8	7.6	3.8		3	3.5		2.5			5	4	2	1	2	1	4							
G6416	A	A4	5	8	9	8.7	3	7	6.3	6.5	5.3	3	1.5	7.5	7	5	3.5	6	1	8	5	2.5	1	large	dark red	s	
G9603	A	A4	7.5	7.8	8.5	5.3		4.5	4.5		6			7	7	2	5	5	1	4							
ICA_CALIMA	A	A4	6.4	7.4	9	5.3	3	4.5	3	4.5	4		1.5	5	7	1	1	5	1	4		1	1	large	red with light brown mottles	s	
KAT_B1	A	A4	5	5	8.2	2	7.5	6.5	5.5	6	7.5		1	6	7	5	8	5	2	8		1	1	small	yellow		
KAT_B9	A	A4	5	5.2	8.8	9	7.5	6.5	7	6	6.5		1	8	7	6	6	5	1	8		1	1	small	dark red-brown	s	
LPA_467	A	A4	5.4	6.7	9	3.8		6.5	6.5		6			8	7	5	5	7	3	6							(SUG47xSEA5)x(SUG47xUSA63)xSAB682
LSA_142	A	A4	4.5	4.7	9	2.7		4.5	5.5		1.5	1		6	2	5	1	5	1	4	1						G12666xG14016
MAB_376	M	A4	6.8	5	2.3	8.7	4.1	5	4.5	2.5	2.5	2	1.5	3	4	6	1	5	1	5	3	1.5	2	small	black-red	s	RAB619X(G9836xRAB655)
MAZ_032	A	A4	3.3	2.6	2.8	3.5	2.7	4.5	4.5	3.5	4	2.5	1	6	6	3	2	4	3	5	2	1	1	large	red	s	(CAL143xSAB620)x(RMA70xRAZ167)
TAL_SUGAR	A	A4	4.3	4.2	9	5	4.5	3	5	5	3	3	1	7	5	3	1	2	3	4	3	1	1.5	large	light-brown with dark-red stripes	s	
NUA_035	A	A4	6	8.3	9	5.2	3	5.9	6.2	7	4.7			7.3	6.3	5.1	3.3	5.7	1.8	6.6		1	1	int./large	dark-red with light brown mottles/calima		CAL96X(CAL96XG14519)
NUA_045	A	A4	8.3	5.6	9	8.3	3.4	6	5.5	5	2.5	3	2	8	4	3	1	5	2	7	4	1	1	large	red white mottles/calima		
SAB_645	A	A4	4.8	7.2	8.5	4.8	4	4.5	5.5	6	2.5		2.5	7	4	4	1	5	1	4		1	1	large	red wit white mottles/calima	s	
SAB_711	A	A4	7.7	5.5	7.6	5	3.3	5	6.5	6	4.5		3	8	6	5	3	4	2	6		1	1	int./large	white	s	(ABA58xICAQUIMBAYA)F1xSAB258
SAB_712	A	A4	6.9	4.7	4.8	6.3	4	5.5	6	6.5	4.5		2	7	6	5	3	5	2	6		1	1	small	white	s	(ABA58xICAQUIMBAYA)F1xSAB258
SEL_1513	A	A4	5.6	7.8	9	8.7	6	6.5	6	4.5	3.5		1	7	6	5	1	6	2	7		1	1	int./large	dark red-brown		L.CATRACHITAI-17AX(L.CATRACHITAI-17AXBRB153)
SEL_1516	A	A4	8.3	7	8.8	8.8	6.6	8	6.5	4.5	5.5		1	8	7	5	4	7	1	9		1	1	large	brown	s	G76X(G76XBRB189)
SEL_1517	A	A4	6.6	7.2	9	6.7	5.8	5.5	3.5	5.5	3.5		1.5	3	5	4	2	7	1	4		1	1	int./large	brown	s	G76X(G76XBRB189)
SEL_1518	A	A4	4.8	8.8	8.7	5.8	5.9	7.5	5.5	6.5	5		1.5	7	7	4	3	7	2	8		1	1	large	brown	s	G76X(G76XBRB189)
SEL_1519	A	A4	5.6	9	9	6	7.3	7	5	5	4.5		1	8	6	2	3	7	1	7		2	1	large	beige	s	G76X(G76XBRB189)
SEL_1520	A	A4	3.8	6	9	9	7	7	6.5	5	4		1	7	7	6	1			7		1	1	large	light-brown	s	G76X(G76XBRB211)
SEL_1521	A	A4	4.3	8.7	9	9	4.7	8	5.5	7.5	4		1.5	7	7	4	1			8		1	1	large	beige	s	G76X(G76XBRB211)
SEL_1533	A	A4	4.5	7	8.6	5.8	2	6.5	8	6	6.5		1.5	9	8	7	5	7	2	6		2	1	int.	red	s	G76X(G76XBRB186)
SEL_1545	A	A4	5.2	7.3	9	7.7	2.8	3.5	4.5	4	3.5	1.5	2	7	3	2	4	4	2	3	1	1.5	2	large	var. red and light-red	s	MONTCALMxBRB191
SEL_1560	A	A4	5	8.5	7.5	6.8	4.4	5	5.5	4	2.5	7	2	7	4	4	1	5	5	5	9	1	1	int./small	dark-red	s	(L.CATRACHITAxINIA17)AxAFR298
SEL_1561	A	A4	6.8	8.4	9	4.2	3.8	5	5.5	6.5	3.5		3.5	6	6	5	1	5	3	5		1	1	int./small	dark-red	s	(L.CATRACHITAxINIA17)AxAFR298
SEL_1562	A	A4	7.2	9	7.9	6	3.8	5	4.5	3.5	3	3.5	1.5	6	4	3	2	5	2	5	5	1	1	int./small	red	s	(L.CATRACHITAxINIA17)AxAFR298
SEL_1563	A	A4	7.5	8.6	9	3.3	4.3	4.5	4.5	4	3.5	3	2.5	6	6	3	1	4	3	5	3	1	1	int./small	dark-red	s	(L.CATRACHITAxINIA17)AxAFR298
SEL_1564	A	A4	5.4	8.6	5.8	5.4	3.9	4.5	6	6	5	2.5	2	6	6	6	4	5	2	4	3	1	1	int./small	dark-red	s	(L.CATRACHITAxINIA17)AxAFR298
SEL_1565	A	A4	5.6	9	8.5	6		5	4.5		3.5			5	6	4	1	5	2	5							(L.CATRACHITAxINIA17)AxAFR298
SEL_1566	A	A4	5.3	6.6	9	5.4	2.7	6.5	5.5	5	6		2	7	6	4	6	7	1	6		1	1	large	dark-red	s	(L.CATRACHITAxINIA17)AxBRB153
SM_26775	AxM	A4	5.3	3.4	9	6		6	6		5	2		6	6	6	4	6	2	6	2						MAB351xCAL96

SM_26785	AxM	A4	9	6.7	6.2	5.3		6	6		3.5	2		6	4	6	3	6	2	6	2						MAB353xNUA45
SM_26796	AxM	A4	7.2	5.8	8.6	4.7		6.5	5.5		4	3		7	6	4	2	7	4	6	2						MAB484xLYANMUNGO90
SM_26819	AxM	A4	8	7.8	6.9	5		6.5	6.5		4.5			8	7	5	2	7	5	6							MAZ32xMAB484
DAB_252	A	A5	4.6	5.3	9	5.5	3	6.5	7	7.5	6.5		3	8	8	6	5	5		8		1	1	large	red with light-brown mottkes	s	(SAB628xCAL143)XSAB659
DAB_573	A	A5	5	8	8.7	4.5	3.7	5	3.5	6.5	4		1.5	6	7	1	1	5	1	5		1	1	large	red	s	PVA773xAFR298
DAB_586	A	A5	6.7	7	9	4.5	5.8	7.5	4.5	6.5	4			8	7	1	1	7	3	8		1	1	large	dark-red	s	SEQ1004xBRB266
DAB_604	A	A5	5.8	8	8.8	3.2	4.3	7	5.5	5.5	5		2.5	7	6	4	4	7	3	7		1	1.5	large	dark-red	s	SUG47xRAA30
G5164	A	A5	7.6	9	7.2	4.7	2.9	5	5	7	5	3	3	7	6	3	4	5	3	5	3	1	2	int./small	light brown with dark-brown-red spots or stripes	s	
LPA_714	A	A5	7	7.3	2	2.3	5.2	5	4.5	6	3.5	2.5	2.5	5	6	4	1	6	3	4	2	1	1	large/int.	red	s	(SUG47xSEA5)x(PVA773xUSA63)xCIM9314-36
RAA_030	A	A5	9	8.8	6.8	5.7	5.2	6.5	6	5.5	5.5		3	7	7	5	4	5	2	8		1	1.5	large	dark-red	s	AFR291x(DRK9xAFR188)
SEL_1542	A	A5	4.3	5	8.5	7.2	4.2	6	5	6	3.5		1	7	6	3	1	7	1	5		1	1	large	brown	s	MONTCALMxBRB191
SEL_1543	A	A5	4	4.7	8.2	8.5	3.7	6	6.5	3.5	4		1	8	5	5	3	6	2	6		1	1	large	brown	s	MONTCALMxBRB192
SEL_1544	A	A5	5	7.4	7.7	7.2	3.7	7	6	7	3.5	2.5	4	7	6	5	1	6	2	8	3	1	1	large	dark red-brown	s	MONTCALMxBRB190
SEL_1552	A	A5	5.2	7	3.8	6.3	4.3	6	6.5	6	3.5	2.5	1	8	6	5	1	6	3	6	2	1.5	1	large	dark-red	s	DRK57xBRB217
SEL_1553	A	A5	6	6.5	3.8	7	3.5	6	7	7.5	5.5		2	8	6	6	5	6	1	6		1.5	1	large	dark-red	s	CHOCHOxBRB189
SEL_1554	A	A5	6.3	7.4	8.3	7	8.4	7	7	7	5.5		1.5	8	6	6	5	7	1	7		1	1	large	light brown-red	s	REDKLOUDxBRB191
SEL_1555	A	A5	6.6	9	9	7	5.8	7.5	7.5	6.5	5.5		1.5	8	7	7	4	7	1	8		1.5	1	large	red	s	REDKLOUDxBRB192
SEL_1556	A	A5	6.8	6.2	8.5	8.7	3.8	7.5	6	8	6		1.5	7	7	5	5	7	1	8		1	1	large	brown	s	REDKLOUDxBRB189
SEL_1557	A	A5	4.3	5.2	9	4.5	5	7	6	6.5	7		1	7	7	5	7	7	1	7		1	1	large	light brown-red	s	REDKLOUDxBRB190
SEL_1558	A	A5	2.6	7.6	5.6	5.2	3.6	7.5	6.5	7	5.5			7	6	6	5	8	1	7		1	1	large	brown	s	REDKLOUDxBRB192
SEL_1559	A	A5	4.2	6.7	7.5	3.2	2.7	6.5	4.5	5	3.5		1	7	6	2	1	6	1	7		1	1	large	red	s	RAA15xBRB189
SUG_131	A	A5	4.8	5	4.8	4.2	4	3.5	4	5	2	1.5	1	6	3	2	1	4	2	3	1	1	1	large	white/beige with red stripes	s	
AFR_702	A	M/A	3	4.4	2	7.5	2.1	1.5	2	5	1.5		2	2	2	2	1	2	2	1		1	3		dark-red	o	MutikiredxAFR308
AFR_703	A	M/A	5.3	4.2	1.4	4.6	2.1	2.5	3.5	4	2	1.5	1.5	4	2	3	2	3	2	2	1	1.5	2.5	large	dark-red	s	MutikiredxAFR309
BAT_332	M	M/A	4.5	4.8	1	7.7	5.6	6.5	3	2	1.5	3.3	1.5	2.7	2	3.3	1	6.5	4	6.5	2.5	1.5	1.5	very small	light-brown	o	G4525xG4485
BAT_841	M	M/A	6.7	8.6	1.2	6.3		7				5						7	5								G4122xG1320
BEAVER_3	M	M/A	8.7	9	1	8.5	8.3	6.5	6	4.5	4	5	1	5	5	7	3	7	5	6	5	1	1	small	dark red-brown	s	
DAB_049	M	M/A	8.8	7.9	2	5.8	7.3	7.5	6.5	6	6		2	7	8	6	4	8	4	7		1	2	small	dark-red with brown mottles	s	SEQ11xTALSUGAR
DAB_578	A	M/A	6	4.2	3	3.7	3.6	4	3	3.5	2		1.5	3	3	3	1	3	3	5		1	1	large	red with white mottles but not calima	s	PVA773xRAA30
DAB_603	A	M/A	3.2	3.8	4.2	3	2.1	3.5	3.5	4	4		2	3	5	4	3	5	4	2		1	1	int./large	red	s	SUG47xRAA30
DICTA_17	M	M/A	9	8.8	2.5	8.3		8	6.7		5.7			6.6	5.9	6.9	5.4	8	3.8	8							(MCD2004x(BAT1225xAB136))
FLOR_DE_MAYO	M	M/A	9	9	1	9	8.6	7	7	3.5	5.5	3	2.5	6	5	8	6	7	4	7	2	1	3	int./large	brown-violet	s	
G13936	A	M/A	8.5	8.7	3.2	9	7.6	7.5	6	6	3.3	5	1.5	5.5	4.5	6.5	2	7	5	8	5	1	3.5	small	black-brown	s	
G1805	M	M/A	2.2	1	1.5	4.3	8.5	6	2	2	2	4	1.5	3	3	1	1	7	7	5	1	1	3	small	white/beige	o	
G18970	M	M/A	7.7	6.7	1.7	2.7		5	6		1.5	3		4	2	8	1	6	4	4	2			small	brown	s	
G2858	M	M/A	9	9	1	9	4.2	8	7.5	7.5	7.8			7.5	7.3	7.7	8.3	7.5	7	8.5		1	2	int./large	light-brown with green-brown spots/mottles	s	
G4090	M	M/A	7.8	8.4	1.8	8.8	8.8	7.5	8	6	7		1.5	8	7	8	7	7	1	8		1	2	very small	red	s	
G4691	M	M/A	7.2	2.7	1.5	3.5		6	2.5		1.5	1		3	2	2	1	7	1	5	1			small	light-brown	o	
G5207	M	M/A	7.2	1.2	1.2	2.8		3.5	6		2.5	1.5		6	3	6	2	5	2	2	1			small	black	o	
G5694	M	M/A	8.4	7	1	4.5	3.7	6.8	5.7	5.5	5		1.5	5	5	6.3	5	5.5	3	8		1	1.5	very small	black	o	
G855	M	M/A	4.8	2.7	1	7.7	4.5	6.5	2	2	3		1	2	5	2	1	6	5	7		1	3.5	small	brown	o	
ICA_PIJAO	M	M/A	6.8	8.3	1	8.8	5.1	7	6	5.5	3.5	5.5	1.5	6	6	6	1	7	6	7	5	1	1.5	very small	black	o	PorrilloSintéticoxMéxico11

INB_820	M	M/A	6.3	4.3	1	9		6.5	3		1.5	6		2	2	4	1	7	7	6	5			small	brown-cafe	o	INB108xINB605
MAB_344	M	M/A	8.8	3.2	1	1.5	1.5	6	5.5	2	4	5	1.5	4	6	7	2	6	7	6	3	1	1.5	small	dark-red	s	TIO CANELA75X(G4691xG10613)
MIB_216_2	M	M/A	8.6	7.8	1.5	5.6		6.5	5.5		3	2.5		6	4	5	2	6	3	7	2						FEB226xINB150
NUA_365	A	M/A	6	4.3	6.2	9	4.5	5	7	7	3		2.5	6	5	8	1	3	1	7		1.5	1	large	red with white mottles/calima	s	NUA35x(CAL96xG23823E)
PAN_072	M	M/A	9	9	1	7.5		6.5	6.3		4	6.5		6	4.5	6.5	3.5	6	6	7	7			small	white	s	BAT338xXAN86
SEL_1570	M	M/A	4.2	5	1	5	6.4	6	6	4.5	3.5	3.5	2	5	3	7	4	7	4	5	3	2	2	small/int.	black	o	(SMC33xSMN38)X(SEF100xSMC140)
SEQ_011	M	M/A	8.4	8.6	1.2	6.3	7.8	8	7	6	5	4	2	7	7	7	3	8	4	8	4	1.5	2	small	violet with light-brown mottles	s	BAT477xSCRISTOBAL83
SIN_524	M	M/A	4.2	3.8	1	9	4.4	6.5	2	2.5	1.5			2	2	2	1	6	2	7		2.5	1	small	black	o	ICTA LIGEROxINB841
SMC_184	M	M/A	3.5	3	1.2	5.7	6.4	4.5	2.5	2.5	2	1	1	2	2	3	2	4	1	5	1	1.5	2	small	brown with darker stripes	o	(SMC124xSMC47)X(SMC130xSMC21)
SMC_230	M	M/A	2	3.3	1	5.2		3.5	2		1.5	1.5		2	2	2	1	6	2	1	1						(SMC124xSMC47)X(SMC130xSMC21)
SMN_045	M	M/A	8.3	1.8	1	1	2.1	1.5	7	2.5	5	1	1.5	7	5	7	5	2	1	1	1	1	2.5	small	black	o	(SCR16xMAB766)X(MIB780xSMR49)
SMR_084	M	M/A	9	7.7	1	4.5	3.9	7.5	7	5.5	5.5	4	2	7	6	7	5	7	5	8	3	2	3	small	dark dark-red	s	(SCR2xSMR42)X(MIB755xSMC16)
AAB_001	AxM	M1	2.7	3.6	1	3	6	1.5	2	2.5	1.5	2.5	1	3	2	1	1	2	4	1	1	1.5	1.5	int./large	dark-red	s	SAB686xMAB348
AAB_001_b	AxM	M1	3	3.5	1.2	2.3	5.3	1	2.5	2.5	1.5	2	1	3	2	2	1	1	3	1	1	1	1.5	small	dark-red	s	SAB686xMAB348
AAB_004	AxM	M1	1.8	1.5	1	1.2		1	1.5		1			2	1	1	1	1	1	1							NUA45xMAB348
AAB_005	AxM	M1	1.3	1.8	1	2.2	3.2	1	1.5	2	1		1.5	2	1	1	1	1	2	1		1	1	small	red	s	AFR298xMAB349
AAB_005_b	AxM	M1	2.2	1.3	1	1.8	3.5	1	2	2	1	1.5	1	2	1	2	1	1	2	1	1	1	1	small	red	s	AFR298xMAB349
AAB_006_b	AxM	M1	1.5	1.2	1	2		1	1.5		1.5	1.5		2	2	1	1	1	2	1	1						MAB352xAFR298
AAB_007	AxM	M1	2.2	2.7	1	2.7	4.8	3	1.5	2	1.5	1	1	2	2	1	1	5	1	1	1	2	2	small	black	o	MAZ32xMAB348
AAB_007_b	AxM	M1	3	2	1	3.2	5.5	1	1.5	3.5	1	1	1.5	2	1	1	1	1	1	1	1	1.5	2	int./large	var. brown and dark dark-red	s	MAZ32xMAB348
AAB_008	AxM	M1	2.8	2		1.8		1	1.5		1.5			2	2	1	1	1	1	1							MAB349xNUA45
AAB_008_b	AxM	M1	1	1.2	1.3	1.6	2.8	1	1.5	2	1.5	2	1.5	2	2	1	1	1	3	1	1	2	3	int./large	var. dark red with white mottles		MAB349xNUA45
AAB_009	AxM	M1	2	3.5																							NUA45xMAB348
AAB_009_b	AxM	M1	2.8	2	1.5	1.5		1	2		1			2	1	2	1	1	2	1							NUA45xMAB348
AAB_010_b	AxM	M1	2.8	2.2	1	1.8		1.5	2		1.5	2		2	2	2	1	2	3	1	1						MAB352xNUA45
AAB_011	AxM	M1	4.7	2	1	1	2.7	1.5	3	2	2	2	1	2	3	4	1	2	3	1	1	1.5	2	int./large	dark-red	s	MAB484xSAB686
AAB_012	AxM	M1	2	1.2	1	1.7	3.7	1	1.5	2	1		1.5	2	1	1	1	1		1	1	2.5	1	small	dark-red	s	MAZ32xMAB354
AAB_012_b	AxM	M1	2.3	1	1.2	2		1	1		1	2		1	1	1	1	1	3	1	1						MAZ32xMAB354
AAB_013	AxM	M1	2.3	2.8	1	4		3	2		2	2		2	3	2	1	1	2	5	2						SAB686xMAB348
AAB_014	AxM	M1	1.5	1.6	1	2		1	1.5		1.5			2	2	1	1	1	2	1							MAB349xNUA184
MAB_348	M	M1	2	1.6	1	2.5	8.1	1	1.5	2	1.5			2	2	1	1	1	2	1		1	1.5	small	dark brown-red	s	(MD23-24x(G4691xG10474))X(G4090x9824-56-2)
MAB_349	M	M1	1.7	1.3	1	2.5	4.8	1	2	2	2		1	2	3	2	1	1	2	1		1	2	small	dark brown-red	s	(MD23-24x(G4691xG10474))X(G4090x9824-56-2)
MAB_350	M	M1	2.6	2.2	1	2.7	7.2	1.5	3.5	2	3		1.5	4	5	3	1	2	2	1		1	2	small	dark brown-red	o	(MD23-24x(G4691xG10474))X(G4090x9824-56-2)
MAB_351	M	M1	1.2	1.7	1	1.6	6.1	1	1.5	2	2		1	2	3	1	1	1	2	1		3	2	small	red	s	(MD23-24x(G4691xG10474))X(G4090x9824-56-2)
MAB_354	M	M1	2.4	2.3	1	2	4.8	1.5	1.5	2	1		1.5	2	1	1	1	2	3	1		1	2	small	dark-red	s	(MD23-24x(G4691xG10474))X(G4090x9824-56-2)
MAB_359	M	M1	1.3	3	1	2.3	4	2.9	2	2.5	1.3	2	1	2.4	1.4	1.7	1.2	3.6	2.4	1	1	1.5	2	small	black	o	(FEB212x(G4691xG10474))X(DOR500x(DOR390x(DOR390xSAM1)))
MAB_373	M	M1	1.3	1.8	1	2.5	4.2	1.5	1.5	2	1.5	3	1.5	2	2	1	1	2	4	1	2	1	1.5	small	black	s	TIOCANELA75X(G9836xRAB655)
RAI_044_2	AxM	M1	3.5	1.8	1	4.8		3.5	4.5		2	1		4	3	5	1	2	1	5	1						G5686xG10474
RAI_044_3	AxM	M1	2.3	2.3	1	4	5.3	3	6	2	1.5	1.5	1	5	2	7	1	1	1	5	2	2	1.5	int./large	dark-red with red-brown mottles	s	G5686xG10474
RAI_060_2	AxM	M1	2.2	1.8	1.3	9	5.1	2.5	3	2	2.5	1.5		3	4	3	1	1	2	4	1	3.5	1.5	small	dark dark-red	s	G5686xG10474
AAB_002	AxM	M2	7.8	1.8	1	1		1	4.5		1.5			5	1	4	2	1		1	1						MAB484xAFR298
AAB_011_b	AxM	M2	5.3	3.4	3.2	2.4	3.5	6.5	4	3	3.5	1.5	1	4	4	4	3	6	2	7	1	1.5		large	var. red, light dirty red and red with white mottles	s	MAB484xSAB686

G10613	M	M2	8.5	2.3	1	1.6	2.3	1	5	2	3.5	2.5	1	4	4	6	3	1	4	1	1	1.5	4	small	white	s	
MAB_089	M	M2	9	2	1	1	2	2	6	2		2	1	7		5	1	2	3	2	1	1	1.5	small	red	s	TIOCANELA75X(G4691XG10613)
MAB_090	M	M2	9	1.7	1	1	2.3	1	5	2.5	3	2.5	2	5	5	5	1	1	3	1	2	1.5	2.5	small	red	o	TIOCANELA75X(G4691XG10613)
MAB_093	M	M2	9	3.3	1	1.5	2.3	1	6	2.5	5	2	1	6	6	6	4	1	3	1	1	2	1	small	black	int./o	(FEB212x(G4691xG10613))x(DOR390x(DOR390x(DOR390xSAM1)))
MAB_094	M	M2	8.2	2.8	1	1.5	3	1	6.5	2	1.5		1.5	6	1	7	2	1		1	1	1.5	1	very small	black	int./o	(FEB212x(G4691xG10613))x(DOR390x(DOR390x(DOR390xSAM1)))
MAB_095	M	M2	9	3.2	1	1	2.8	1.5	6.5	2.5	2.5	2	1.5	6	2	7	3	2	3	1	1	2	1	very small	black	o	(FEB212x(G4691xG10613))x(DOR390x(DOR390x(DOR390xSAM1)))
MAB_096	M	M2	9	7.7	1	1	2.9	4	6.5	2.5	3	1.5	1	7	5	6	1	7	2	1	1	2	1	very small	black	o	(FEB212x(G4691xG10613))x(DOR390x(DOR390x(DOR390xSAM1)))
MAB_097	M	M2	8.8	3.3	1	1	2.9	2	6	2	3.5	1.5	1	5	4	7	3	3	2	1	1	1.5	1	very small	black	o	(FEB212x(G4691xG10613))x(DOR390x(DOR390x(DOR390xSAM1)))
MAB_098	M	M2	8.8	3.4	1	1	2.1	1.5	6.5	3	3	2	2	6	3	7	3	2	3	1	1	1.5	1	very small	black	o	(FEB212x(G4691xG10613))x(DOR390x(DOR390x(DOR390xSAM1)))
MAB_099	M	M2	7.4	3	1	1	2.1	2	5.5	2.5	2.5	3	1	6	4	5	1	3	5	1	1	1.5	1	very small	black	o	(FEB212x(G4691xG10613))x(DOR390x(DOR390x(DOR390xSAM1)))
MAB_100	M	M2	9	3.7	1	1	2.3	1	6	3.5	4	2	1	6	3	6	5	1	3	1	1	1	1.5	very small	black	o	(FEB212x(G4691xG10613))x(DOR390x(DOR390x(DOR390xSAM1)))
MAB_163	M	M2	8.5	2.8	1	4.3		4.5	6.5		6	2		6	5	7	7	3	1	6	3						TIOCANELA75X(G4691xG10613)
MAB_345	M	M2	9	1.7	1	1.3	2.5	1.5	6.5	2	3	1.5	1	6	4	7	2	2	2	1	1	1	1.5	small	white	o	TIOCANELA75X(G4691xG10613)
MAB_346	M	M2	9	3.8	1	1.3	2.4	1	5.5	3	3.5	1.5	1	5	4	6	3	1	2	1	1	1.5	1	small	light-brown with dark-brown stripes	o	FEB212X(G4691xG10613)
MAB_347	M	M2	9	3.5	1	1		1	6		2.5	1.5		5	4	7	1	1	2	1	1						FEB212X(G4691xG10613)
MAB_484	M	M2	8	2.5	1	1	3.3	1.2	5.9	2	3.6	1.7	1	5	4.7	6.5	2.8	1.2	2	1.1	1.4	2.5	1	small	red	s	(MAB163xSER31)X(SEN22xSER7)
MAB_766	M	M2	9	6.9	1	1.5	2.8	3.5	6	2	1.5	3	1.5	6	2	6	1	5	4	2	2	1	2	small	black	o	((MAB484xMIB158)xMIB475-1)-3XMIB602
SER_320	M	M2	8.5	2.6	1	1.2	2	1	7	3	3.5		1.5	7	6	7	1	1	2	1		1	1.5	small	dark red	o	(SER155xRCB234)X(MIB451xMIB487)
SMC_051	M	M2	5.6	1.5	1	1	2.2	1	5	2	2	2	1	4	3	6	1	1	3	1	1	1.5	1.5	small	brown with darker stripes	o	(SCN7xSMB14)X(SER158xMIB780)
SMN_031	M	M2	7	1.7	1	1	2.7	4.5	6.5	3.5	4	2.5	1.5	6	5	7	3	6	4	3	1	2	2	small	black	o	(SCR16xMAB766)X(MIB780xSMR49)
SMN_033	M	M2	7.8	1.3	1	1		1	6		4.5	1.5		6	4	6	5	1	2	1	1			small	black	o	(SCR16xMAB766)X(MIB780xSMR49)
SMN_042	M	M2	7.3	1.3	1	1	2.1	1	6.5	3	2.5	2.5	1.5	6	4	7	1	1	3	1	2	1	2.5	small	black	o	(SCR16xMAB766)X(MIB780xSMR49)
SMN_047	M	M2	9	1.8	1	1	2	1	7	2	3.5	1.5	1.5	7	3	7	4	1	2	1	1	1	2.5	small	black	o	(SCR16xMAB766)X(MIB780xSMR49)
SMN_049	M	M2	9	1.7	1	1	2.5	1	7	2	4	2.5	1.5	7	4	7	4	1	4	1	1	1	2	small	black	o	(SCR16xMAB766)X(MIB780xSMR49)
SMN_051	M	M2	8.8	1.3	1	1.2	2.9	1	7	3.5	3	2	1	7	5	7	1	1	3	1	1	1.5	2	small	black	o	(SCR16xMAB766)X(MIB780xSMR49)
BFS_057	M	M3	7.8	4	1	1.8	3	2.5	6	2.5	6	1.5	2	5	6	7	6	2	2	3	1	1	1	small	dark red	o	(SER176xRCB591)x(SXB407xSER118)
G5653	M	M3	6.5	2.2	1	1.7	3	1	6	2	4.5	2	1	6	5	6	4	1	3	1	1	1.5	3	small	red	s	
MAR_001	M	M3	9	2	1	1.3	3.1	2	5.8	2	3.5		1	5	6	6.5	1	2.8	4.2	1		1.5	2	small	brown	o	(BAT85x(A83xXAN112))X(G15416X(A442xG811))
MAR_002	M	M3	9	3.4	1	2.5	2.6	1	4.5	2	5	1.5	1.5	4	6	5	4	1	2	1	1	2	3	small	light-brown with green-brown stripes	s	A252xG5653
MEXICO_54	M	M3	8.1	4.5	1	1.7	2.3	1.3	6.3		6			5.7	6.3	7	5.7	1.5	4	1				int/small	light-brown	s	
RCB_591	M	M3	9	3	1	1.7	2.3	1.5	6	2	4	2	1.5	6	5	6	3	2	3	1	1	1	2	small	dark-red	s	(NCB228xRCB224)xSXB244
SCR_066	M	M3	7	2.4	1	1	2	1	5.5	3	3.5	1	1	5	6	6	1	1	1	1	1	1.5	1	small	red	s	(SMC17xMIB396)F1X(SCR8xSMC37)
SEC_035	M	M3	6	3.8	1	1.8		1.2	6.5		5.5			6	5.8	7	5.3	1.5	2.3	1							(SMC21xSMR59)X(SMC33xSCR16)
SMC_188	M	M3	9	5.3	1	2	2.1	1.5	5.5	2	3	1.5	1.5	5	3	6	3	2	2	1	1	1	2	small	brown	o	(SMC33xSCR9)F1X(SEF38xSMC143)
SMR_045	M	M3	8.7	2.5	1	2	2.3	1.5	6.5	2	6	2	1	6	6	7	6	2	3	1	1	1.5	2	small	dark-red	s	(SER125xSMB6)X(SXB743xMIB499)
SMR_069	M	M3	9	4.3	1	2.5	1.7	2	6	2.5	3.5		1	7	6	5	1	3	1	1		2	1.5	small	dark-red	s	(ALB34xSMR40)X(SER48xMIB778)
SMR_081	M	M3	8.8	3.7	1.5	2.2	2	1	6	2.5	4	1	1	6	4	6	4	1	1	1	1	1	2	small	red-brown	s	(SMC13xSCR39)X(MIB755xSMR47)
SMR_120	M	M3	4.5	5	1	2.7	2.3	2	5.5	2.5	4	1.5	1.5	4	4	7	4	3	2	1	1	1.5	1.5	small	dark-red	s	SMC48X(SMC16xBFS32)
SMR_121	M	M3	5.4	5.1	1	3	2.2	2	6	2	3.5	3.5	1.5	6	6	6	1	3	5	1	2	1	2	small	dark-red	s	SMC48X(SMC16xBFS32)
SMR_155	M	M3	8.5	4.4	1	1.8	4	2	6	2.5	2	2.5	1	6	3	6	1	3	4	1	1	1.5	1	small	red	s	(SMC40xSCR16)XSMR72
G3680	M	M4	9	2.4	2.5	1		5	6		4.5	2		6	4	6	5			5	2						
GGR_017	M	M4	8.8	1.3	1	1	2.6	1	7.5	3	4.5	2	1.5	8	6	7	3	1	3	1	1	1	2	small/int.	red-brown	s	

GGR_029	M	M4	8.5	1	1.2	1	2.3	3.5	6.5	4.5	3.5	1.5	2	6	5	7	2	2	2	5	1	1	2.5	small	light-brown	s	RCB593X(SEA15xG13614)
MAB_375	M	M4	8.2	7	1.3	7.8	4.3	5.5	4.5	3.5	3		2	3	5	6	1	6	2	5		1	2	small	red	s	RAB619X(G9836xRAB655)
RCB_592	M	M4	8.7	1.6	1	1	2.3	1.5	6.5	4	6	1	1	5	6	8	6	1	1	2	1	1	2	small	dark-red	s	(NCB228xRCB224)xSXB244
RCB_593	M	M4	8.3	1.2	1	1	2.2	2.5	6.5	3.5	5	2	1	6	4	7	6	4	3	1	1	1	2	small	dark-red	s	(NCB228xRCB224)xSXB244
SCR_040	M	M4	8.2	1.2	1	1	3.1	1	6.5	3.5	6	1.5	1	6	6	7	6	1	1	1	2	1	2	small	dark-red	s	SER48xRCB593
SEF_040	M	M4	7.6	1.3	1	1		1	6		5	1		6	6	6	4	1	1	1	1			small	dark-red	s	(ALB74xINB841)XRCB593
SEL_1569	M	M4	6.7	2.2	1	1.8	2.3	3.5	5.5	3.5	5.5	2.5	1.5	6	6	5	5	2	3	5	2	1	2	small	dark-red	s	(SMC41xSER326)X(ALB91xSCR16)
SEN_086	M	M4	7.2	3.3	1	2	2.3	2	5.5	2	3.5		1.5	6	6	5	1	3	3	1		1.5	1	small	black	s/i	C-20X(VAX6xSER16)
SER_339	M	M4	7.5	5.7	1	3.3	7.1	6	6.5	5	4.5	5	2.5	6	5	7	4	7	5	5	5	1.5	1	int./small	red	s	(SMC21xSMR57)X(SCR8xSMC13)
SER_385	M	M4	8.5	6.2	1	3.2	5.6	5	7	4.5	4	4.5	2	7	6	7	2	5	5	5	4	1	2.5	small	dark-red	s	(SMC47xSMB33)X(SEF82xSMC140)
SMC_122	M	M4	8	1.5	1	1.2	2.5	1.5	5.5	4	3.5		2	6	6	5	1	2	3	1		1	2	small	cafe	o	(MAB766xSMR46)X(MIB755xRCB593)
SMC_175	M	M4	8.8	8.4	1	4	5.4	7.5	7	6	7.5		3	6	7	8	8	7	5	8		1	2	small	white	o	(SMC47xSMN40)F1X(SCR16xSMC21)
SMC_179	M	M4	7.8	5.8	1	4.5	6.5	6	6	3	5	4.5	1	5	5	7	5	7	7	5	2	1	2.5	small	light-brown	o	(SMC40xSCR16)F1XSMC140
SMC_203	M	M4	8.5	6.4	1	2.7	3	5.5	7	3.5	6	2	2.5	7	6	7	6	6	2	5	2	1	2	small	brown with small dark brown spots	s	(SMC47xSMN40)F1X(SCR16xSMC21
SMC_228	M	M4	5.2	8.8	1	7.5	4.4	7.5	6	5	4	3	1.5	6	5	6	3	7	3	8	3	1	2	small	light-brown with small violet spots	o	(SMC47xSMN40)X(SCR16xSMC21)
SMC_229	M	M4	6.3	4.5	1	1.8	3.9	5.5	6.5	4.5	5.5		3	7	6	6	5	6	2	5		1	2.5	small	white	o	(SMC47xSMN40)X(SCR16xSMC21)
SMN_064	M	M4	8.3	4.8	1	2.7	3.7	6	6.5	6.5	3.5	1.5	2	6	6	7	1	6	1	6	2	1	2	small	black	o	(SMC33xSMN10)X(SMC141xSEF31)
SMR_130	M	M4	8.4	7.6	1.2	7	4	8	6.5	5	3.5	4.5	2	7	6	6	1	8	6	8	3	1	1.5	small	dark red	s	(SMC17xSCR16)XSMR57
SXB_743	M	M4	8.8	1.7	1	1.2	2.2	1.5	6.5	4	5.5	1		6	6	7	5	2	1	1	1	1.5	2	small	dark red	s	(NCB228xRCB224)xSXB244
A_154	M	M5	8.4	7.4	1	7.5	2.8	5	3	2	2	3.5	1.5	2	3	4	1	5	4	5	3	1.5	1	very small	beige	o	INT248xA30
A_801	M	M5	3.8	4	1	5	9	4.5	3	2.5	3.5	3	2	4	6	2	1	5	3	4	3	2	1.5	small	beige with green brown stripes	o	EMP250X(A769X((A429xXAN252)X(V8025xPINTOUI114)))
AAB_015	AxM	M5	7	7.2	1	6		5	2.5		2	2.5		2	3	3	1	6	3	4	2						MAZ32xMAB353
AQB_147	M	M5	3.2	1.2	1	6	5.3	2.5	2	2	1.5	2	1.5	2	2	2	1	4	3	1	1	2.5	2	small	brown with dark stripes	o	TAR4X((A686xVAX4)x(TIO CANELA75xA801))
BAT_093	M	M5	8.2	8	2	9	4	5.5	3.5	4	4		1.5	5	6	2	2	6	4	5		1.5	1.5	small	yellow	s	(G3709xG1320)x(G3645xG5478)
DOR_364	M	M5	9	9	1	9	5.8	7	6.5	2	4.5	4	1.5	6	5	7	4	7	5	7	3	2.5	1.5	small	red-black	s	BAT1215x(RAB166xDOR125)
MAB_300	M	M5	5	3.8	1	6	4.9	4	3	3	2.5	1.5	2	3	4	3	1	3	2	5	1	2.5	2	small	brown with darker stripes	o	FEB216X((A806X((MAR1XG4032)X(A240XG5686)))
MAB_374	M	M5	7	6.8	1.5	2.8	2.5	7		2.5		4	1.5	7	6			7	4			1.5	2	small	red	s	TIO CANELA75X(G9836xRAB655)
MD_23_24	M	M5	7	7.3	1.2	9	6	6	3	2.5	2	5	1.5	2	3	4	1	7	5	5	5	1	1	very small	dark-red	s	
NXB_080	M	M5	2.6	2	1	4.7	6.1	2.5	2	2.5	1.5	3	1.5	2	2	2	1	4	5	1	1	1.5	2	small	light brown with green brown-dark stripes	o	(A801xMAR1)x((A321xCARIOCA)x(A429xXR-12307-1))
SCR_082	M	M5	1.8	2.7	1	2.6	6.9	1.5	4.5	4.5	2	1.5	1.5	3	3	6	1	2	2	1	1	1	2.5	small	white	o	(SMC47xSMN40)X(SCR16xSMC21)
SEC_071	M	M5	7.6	8.8	1	4	6.8	7	7	6.5	6	6	2.5	6	6	8	6	7	6	7	6	1	2	small	red-violet	o	(ALB6xSCR9)XSMC141
SEL_1567	M	M5	6.3	9	1	4.8	4.5	7	6.5	6	5.5	6.5	2	7	5	6	6	7	7	7	6	1	2	int./small thin	beige	o	(SMC47xSMB33)X(SEF82xSMC140)
SEL_1568	M	M5	5	5.2	1	5.8	6	4.5	3.5	2.5	2	2.5	2.5	3	3	4	1	6	3	3	2	1	2	small	beige with dark-brown stripes	o	(ALB6xSCR9)XSMC141
SER_022	M	M5	7.2	5	1	3.3	5	3.5	2.5	4	4.5		1.5	2	7	3	2	6	1	1		1	1	very small	dark-red	s	(SEA22x(TLP35xG21212))XEAP9504-30-B
SMN_065	M	M5	2.6	3.8	1	3.4	6	3.5	2	2	1	1	1	2	1	2	1	6	1	1	1	1	2	small	black	o	(SMC47xSMN40)X(SCR16xSMC21)
SMR_138	M	M5	2.8	4	1.2	6.2	8	2.5	1.5	2.5	1	2	1	2	1	1	1	4	3	1	1	1	2	small	dark-red	o	(SMC47xSMN40)X(SCR16xSMC21)
SMR_139	M	M5	2.8	3	1	5.2		3	1.5		1	1.5		2	1	1	1	3	2	3	1						(SMC47xSMN40)X(SCR16xSMC21)
SMR_150	M	M5	2.8	3	1	3.2	3.3	1.5	1.5	6	1.5	2	1	2	2	1	1	2	3	1	1	1	1	large	dark-red	s	(SMC47xSMN40)X(SCR16xSMC21)
SMR_158	M	M5	2.3	3.2	1	5.6	8	2	1.5	2	1.5	2	1	2	2	1	1	3	3	1	1	1	2	small	dark-red	o	(SMC47xSMN40)X(SCR16xSMC21)
SMR_165	M	M5	1.3	3	1	3.3	7	2.5	2	3	1	1.5	1	2	1	2	1	3	2	2	1	1	2	small	dark-red	o	(SMC47xSMN40)X(SCR16xSMC21)
SXB_184	M	M5	2	3	1	2.3	7.6	2	2	2.5	1	1.5	1.5	2	1	2	1	3	2	1	1	1.5	2	small	light-brown with dark-brown stripes	o	(SAM6xTIO CANELA75)X(NXB80xG21212)
SXB_412	M	M5	6	5.6	1	4.2	2.8	4.5	4	3	4	3	1.5	4	6	4	2	5	3	4	3	1.5	1	small	light-brown	o	(A686xA774)X(NXB80xSEA15)

AAB_006	AxM	NA	1	1.2	1	1	5.4	1	1.5	2	1		1	2	1	1	1	1	2	1		1	1	int./small	dark-red	s	MAB352xAFR298
AFR_612	A	NA	3.6	3.5	3.3	4.8	2.3	3	2	5.5	1.5	2	2.5	3	2	1	1	4	3	2	1	2	1	large	red with white mottles/calima	s	G20554x(PAD3xG20554)
ALB_252_05	M	NA	9	9	2.2	7	6.2	7	6.5	7	3	5	2	6	5	7	1	7	6	7	4	1	2	int./large	red	o	((VAX1xBRB191)xG21212)x(RAB655xG22041)
ASC_112	M	NA	9	9	1	5.8	3.3	6	2.5	2.5	2.5		1	2	4	3	1	6	3	6		1	2.5	very small	black	o	ICTA ALTENSEx(ICTA HUPUx(ASC72xASC77)
BRB_191	A	NA	5.5	4	2	3.7	2.1	4.5	3	6.5	1.5	1.5	2	4	2	2	1	5	2	4	1	1	1	large	red, most have with white mottles/calima	s	CAL143x(CAL122xMCR2511)
DAB_525	A	NA	7.8	5.6	8.4	3.7	3.2	7	5	4.5	7.5		1.5	7	8	3	7	7	1	7		1	1	intr large	red	s	(KATB1xSAB618)X(SAB623xSAB627)
DAB_600	A	NA	5.8	5.6	9	7.2	4.7	7	6	5	3		3	7	4	5	2	7	3	7		1	1	large	dark-red	s	SUG47xAFR298
DAB_613	A	NA	8	7.4	2.5	6	6	5.5	5.5	7.5	4		1.5	7	5	4	3	5	3	6		1	1	large/int.	dark-red	s	SEQ1004xBRB266
DAB_910	A	NA	6.3	7.2	7.2	6.3	6.1	7.5	6.5	6.5	5		1	7	6	6	4	7	3	8		1	1	large	red-brown	s	SAB645xKATB9
DAB_917	A	NA	4.3	9	8.8	2.7	3.2	5.5	5	4	6		1	7	6	3	6	5	1	6		1	1	int./large	red with white mottles	s	SAB650xKATB9
DAB_935	A	NA	4	4.5	8.4	4.2	3.7	3.5	4	6	2.5		1.5	6	4	2	1	5	2	2		1	1	int./small	white	s	SAB713xKATB1
G10474	M	NA	2.2	1.8	1	2.2	3.5	1	2.5	2	1	1		3	1	2	1	1	1	1	1	2	4	small	red	s	
G10909	M	NA	2.8	1.5	1	3.3	1.9	2.5	1.5	2	1.5		1	2	2	1	1	3	6	2		1.5	4	small	dark dark-red	o	
MAB_352	M	NA	1	1	1	1.8	4.1	1	1.5	2	2.5	1	1	2	3	1	2	1	1	1	1	1	2	small	dark-red	s	(MD23-24x(G4691xG10474))X(G4090x9824-56-2)
MAB_353	M	NA	2.4	2.3	1	2.2	8.4	1.5	1.5	2	1	1.5	2	2	1	1	1	2	2	1	1	1	1.5	very small	dark-red	s	(MD23-24x(G4691xG10474))X(G4090x9824-56-2)
RAI_037_2	AxM	NA	5.2	5.7	1.7	3	5.7	4	2.5	5.5	2	2		4	3	1	1	7	3	1	1	2.5	1	very small	var. dark dark-red and brown	s	G5686xG10474
RAI_037_3	AxM	NA	8.8	4	1		6.1	7		5		3						7	3			4	1	very small	black	s	G5686xG10474
RAI_044_4	AxM	NA	4	2.2	1.2	9	3.2	2	6	2.5	2.5	1	1	6	4	6	1	1	1	3	1	1.5	3	small	red with light-red mottles	s	G5686xG10474
RAI_060_1	AxM	NA	4.2	4	1.5	1.5	6.4	1	2.5	3	2.5	1	1.5	2	4	3	1	1	1	1	1	2.5	1	small	black	s	G5686xG10474
RAI_062	AxM	NA	2.7	2.2	1.2	3.3	3	1	3.5	2	3	2	1	5	4	2	2	1	3	1	1	1	3	int./small	brown-red	s	G5686xG10474
RAI_072	AxM	NA	1.3	2.1	1	2.7	5.5	2.5	2	2	1	1.5	1	2	1	2	1	2	2	3	1	1	3	large	brown	s	G5686xG10474
RAI_072_1	AxM	NA	2	1.4	1.5	6.3		5	3.5		2	1		4	3	3	1	5	1	5	1						G5686xG10474
RAI_072_2	AxM	NA	1	1	1	9	3.7	3	4.5	2	2	1		3	3	6	1	1	1	5	1	1	3	small	light-brown	s	G5686xG10474
RAI_072_3	AxM	NA	3	3.1	1		3.1			2			1									1	3.5	small	brown	s	G5686xG10474
RAI_097	AxM	NA	2.5	1.2	1	3.3	5.1	2	4.5	2	2	1.5	1	5	3	4	1	3	2	1	1	1.5	4	int./small	brown mottled, barely visible	s	G5686xG10474
RAI_133_1	AxM	NA	9	6.2	1.5	3	5.8	3	1.5	5	1.5		1	2	2	1	1	5	2	1		2.5	3.5	small	red (some with fine dark red stripes)	s	G5686xG10474
RAI_133_5	AxM	NA	8.5	5.4	2.7	1	6.1	3	1.5	3	1.5	1.5	1	2	2	1	1	5	2	1	1	3.5	3.5	small	cream/light brown with brown mottles	s	G5686xG10474
RAI_133_6	AxM	NA	9	5	2.2	3.7	8.5	5.5	2.5	6	3	2	1	3	5	2	1	6	2	5	2	1	3	small	brown	s	G5686xG10474
SCN_022	M	NA	6	7.3	1	6	5.7	7	6	3.5	3.5	4.5	2	6	5	6	2	7	5	7	4	1	2	small	black	s	(SMC33xSMN53)F1X(SEF31xSMC21)
SEL_1512	A	NA	7.7	7.7	5.3	4.8	4.5	5.5	6	5.5	6.5		2	6	7	6	6	6	1	5		1	1	int./large	dark-red	s	L.CATRACHITAI-17AX(L.CATRACHITAI-17AXBRB153)
SEL_1523	A	NA	3.8	6.7	8.4	3.5	3	7	6	6	6.5		2	7	7	5	6	6	1	8		1	1	large	light-brown	s	G76X(G76XBRB186)
SEL_1539	A	NA	4	4	1	5.2	2	2	4	5	2	1	1.5	3	2	5	2	3	1	1	1	1	2.5	large	red	s	MONTCALMxBRB182
SEL_1551	A	NA	6.3	6	8.8	4.3	6.7	7.5	4.5	2	5.5		1	6	7	3	4	7	2	8		1	2	small	red	s	DRK57xBRB216
SER_389	M	NA	9	2.8	1	1.3	3.1	6	7	4.5	4.5		2	7	4	7	5	7	2	5		1.5	2.5	small/int.	dark-red	s	(SMC47xSMN40)X(SCR16xSMC21)
SM_26774	AxM	NA	6.2	6.9	9	6		6.5	6.5		6.5			7	5	6	8	6	1	7							MAB352xCAL96
SM_26779	AxM	NA	9	6.4	3	7.3		7	6.5		5	3		7	6	6	4	7	2	7	4						MAB349xNUA45
SMC_081	M	NA	7	2.5	1	1.7	2	2	6	2	3	2	1.5	5	4	7	2	3	3	1	1	1.5	1.5	small	brown	o	(SCB790xSMB14)X(SER155xMIB755)
SMC_195	M	NA	7	8.4	1	3.8	6	6	5	4	2	4	1	4	3	6	1	6	6	6	2	1.5	2	small	brown	o	(SMC33xSMN38)F1X(SEF100xSMC140)
SMN_039	M	NA	1.2	1.8	1	5.7	6.6	3	2	2	1	2	1	3	1	1	1	5	3	1	1	1	3	small	black	o	(SCR16xMAB766)X(MIB780xSMR49)
SMR_149	M	NA	2.7	3.3	1	4.3	6.1	2	1.5	2	1	1.5	1	2	1	1		2	2	2	1	1.5	2	small	dark-red	s	(SMC47xSMN40)X(SCR16xSMC21)

Supplementary material 2: TASSEL 5 command line script and graphical user interface step by step explanation.

GWAS analysis using the command line with shell script named runTASSEL_MLM.sh

---

```
#!/bin/bash

HMP=/Users/mnay/test/extBALSIT_hmp.txt
PCA=/Users/mnay/test/extBALSIT_PCA1.txt
Kinship=/Users/mnay/test/extBALSIT_kinship.txt
Tassel=/Users/mnay/test/TASSEL5/run_pipeline.pl
PHEN=$1
OUT=$2
LOG=$3

#calculate kinship
 ${Tassel} -h ${HMP} -sortPositions -KinshipPlugin -method Centered_IBS -endPlugin -export extBALSIT_kinship.txt -exportType SqrMatrix

 #calculate PCA
${Tassel} -fork1 -h ${HMP} -sortPositions -PrincipalComponentsPlugin -covariance true -ncomponents 2 -reportEigenvalues true -reportEigenvectors true -endPlugin -export extBALSIT _PCA.txt -runfork1

#calculate genome-wide association
 ${Tassel} -Xms512m -Xmx10g -log ${LOG} -fork1 -h ${HMP} -sortPositions -fork2 -r ${PHEN} -fork3 -q ${PCA} -fork4 -k ${Kinship} -combine5 -input1 -input2 -input3 -intersect -combine6 -input5 -input4 -mlm -mlmVarCompEst P3D -mlmCompressionLevel None -export ${OUT} -runfork1 -runfork2 -runfork3 -runfork4
```

Specify the correct location of genotype and the tassel software, then run the program in the command line using the following command.

```
./runTassel.sh phenotype_file.txt results_gwas log_file_mlm
```

with:

phenotype_file.txt:	specify where the phenotypic data are stored
results_gwas:	File name of the output file
log_file_mlm:	file where the progress of the analysis is reported

The same GWAS analysis conducted with the TASSEL graphical user interface:

1. Download TASSEL (https://www.maizegenetics.net/tassel) and open the Tassel.jar file
2. In the TASSEL 5 GUI, choose the option Workflow and click on the MLM (PCA+K) option
3. A new window, which is guiding you through the analysis process appears. In the first step it asks you to import the genotypic file. Select hapmap format (or the format you have your genotypic file in) and tick the option sort position, then click ok.
4. In the next step, specify the location where this file is stored. Check if you agree with the default settings and click 'Filter'.
5. Principal component analysis is used to correct for population structure. In this step, the number of principal components you want the model to consider can be determined. Insert '2' as the number of components as a starting point, but this value can be varied.
6. In the next step, the method of kinship calculation is determined. Use the standard 'Centered IBS' and leave the standard maximum number of alleles at 6.
7. Import the phenotypic data into the model. Leave the standard 'make best guess' and click 'ok'. Specify the location on your computer where the phenotypic file is stored.
8. For the 'MLM Options' use 'No Compression' and the P3D variance component estimation
9. Either specify where TASSEL should save your results or just press 'Okay' (recommended) and the program starts calculating the associations. The results table can either be analyzed in the TASSEL program or exported as a table and analyzed in R.

Supplementary material 3: R script that implements haplotyping at the provided interval

Supplementary material 4: R script that produces as boxplot comparing the disease scores of plants of both alleles at a SNP

```
#R Script
############################################################
## Complete analysis of GWAS, haplotyping and SNPs for Angular leaf spot
#preparation
setwd("… add your work directory here…")
library(reshape2)

##Upload genotypic and phenotypic data
pheno <- read.delim("phenotypes_extBALSIT.txt", na.strings="NA") #import phenotypes in tassel format
geno <- read.delim("extBALSIT_hmp.txt", na.strings="N") #import genotype file in hapmap format

# check them all
head(pheno)
head(geno)[1:15]

#####################################################################
##### Supplementary material 3: Haplotyping #####
#####################################################################

#First, code function that are used in subsequent analyses

# FUNCTION 0: Aid function to transpose genotype dataframe
geno_t<-function(genotypes) {
        genotypes<-genotypes[,-c(2:11)] # remove hmp-specific information
        snps<-genotypes[,1] # transpose and ensure that row and column names are correct
        smpl<-names(genotypes)
        t.genotypes <- as.data.frame(t(unname(genotypes[,2:ncol(genotypes)])))
        colnames(t.genotypes) <- snps
        line<-smpl[2:length(smpl)]
        t.genotypes<-cbind(line,t.genotypes)
 t.genotypes}

#FUNCTION 1: Clustering of the haplotypes at interval
# input is genotyping file and interval of snps
clustering_alleles<-function(geno, snps) {
        t.geno<-geno_t(geno) #apply function coded above
        #define interval that will be clustered in haplotypes
        int<-t.geno[,c(which(names(t.geno)==snps[1]):which(names(t.geno)==snps[2]) )]
        rownames(int)<-t.geno$line
        keep<-which(apply(int, 1, function(x) sum(is.na(x)))<(dim(int)[2])/2) #delete genotype that have
        more than data 50% missing data max
        keep<-unname(keep)
        print(rownames(int[-keep,])) # print lines with more than 50% missing data
        int<-int[keep,] # only keep lines which have more than 50% missing data
        for (x in 1:(dim(int)[2])) {int[,x] <- as.numeric(int[,x]) } #change genotypic info to numeric
        int<-apply(int, 2, function(x) {ifelse(x==2,-1,x)}) # recode so 2 is not -1, 1 stays 1
        di<-dist(int, method = "euclidean") # calculate euclidian distance between genotyes
        hc <- hclust(di, method = "ward.D2") # cluster with the ward.D2 method
        png(paste("Test_plot_clustering",snps[1],snps[2],".png"), height = 500, width = 1500) ## plot
         plot(hc)
        dev.off()
         int } #output is file that contains the recoded (1/-1) genotypes at the specified interval
```

```
#FUNCTION 2: Plotting the haplotype specific ALS resistance
# input are the output of FUNCTION 1, the number of clusters (determined by looking at the tree plot output of FUNCTION 1).

plot_clusters<-function(int, nr.clusters, pheno) {
        # redo clustering on genotype file of interval as aboce
        di<-dist(int, method = "euclidean")
        hc <- hclust(di, method = "ward.D2")
        cu<-cutree(hc, nr.clusters) # cut three to get the number of cluster specified
        cu<-as.factor(cu) #convert cluster number to factor
        geno_cluster<-cbind.data.frame(cu,int) #add cluster number to data frame
        geno_cluster$cu<-as.factor(geno_cluster$cu) #again, convert to factor

        #possible to name and reorder clusters here. #example with 11 cluster
        #levels(geno_cluster$cu)<-c("M5", "M1", "M2", "A1", "A4", "M/A", "M3", "A5", "A3", "M4", "A2")
        #geno_cluster$cu<-factor(geno_cluster$cu, c("M1","M2", "M3","M4","M5", "A1", "A2", "A3", "A4", "A5", "M/A"))

        print(table(geno_cluster$cu)) #print number of lines in each cluster
        names(pheno)[1]<-"line"
        line<-as.factor(rownames(geno_cluster)) #merge the cluster factor with the genotypic data
        geno_cluster<-cbind(line, geno_cluster)
        ht<-merge(pheno, geno_cluster, all.x=T, by="line")

        #plot the haplotype-specific phenotype and list lines and the corresponding cluster
        for(n in 2:length(pheno)) {
                png(paste0("Haplotype_",names(ht)[n] ,snps[1],snps[2],"_K",nr.clusters,".png"), height = 400, width = 900)  ## change plot size here
                boxplot(ht[,n]~ht$cu, data = ht, main=names(ht)[n], col=c("#00441B", "#006D2C", "#238B45", "#41AE76", "#66C2A4", "#99D8C9","#A50F15" ,"#CB181D" ,"#EF3B2C" ,"#FB6A4A", "#FC9272" ,"#FED976" ))
                dev.off()}
         for(n in 1:length(levels(geno_cluster$cu))) {
                print(paste(levels(geno_cluster$cu)[n]))
                 print(rownames(geno_cluster[which(geno_cluster$cu== levels(geno_cluster$cu)[n]),])) }

        ht}

### RUN THE ANALYSIS

## step 1: decide on interval. for example
# Phg-2 chr8 interval
snps<-c("Chr08pos61150549.1", "Chr08pos62934224.1")
# or phg4 interval
snps<-c("Chr04pos45663963.1", "Chr04pos46152634.1")

## step 2: run this and check test plot of clustering (in working directory)
# and decide on number of clusters for subsequent analysis
int_genotype<-clustering_alleles(geno, snps)

## step 3:Plots of the haplotype specific effects are now in the working directory
gp_hapl<-plot_clusters(int_genotype, 11, pheno)

# check which lines are in haplotypes 3 and 5
gp_hapl[which(gp_hapl$cu=="3"),"line"]
gp_hapl[which(gp_hapl$cu=="5"),"line"]
```

```
############################################################
##### Supplementary material 4: Boxplots #####
############################################################

#check if loaded correctly
head(pheno)
head(geno)[1:15]

# FUNCTION 1: Combine genotypic and phenotypic information
genopheno<-function(genotypes, phenotypes) {
        genotypes<-genotypes[,-c(2:11)] # remove hmp-specific information
         snps<-genotypes[,1]
        smpl<-names(genotypes)
        t.genotypes <- as.data.frame(t(unname(genotypes[,2:ncol(genotypes)])))
        colnames(t.genotypes) <- snps
        t.genotypes$line<-smpl[2:length(smpl)]
        t.genotypes$line
        names(phenotypes)[1]<- "line"
        output<-droplevels(merge(phenotypes, t.genotypes, by="line")) # merge with phenotype data
         output}

# FUNCTION 2: Plot snp effect in the different experiments
MAS_snps<-function(snp_test) {
        for (snp in snp_test) {
        data1=gp[which(gp[,snp]==levels(gp[,snp])[1]),] #susceptible SNP
        data2=gp[which(gp[,snp]==levels(gp[,snp])[2]),] # resistant SNP
        data<-rbind(data1[,c( 1:length(pheno), which( colnames(data1)==snp ))], data2[,c( 1:length(pheno),
        which( colnames(data2)==snp ))])

        #if the wrong box is red, recode as below
        #data[,  which(  colnames(data)==snp)]<-factor(data[,  which(  colnames(data)==snp)],levels(data[,
        which( #colnames(data)==snp)])[c(2,1)])

        data<-melt(data, id.vars=c("line", snp)) # convert data to the long format

        png(paste0( snp, ".png"), height=400, width=600)  #plot
         par(mar = c(10,4,4,2) + 0.1, cex=1.2)
         boxplot(value~data[,snp]+variable, data=data, yaxt="n",xaxt="n",   col= c("#ca8481","#b4d878"),
        main= paste( snp) )
         axis(2, at=1:9, labels=F)
        axis(2, at=c(1,3,5,7,9), labels=T, cex.axis=1.5)
        abline(h=3, lty="dashed")
        text(x =  2*seq_along(levels(data$variable))-0.4, y = par("usr")[3] - 2.5, srt = 45, adj = 1, cex=1.5,
        labels = levels(data$variable), xpd = TRUE)
        abline(v=c(2.4,4.5,6.5,8.5,10.5,12.5,14.5, 16.5, 18.5),lty=1, col="grey")
        axis(1, labels = rep(c(levels(gp[,snp])[1], levels(gp[,snp])[2]), 10), at = 1:20, tick = T)
        dev.off()  }
        }

## Run analysis
gp<-genopheno(geno, pheno)

#Plot and save plots in the working directory
MAS_snps(c("Chr08pos61681984.1","Chr08pos61682023.1","Chr08pos61682035.1"))
MAS_snps("Chr08pos61681984.1")
```

Supplementary material 5: DNA sequence for molecular marker development. Listed are DNA sequences 50bp before and after the SNP to be used for in-house or outsourced marker design.

Phg-4 on chromosome 4

Chr04pos46703147.1 >Chr04:46703097-46703197
CGCCGTCGCAGCATCCGGGGTCATTCCGTTGCCGGTTGCATCACGGCGA**[A/G]**TATGT
ATGGCGTGGTAGAGCTATTAGGTAGTTGGATGTATCCAATAATAA

Chr04pos46934061.1 >Chr04:46934011-46934111
CCCTAACTAGAACCAAATCATCATCACCATAATTTTCCACAATCCG**[C/T]**GGCCA
CGGCATCACACTTTAGCCAGTCCAATTGCAGCAGTACGTAATTTT

Chr04pos46727398.1 >Chr04:46727348-46727448
AAATCGCAGCGTTGAACCATTTCGGGGAGAATCCTCGACCGAGCAGGCC**[A/G]**TCCA
ACATGTCTGAAGATCGGACTCGGTGGCTGAACTCCGCGAACGATTC

Phg-2 on hromosome 8

M1
Chr08pos61901182.1 >Chr08:61901132-61901232
TTTACATCTCACTATCTATTGGAAACGCAAATGGACTGTCTGGGAGGTT**[G/T]**AGTCC
TTCATAGCTGGATAAAATTCAAAACACAAATTACATGATCTTTAG

M2
Chr08pos62188623.1 >Chr08:62188573-62188673
CAGGCCAAGACATATCCAGTTTGCAAGTTAATTCTCTGTATAACCTTAC**[C/T]**CCTCA
GGGGCAACATCTCACCTTTCCCCCATCACAGGCTGCTCATGGAGC

M3
Chr08pos61828096.1 >Chr08:61828046-61828146
CATGCAGCAATGAAAAGCCAATGTATTTAGTAAATAGTTTAATGAAAAA**[C/A]**CAAA
AGGAAGACATATGCATGCCTCAAGGTTACGCTGGGTTGGATCTAGT

Chr08pos61878388.1 >Chr08:61878338-61878438
CTTTGCTTACGCTCAATAATAGTGGGTTGCTTACATCACTAGCTGATAA[**C/A**]ACCATT
CAATTAGTTTAGGTTTATTGGTTTACTCAGAAGATTATATATGT

Chr08pos61880092.1 >Chr08:61880042-61880142
CGAAGGTATGACAATTTGGTTGAAGCTCTGAATTTCTTCAGCATGTGTG**[C/T]**TCGGTT
GCATTGCTGCTGCTGAGTTTTTTCCTTTTCATTGTGTAGAAAAA

M4
Chr08pos61681984.1 >Chr08:61681934-61682034
TGTGCAATTGCAACACAGAGCAGCCATGGGGCACTACAGTGACATCCAG**[T/C]**GTCTC
TCAATAGCTTTATAGTGGCACAATAGTGCTATTGTTGAAAAATTC

Chr08pos61682023.1 >Chr08:61681973-61682073
TGACATCCAGTGTCTCTCAATAGCTTTATAGTGGCACAATAGTGCTATT**[G/A]**TTGAA
AAATTCCTTGGATCCAAGATCTTAACTCCATCCACTAGTGCAGCT

Chr08pos61682035.1 >Chr08:61681985-61682085
TCTCTCAATAGCTTTATAGTGGCACAATAGTGCTATTGTTGAAAAATTC**[C/G]**TTGGAT
CCAAGATCTTAACTCCATCCACTAGTGCAGCTGCTATACAGCAT

M5 & M1

Chr08pos61388457>Chr08:61388407-61388507
CATTTCCTTCAGTTGAAAGCCTAACTGCTGCAACTAAGGATCCCATTCC**[C/T]**CCGTCA
TCTGTACTCAAGCAATTGGCTATAGCTGTTGAGTAAGTTGTAAT

Chr08pos61502023>Chr08:61501973-61502073
CCATGTAGCCACTAACCGAAGCATAATAGCCTTTAGCCACTCCAACACT**[G/C]**TCATC
ATATTTCCCCTTGTACTTGTCACCAAACTCAAACAACTACAACGA

Chr08pos61533289>Chr08:61533239-61533339
CAGTTAAGGTTAACAATCTTAGGGTTATGAATATGTGCAGCAACCAAGG**[T/C]**TTTGT
CACAGTTATCAGCACCATCACCAGCAATTCTGGCATCATAGTTTG

A1

Chr08pos61825787.1 >Chr08:61825737-61825837
GCAAGAATAATAATAAGATAAGATAAATGAGCTTTACATGATAACTAAT[**C/A]**GCAA
AACTTTCTGGGAATTTTCTATAGCACATACTTTTGAAACATTATCT

Chr08pos61879951.1 >Chr08:61879901-61880001
GGTCAGTTGTCCCATGCTGCTGAGCCCAACAGTGCTTCTGAGCCATCAC**[A/C]**TGTAG
CAACAAATGGTGATGGTTTAGAGGGTGTAGGGTTTGTGTCAAACA

Chr08pos62191492.1 >Chr08:62191442-62191542
TGTTTGTTTTTTCTTGTATTATATGGCTCAGACAATGGTGCTGCTGTTA**[A/G]**TATTTAC
ACTCAAATCTGTGCCGTCTTCACCTGTTGATTTCGGCTTCGGT

A2

Chr08pos61828125.1 >Chr08:61828075-61828175
GTAAATAGTTTAATGAAAAACCAAAAGGAAGACATATGCATGCCTCAAG**[G/A]**TTAC
GCTGGGTTGGATCTAGTAAGTCCCCAATTTGTTCATTGTATATCTG

Appendix 2: Article in the Annual Report of the Bean Improvement Cooperative

Angular leaf spot resistance – GWAS of field and greenhouse screenings in Colombia

Michelle Maria Nay[1], Hector Fabio Buendia[2], Ana Elizabeth Portilla[2], Bruno Studer[1], Bodo Raatz[2]

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Introduction

Angular leaf spot (ALS) caused by the fungus *Pseudocercospora griseola* is an important disease in common bean (*Phaseolus vulgaris* L.) and causes high yield losses in the tropics and subtropics. ALS resistant common bean lines have been characterized and resistance loci were repeatedly found on chromosomes 1, 4, 8 and 10 [1-4]. However, due to the high diversity of the pathogen and the pathotype-specificity of the resistance in common bean, efficient and durable resistance is difficult to achieve [5]. This work aims at finding resistance loci specific to Colombian isolates of *P. griseola* in the field and greenhouse.

Materials and methods

To study ALS pathotype-specificity, a panel of 316 common bean lines, named extBALSIT, was evaluated in the greenhouse with a single isolate and in the field with a mix of isolates. In the greenhouse, a single isolate of the race 63-47 was used and in the field, a mix of five isolates previously collected in Darien with the races 63-0, 31-47 (2x), 5-47 and 15-44 was used. The panel was assembled to contain a collection of the most resistant plant material available at CIAT, including the Bean ALS International Trial (BALSIT) panel of previously characterized resistance sources, breeding material with phenotypic variability for ALS response and susceptible checks. Disease severity was evaluated with the CIAT standard scale ranging from 1 (no disease symptoms) to 9 (very severe disease symptoms and defoliation). The common bean lines were genotyped-by-sequencing (GBS) using the restriction enzyme ApeKI [6] and SNPs were extracted using the NGSEP pipeline [7], filtering for a minimum quality score of Q40, maf < 0.5, 20% missing data and removing heterozygote values. Genomic positions correspond to the v2.1 of the *P. vulgaris* reference genome [8]. Genome-wide association studies (GWAS) were conducted with the TASSEL 5.0 MLM model using PCA to correct for population structure and the K matrix to correct for kinship [9].

Results and discussion

The extBALSIT panel was tested in the greenhouse with race 63-47 of *P. griseola*, and in the field in Darien (Colombia). Phenotypic results showed a different distribution of resistant and susceptible lines between the experiments, indicating that the resistance in the panel is pathotype-specific (Figure 1).

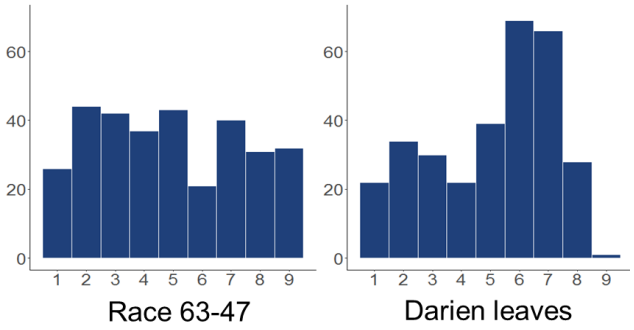

Figure 1: Histogram of mean angular leaf spot scores of the 316 lines in the extBALSIT panel tested in the greenhouse with race 63-47 and in the field in Darien.

After filtering, GBS resulted in 22,765 SNPs that were tested for their association with disease resistance scores. A major resistance locus on chromosome 8 was identified in both trials (Figure 2). This locus at the end of chromosome 8 coincided with the previously characterized resistance locus *Phg-2*, found in the Mesoamerican common bean background [3]. Our results demonstrate the high importance of the *Phg-2* locus in conferring ALS resistance to Colombian pathogen isolates.

Resistant and susceptible lines were best distinguished with the T/G SNP at position 61,901,182 on chromosome 8 (Figure 3). The T allele at this position originated from genotype G10474 and was contained in several MAB lines (MAB 348-351, 354, 373). These lines showed broad-spectrum resistance to race 63-47 in the greenhouse and to a mix of five races in the field in Darien. The locus, however, did not explain the resistance completely and there were probably other resistance loci present, which could not be detected using this panel. This SNP constitutes a promising candidate for the development of molecular markers and its use in marker-assisted selection.

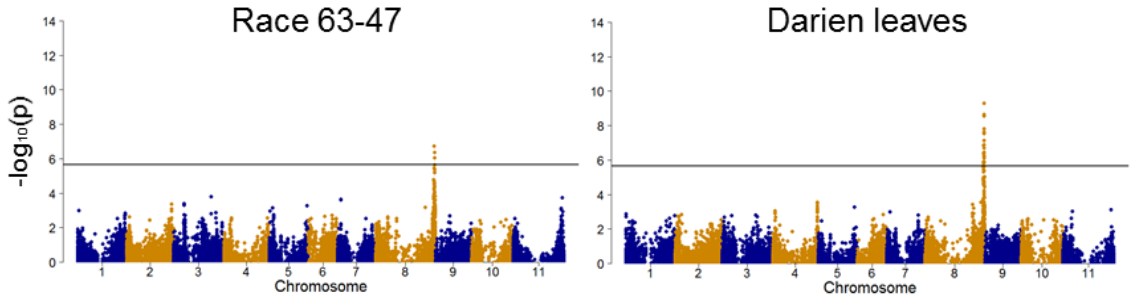

Figure 2: Genome-wide association studies results of angular leaf spot disease resistance on leaves with race 63-47 in the greenhouse and with a mix of races in the field in Darien. The significance threshold was Bonferroni-corrected with $\alpha = 0.05$.

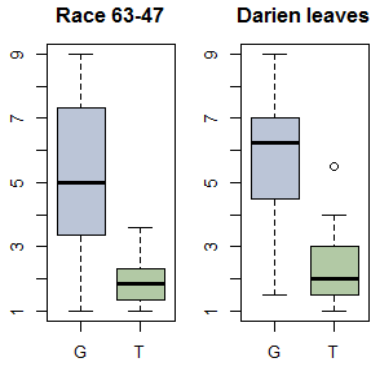

Figure 3: Selected SNP marker tagging the resistant lines. Shown are ALS scores of the lines with nucleotide G (n=200) and T (n=25) at position 61,901,182bp on chromosome 8. On the y-axis, the angular leaf spot resistance is given on a 1 (resistant) to 9 (susceptible) scale.

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Appendix 3: ETH Global blog post

Magic Beans –

Breeding Disease Resistant Beans for Smallholder Farmers

by Michelle Nay

Diverse bean grain types collected during field visits. Consumers are very picky about the look of their beans, therefore beans are bred to resemble certain market classes (e.g. red kidney beans, pinto beans, cannellini beans, etc.). All grain types not resembling these are discarded. (photo credit: ETH Zurich/Michelle Nay)

Although I have been working with beans for two years now, I only fully realized the importance and impact of my project when I arrived in Uganda two weeks ago. The traditional meal here is a bowl of beans with starchy side dishes. Very few people can afford meat, so for most people in Uganda this means they eat beans every day: for lunch, for dinner, during the week and on weekends. Most farmers practice subsistence agriculture, and every free piece of land, no matter how small it is, is cultivated. The crop yield on these small patches is very low and plant diseases are a widespread problem in the region. One solution would be to use pesticides, but these are not always available, and they are often expensive and unsustainable. The more feasible solution would be to enhance crop yields. Under the challenging conditions encountered on smallholder farms, it would be more sustainable to breed plants that are relatively resistant to plant diseases.

Bean plant showing characteristic symptoms of Angular Leaf Spot. The fungus causes dark angular spots on the leaves. When an infection is severe, the leaves wither and fall. Plants with withered leaves harvest less energy from the sun and thus produce less or smaller grains, resulting in reduced crop yield. (photo credit: ETH Zurich/Michelle Nay)

Providing the scientific background to breed beans resistant to a fungal disease known as Angular Leaf Spot is the objective of my doctoral studies. Considered a major limitation to bean production in the tropics, Angular Leaf Spot causes yield losses of up to 80%. In my project, I am investigating the genetic basis of disease resistance in beans and developing tools that facilitate resistance transfer to new bean varieties through conventional breeding. To ensure that my findings have a positive effect in farmers' fields, I am working in close collaboration with the International Center of Tropical Agriculture (CIAT, known by its Spanish acronym). CIAT manages large bean breeding programs focused on low-input systems encountered on smallholder farms throughout the tropics. They have developed bean varieties that are stress resistant and perform well in drought, heat and low nutrient environments. To release new varieties, CIAT works with national agricultural programs, and in Africa it coordinates the Pan African Bean Alliance (PABRA), a network of 30 African countries, that is responsible for the dissemination of seeds on the ground.

My project requires extensive fieldwork in Latin America and Eastern Africa, the main bean producing areas of the tropics. Over the past two years, I have been conducting trials in Colombia, where I have been testing over 300 different bean varieties for their resistance to the fungus that causes Angular Leaf Spot. Because pathogen populations differ across continents, the trials also need to be conducted in Africa, and that is why I am currently in Uganda.

In both Colombia and Uganda, my local colleagues warmly welcomed me and made sure that I received full exposure to their culture. Although I have only been in Uganda for a short time, I have already been invited to the 60th birthday party of a colleague's mother. In Colombia, my colleagues loved to go dancing and even at the parties of the research station everyone danced salsa. Caleños (people from Cali, the city I was staying in) learn to dance at about the same time they learn to walk, and fortunately, they were very patient in teaching foreigners, like me, how to dance.

Plants' reaction to Angular Leaf Spot vary. The variety planted in the lowest row is resistant to Angular Leaf Spot, while the varieties in the upper rows have characteristic angular lesions on leaves, and the leaves are turning yellow. (photo credit: ETH Zurich/Michelle Nay)

While I enjoyed exploring Colombia and Uganda, conducting research sometimes poses challenges; but with some creativity and flexibility, I have always found ways to overcome the challenges. One difference working in the field versus ETH is the availability of lab reagents (a substance used in chemical analysis) and services. At ETH, reagents are usually available on campus or can be shipped in less than a week. In Colombia it took 2-3 months for reagents to be delivered. I once had to send three frozen 1.5ml tubes for DNA sequencing to the USA. I packed the three tubes in 20kg of dry ice, but then the package was stuck in customs at the border for a week. Luckily, the shipping company was experienced with this type of cargo: they replaced the dry ice and the samples were saved.


Every small patch of land is cultivated. Left, beans for home consumption are planted on a small patch of land next to the laboratory of CIAT Uganda. Right, Maize is planted next to the parking of the hotel I was staying at during my first nights in Kampala. (photo credit: ETH Zurich/Michelle Nay)

Even though things did not always go according to plan - for example seed shipments were delayed, rainy season was unpredictable, and mice damaged my plants in the greenhouse - somehow we always found a solution to bring experiments to a successful end. The success is due to the support of talented scientists in Colombia and Uganda with whom I had the privilege to work. Meeting them, I realised the opportunities that we have as Switzerland-based scientists. For someone in Colombia or Uganda with a Master's degree and doing similar work, it is difficult to find employment or access opportunities for a PhD position in their field of study. For my fellow ETH Zurich graduates it is comparably easy to find a job that demands their skills.

This experience has made me appreciate, even more, all the opportunities I have been given to travel and learn about how research is conducted in other parts of the world. I hope that at some point in my career I can provide opportunities to scientists that are not as privileged.

About the author

Michelle Nay is a doctoral student in the Molecular Plant Breeding group at ETH Zürich. Her doctoral research is funded by the Engineering for Development (E4D) Program of ETH Global and is conducted in collaboration with CIAT.

Available at https://blogs.ethz.ch/ETHambassadors/2018/06/14/magic-beans/ (Retrieved 14.11.18)

Appendix 4: ETH News article

Advanced breeding paves the way for disease-resistant beans

11.09.2019 | News
By: Peter Rüegg

ETH researchers are involved in the development and implementation of a method to efficiently breed for disease-resistant beans in different regions of the world. Their work will help to improve the livelihood and food security of smallholders in developing countries.

A genetic selection method helps African breeders to grow beans that are disease-resistant. (Photograph: Georgina Smith / CIAT / CC BY-NC-SA 2.0)

For many people in Africa and Latin America, beans are an important staple. Historically described as “the meat of the poor”, beans are rich in protein and minerals, affordable and suitably filling. That is why they are served daily, often with several meals.

In many regions, however, plant diseases severely reduce bean yields. For example, the dreaded angular leaf spot disease can cause yield losses of up to 80 percent – especially in Africa, where smallholders rarely have the opportunity to protect their crops with fungicides.

Genomics-assisted breeding

Working with Bodo Raatz and his team at the International Center for Tropical Agriculture (CIAT), ETH researchers from the group led by Bruno Studer, Professor of Molecular Plant Breeding, investigated the resistance of beans to angular leaf spot disease. Their findings are now enabling disease-resistant bean varieties to

be bred more rapidly and selectively for the world's various bean-producing regions.

Their method is built upon genome analyses of those beans that are potentially suitable for breeding new, resistant varieties. The resulting genetic profiles provide information as to whether the progeny from crossbreeding two varieties will be resistant to the pathogenic fungus's different, locally occurring strains (known as pathotypes).

Genetic profiles created for 316 varieties

Michelle Nay, who carried out the project as part of her doctoral thesis in Studer's group, started by gathering as many different bean seeds as possible from CIAT's seed repository. In total, she collected 316 different varieties that displayed characteristics suitable for breeding resistance to the fungus that causes angular leaf spot disease.

Study author Michelle Nay in a test field in Colombia. (Photograph: courtesy of M. Nay)

Next, Nay planted the beans from her collection in Uganda and Colombia, both in greenhouses and in the field. Her aim was to find out if and indeed how the different varieties react to the fungus's various pathotypes in each country, and then to identify the genetic basis of disease resistance.

Nay also created a high-resolution genetic profile for each of the 316 bean types based on variations in their DNA known as genetic markers, and identified which markers occurred only in the disease-resistant beans. She subsequently used these markers to predict which progeny would be resistant to which pathotypes in a given country, and which ones would be susceptible to disease.

Improvement on conventional plant breeding

“Our method speeds up the breeding process considerably,” Studer says. It’s a big step forward because crossbreeding had previously been a numbers game and involved testing every single plant for its resistance, he explains. Now, on the basis of a genetic test, it is possible to predict a plant’s resistance without testing it in laborious field trials. “This is a huge help in bean breeding and great news for people who rely heavily on beans as a staple of their diet,” Studer says.

The group’s work to provide disease-resistant beans will also help to cut down on global pesticide use. As things stand today, Studer explains, fungicide use is common for bean cultivation in Latin America, but almost non-existent in Africa because many farmers don’t have access to pesticides, or don’t know how to use them safely and efficiently: “Disease-resistant beans are a double win: famers in Latin America can reduce their pesticide use while farmers in Africa can increase their crop yield pesticide-free.”

Simple, inexpensive and open-source technology

CIAT distributes the seeds from this project to various sub-organisations who then supply them to breeders. The analytical method for determining genetic markers is relatively simple and inexpensive to apply, making it viable for use in agricultural laboratories in the countries concerned. It costs less than 0.2 CHF to test a genetic marker, Nay explains, which is an affordable amount for laboratories in less affluent countries. What’s more, all the findings from this study are available through open access. “This way, our work reaches the people who really need these kind of resources,” Nay emphasises.

Nay and Studer worked on this project in close collaboration with CIAT. The global research centre runs the largest breeding programme in the tropics and has several thousand varieties of bean in its seed repository. At its headquarters in Colombia, CIAT breeds new bean varieties, tests the seeds, and, in partnership with the Pan-Africa Bean Research Alliance, makes the seeds available to farmers for cultivation.

In collaboration with CIAT and supported by the World Food System Center at ETH Zurich, Studer and his group will now conduct a follow-up project to refine their breeding method. While the researchers previously focused on markers for one specific disease, the new project will take a more holistic approach as they attempt to use such genome profiles to predict as many plant characteristics as possible.

This research has been supported by the Sawiris Foundation for Social Development.

Beans - meat of the poor

Beans are the staple food in large parts of Africa. (Picture: Georgina Smith / CIAT)

The Angular Leaf Spot disease massively reduces the bean yield. (Photo: M.Nay / ETH Zurich)

The Angular Leaf Spot disease massively reduces the bean yield. (Photo: M.Nay / ETH Zurich)

Reference

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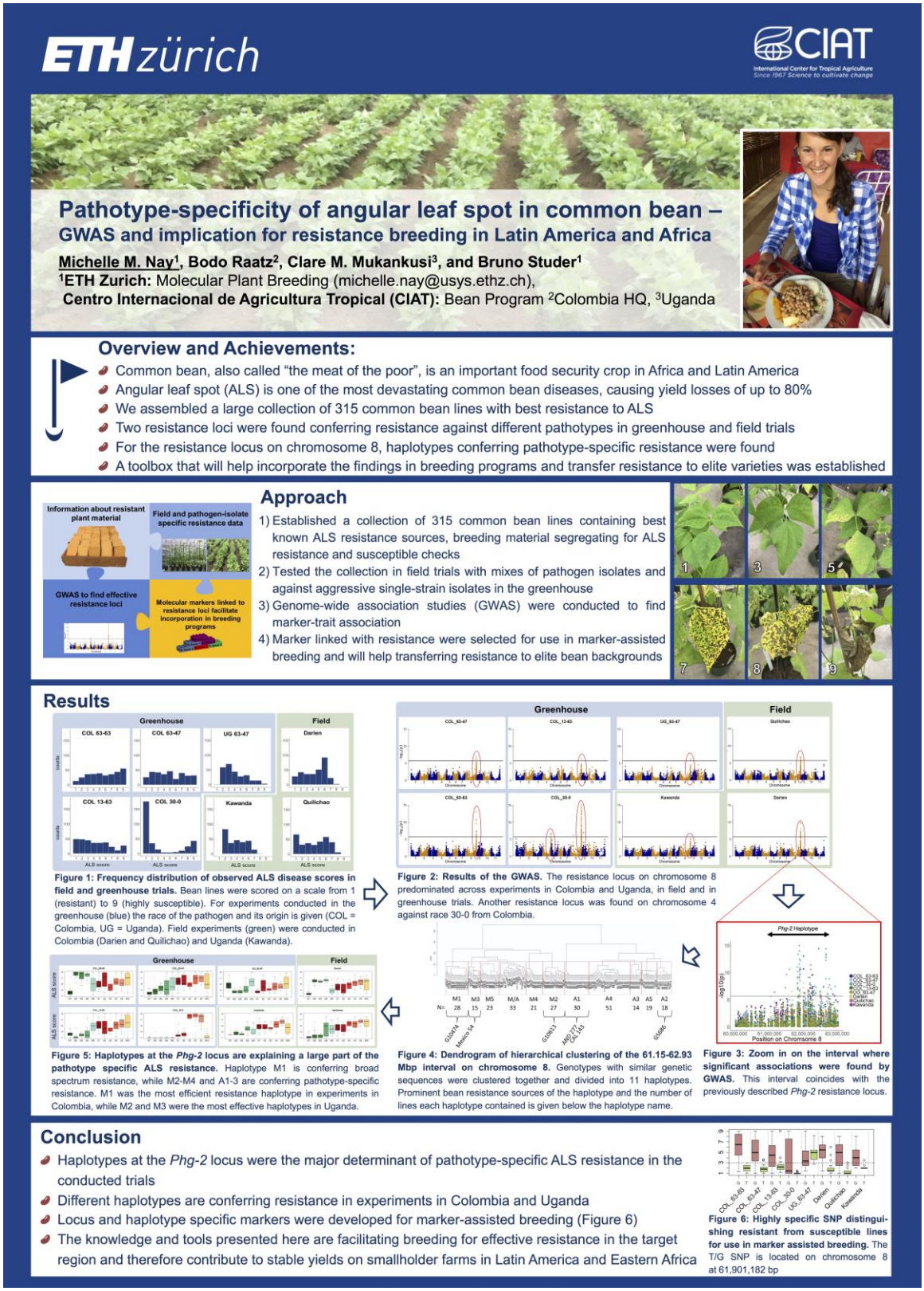
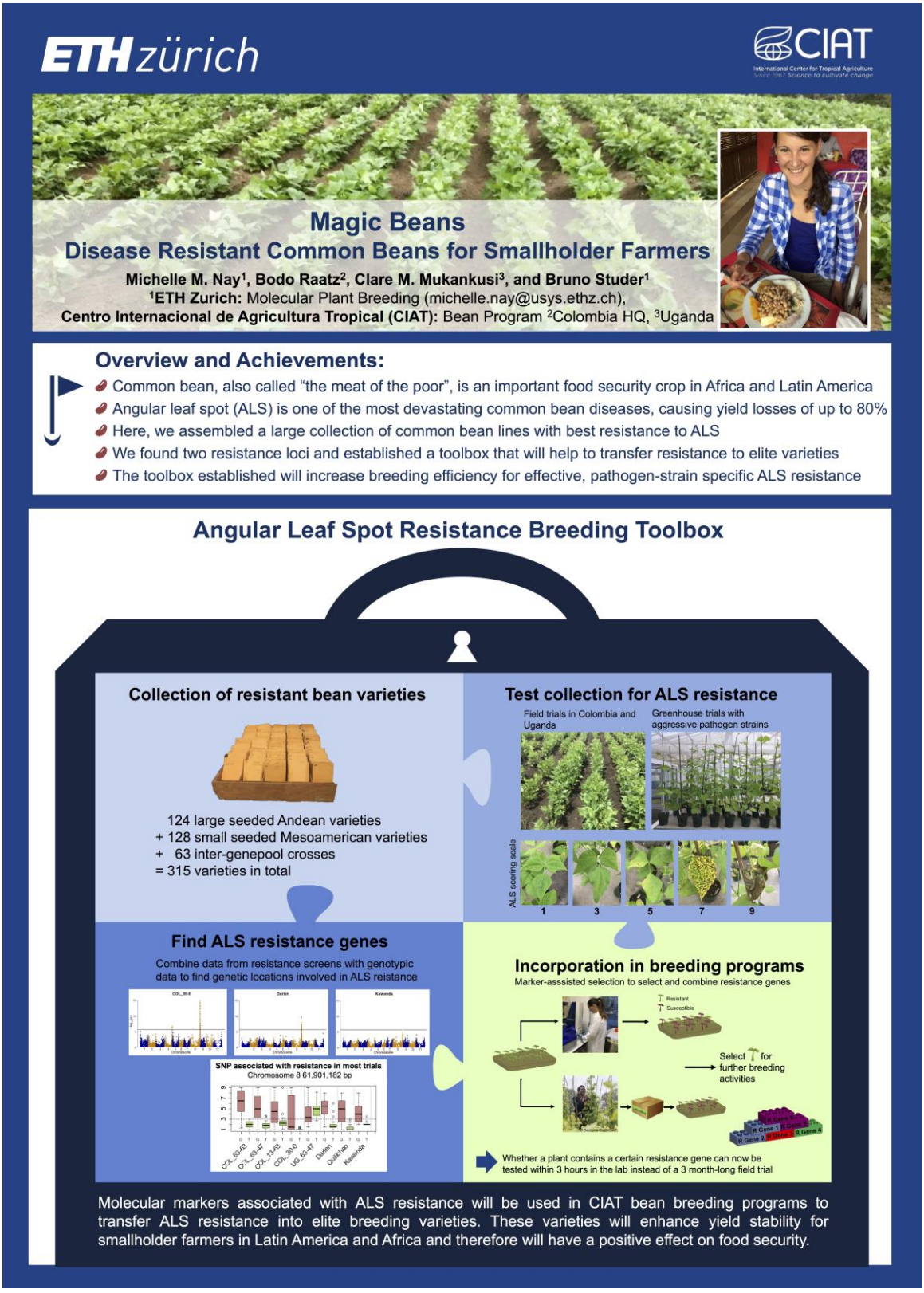
Available at https://ethz.ch/en/news-and-events/eth-news/news/2019/09/advanced-breeding-paves-the-way-for-resistent-beans.html (Retrieved 24.09.19)

Appendix 5: Poster presentations

Poster presented at the Plant and Animal Genome Conference, San Diego, 12-16. Jan 2019.



Poster presented at the World Food System Center Symposium, ETH Zürich, 8. Nov 2018.



Appendix 5: CV

Michelle Maria Nay

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18.09.1990, Swiss citizen

Professional Experience

Feb 2016 – March 2019 **Research associate,** ETH Zürich and CIAT Colombia & Uganda

- Project management of three subprojects in close collaboration with the International Center for Tropical Agriculture (CIAT)
- Conducted extensive field and greenhouse trials in Colombia and Uganda
- Laboratory work, sequencing data analysis, writing publications and presentation of the findings at international conferences

Sept – Nov 2015 **Internship**, International Institute of Tropical Agriculture (IITA) Uganda

- Supporting young professionals to become agricultural entrepreneurs as part of the Humidtropics project of IITA

Education

Feb 2016 – May 2019 **Doctoral student,** ETH Zürich and CIAT Colombia & Uganda

- **Genetic characterization of disease resistance in common bean**
- Genome-wide association study to investigate pathotype specificity of angular leaf spot resistance in common bean
- Resistance gene pyramiding for angular leaf spot resistance
- Interspecific Ascochyta resistance for common bean improvement

Sept 2013 – Jun 2015 **Master of Science, Ecology and Evolution,** ETH Zürich

- Thesis: Forward genetics in perennial ryegrass - A tool to identify beneficial alleles for abiotic stress resistance
- Semester projects focusing on 1) the orphan crop buckwheat and 2) using role playing games to explore coffee farmer's strategy in agroforestry landscapes.

Jun 2014 **Summer School**, University of California, Berkeley, USA

- Three-week environmental leadership certificate course

Sept 2010 – Aug 2013 **Bachelor of Science, Biology,** ETH Zürich

- Broad natural science education
- Laboratory courses in microbiology, ecology and chemistry

Feb – Jun 2013 **Exchange Semester,** University of Princeton, USA