Anthracnose in white lupin

Genetic diversity, virulence and host resistance

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Contents

Summaries

English, German and Dutch

Summary

White lupin (*Lupinus albus* L.) is a grain legume that is known for its high protein content, nutritional quality, efficient nitrogen fixation and unique ability to form specialized cluster roots that support phosphorus uptake. Despite a severe production decline at the end of the past century, white lupin has seen a recent revival to sustain the demand for plant-based protein and reduce Europe's dependency on imported soybean. A major problem in (white) lupin cultivation is anthracnose disease, caused by the globally dispersed, seed- and air-borne fungal pathogen *Colletotrichum lupini*. This PhD thesis aims to provide insights on how to sustainably control anthracnose disease in white lupin in order to support its re-introduction into European cultivation systems. It describes (i) the genetic diversity, phylogeography and virulence of *C. lupini*, (ii) the development of a high-throughput phenotyping protocol to reliably screen white lupin germplasm, (iii) a genome-wide association study identifying resistance candidate genes and (iv) the exploration of effective seed treatments to reduce the primary pathogen inoculum.

Chapter 1 gives a general introduction about the subject of this thesis. Providing background information about the future protein demand, grain legume cultivation in Europe, the history of lupin cultivation and why white lupin would be a suitable addition to imported soybean. It also describes the most important lupin diseases, highlighting the relevance of the highly destructive anthracnose disease. At the end of this chapter, the overall aim and objectives of this thesis are lined out.

Chapter 2 explores the intraspecific diversity of 39 *C. lupini* isolates collected from across the world. Multi-locus phylogeny and morphological characterization shows that diversity is greater than previously reported, distinguishing a total of six genetic groups and ten distinct morphotypes. Highest diversity was found across the South American Andes, indicating it to be the center of origin of *C. lupini*. Results reveal that the current pandemic is caused by strains belonging to genetic group II, which are spread globally, and are genetically and morphologically uniform. Group II isolates were shown to be highly aggressive on tested white and Andean lupin accessions. Isolates belonging to the other five genetic groups were mostly found locally and showed distinct virulence patterns on both white and Andean lupin accessions. Despite its uniformity, it was shown that two highly virulent group II isolates from Chile could overcome resistance of elite white lupin breeding material, stressing the need to implement phytosanitary protocols for international seed transports.

Chapter 3 describes the development of a reliable high-throughput phenotyping tool to identify anthracnose resistance in white lupin germplasm and study pathogen-host interactions. Phenotyping under controlled conditions, performing stem wound inoculation on seedlings, showed to be applicable for high-throughput and its disease scores strongly correlated with two-year Swiss field disease assessments ($r = 0.95$) and yield ($r = -0.64$). Phenotyping a diverse set of 40 white lupin accessions revealed eight accessions with improved resistance to anthracnose, which can be incorporated into white lupin breeding programs. This chapter also describes the

evaluation of a genomic prediction model, based on previously reported resistance quantitative trait loci (QTL), on an independent set of genotypes. Predicted resistance values, however, did not correlate with values observed under controlled or field conditions.

Chapter 4 describes a genome-wide association study (GWAS) to dissect the genetic architecture for anthracnose resistance in white lupin. White lupin genotypes, collected from the center of domestication and traditional cultivation regions, were screened for anthracnose resistance and characterized through genotyping-by-sequencing (GBS). GWAS revealed two significant SNPs associated with anthracnose resistance on gene Lalb_Chr05_g0216161, encoding a RING zinc-finger E3 ubiquitin ligase potentially involved in plant immunity. Further validation experiments are now required to confirm involvement in plant resistance. Population analysis showed a remarkably fast linkage disequilibrium (LD) decay, weak population structure and grouping of commercial varieties with landraces, corresponding to the slow domestication history and scarce breeding efforts in white lupin.

Chapter 5 shows the potential of non-synthetic seed treatments to reduce *C. lupini* infection levels in white lupin. A total of eleven different seed treatments was tested in field trials in Switzerland between 2018 and 2021. Treatments consisted of hot water, steam, electron, long term storage, vinegar, plant extracts and biological control agents (BCAs). The BCAs were tested under controlled conditions for potential antagonistic activity against *C. lupini* during white lupin infection prior to field trials. Results showed that long term storage and vinegar treatments can successfully reduce disease incidence and increase yield to levels similar to those observed for certified seeds. BCA treatments with *Bacillus subtilis, Paraburkholderia phytofirmans* and *Clonostachys rosea* showed significant disease reductions under controlled conditions but not in the field.

Chapter 6 provides an overall conclusion placing the main results of this thesis in a broader context. In order to sustainably and effectively control anthracnose disease in white lupin, an integrative approach, including modern breeding efforts, disease prevention strategies and mixed cropping systems, is recommended. Further research is required to increase our understanding on white lupin-*C. lupini* interaction and to identify genetic regions involved in resistance or virulence, respectively, which could greatly support white lupin breeding. This thesis provides the basis to further explore *C. lupini* population dynamics, virulence and host-speciation, white lupin resistance mechanisms and sustainable ways to control anthracnose disease in order to further facilitate successful white lupin cultivation.

Zusammenfassung

Die Weiße Lupine (*Lupinus albus* L.) ist eine Körnerleguminose mit hohem Proteingehalt, guter Nährstoffqualität, effizienter Stickstofffixierung und, aufgrund spezialisierter Proteoidwurzeln, erhöhter Phosphoraufnahme. Trotz eines starken Produktionsrückgangs Ende des 20. Jahrhunderts erlebt die Weiße Lupine jüngst einen Aufschwung aufgrund der Nachfrage nach pflanzlichem Eiweiß und Europas Abhängigkeit von Sojaimporten. Ein großes Problem im Anbau der (weißen) Lupine ist die Anthraknose-Krankheit, die durch den weltweit verbreiteten, samen- und luftbürtigen Pilzerreger *Colletotrichum lupini* verursacht wird. Ziel dieser Dissertation ist es, Erkenntnisse über Bekämpfungsstragien der Lupinenanthraknose zu gewinnen, um die Wiedereinführung dieser Kulturpflanze in europäische Anbausysteme zu unterstützen. Sie beschreibt (i) die genetische Vielfalt und Virulenz von *C. lupini*, (ii) die Entwicklung eines Hochdurchsatz-Phänotypisierungsprotokolls, (iii) eine genomweite Assoziationsstudie zur Identifizierung von Resistenzgen-Kandidaten und (iv) die Untersuchung wirksamer Saatgutbehandlungen zur Reduzierung des primären Pathogeninokulums.

Kapitel 1 gibt eine allgemeine Einführung in das Thema der vorliegenden Arbeit. Es enthält Hintergrundinformationen über den künftigen Eiweißbedarf, den Anbau von Körnerleguminosen in Europa, die Geschichte des Lupinenanbaus und dessen Potenzial als Ergänzung zur importierten Sojabohne. Zudem werden die wichtigsten Lupinenkrankheiten beschrieben, wobei die Bedeutung der verheerenden Anthraknose-Krankheit hervorgehoben wird. Am Ende dieses Kapitels werden die allgemeinen Ziele dieser Arbeit dargestellt.

In **Kapitel 2** wird die intraspezifische Vielfalt von 39 weltweit verbreiteten *C. lupini*-Isolaten untersucht. Die Multi-Locus-Phylogenie und die morphologische Charakterisierung zeigen, dass die Diversität größer ist als bisher berichtet, wobei insgesamt sechs genetische Gruppen und zehn Morphotypen unterschieden werden. Die größte Vielfalt wurde in den südamerikanischen Anden gefunden, was auf das Genzentrum von *C. lupini* hindeutet. Die Ergebnisse zeigen, dass die aktuelle Pandemie durch weltweit verbreitete und homogene Stämme der genetischen Gruppe II verursacht wird. Isolate der Gruppe II erwiesen sich als äußerst aggressiv gegenüber der Weißen und Anden-Lupine. Isolate der anderen fünf genetischen Gruppen wurden meist lokal gefunden und zeigten sowohl bei der Weißen als auch bei der Anden-Lupine unterschiedliche Virulenzmuster. Trotz ihrer Einheitlichkeit konnte gezeigt werden, dass zwei hochvirulente Isolate der Gruppe II aus Chile die Resistenz von fortgeschrittenem Zuchtmaterial der Weißen Lupine überwinden konnten. Dies unterstreicht die Notwendigkeit der Umsetzung von Pflanzenschutzprotokollen für internationale Saatguttransporte.

Kapitel 3 beschreibt die Entwicklung eines zuverlässigen Hochdurchsatz-Phänotypisierungs-protokolls zur Identifizierung von Anthraknoseresistenz in genetischen Ressourcen der Weißen Lupine. Die Phänotypisierung unter kontrollierten Bedingungen und mittels Wundinokulation des Stängels erwies sich als geeignet für den Hochdurchsatz, und die Krankheitsindizes korrelierten stark mit den Krankheitsbonituren (r = 0,95) und dem Ertrag (r = -0,64) eines zweijährigen

Parzellenversuchs im Feld. Die Phänotypisierung von 40 Weiße Lupine-Akzessionen ergab acht Akzessionen mit verbesserter Anthraknose-Resistenz. In diesem Kapitel wird auch die Evaluierung eines genomischen Vorhersagemodells beschrieben, das auf zuvor berichteten Quantitativen Trait Loci (QTL) für die Resistenz beruht. Die vorhergesagten Resistenzwerte korrelierten jedoch nicht mit den hier beobachteten Werten eines unabhängigen Genotyp-Sets.

In **Kapitel 4** wird eine genomweite Assoziationsstudie (GWAS) zur Entschlüsselung der genetischen Architektur der Anthraknoseresistenz beschrieben. Weiße Lupine Genotypen aus dem Domestikationszentrum und traditionellen Anbauregionen wurden mittels zuvor beschriebenem Phänotypisierungprotokoll auf Anthraknoseresistenz untersucht und mittels „genotyping-by-sequencing“ (GBS) charakterisiert. GWAS ergab zwei signifikante SNPs, die mit Anthraknoseresistenz auf dem Gen Lalb_Chr05_g0216161 assoziiert sind. Dieses Gen kodiert für eine RING-Zink-Finger-E3-Ubiquitin-Ligase kodiert, die möglicherweise an der Pflanzenimmunität beteiligt ist. Die Populationsanalyse zeigte einen bemerkenswert schnellen Zerfall des Kopplungsungleichgewichts (LD), eine schwache Populationsstruktur und eine Gruppierung von kommerziellen Sorten mit Landsorten, was der langsamen Domestikations-geschichte und den geringen Züchtungsbemühungen bei der Weißen Lupine entspricht.

Kapitel 5 zeigt das Potenzial von nachhaltigen Saatgutbehandlungen zur Verringerung der *C. lupini* Infektionsrate. Insgesamt elf verschiedene Saatgutbehandlungen wurden zwischen 2018 und 2021 in Schweizer Feldversuchen getestet. Die Behandlungen umfassten Heißwasser, Dampf, Elektronenbestrahlung, Langzeitlagerung, Essig, Pflanzenextrakte und biologische Bekämpfungsmittel (BCAs). Die BCAs wurden vor den Feldversuchen unter kontrollierten Bedingungen auf ihre potenzielle antagonistische Aktivität gegen *C. lupini* während der Infektion getestet. Die Feldergebnisse zeigten, dass eine Langzeitlagerung und eine Behandlung mit Essig das Auftreten von Krankheiten erfolgreich reduzieren und den Ertrag auf ein ähnliches Niveau wie bei zertifiziertem Saatgut steigern kann. BCA-Behandlungen führten unter kontrollierten Bedingungen, nicht aber im Feld, zu einer signifikanten Verringerung der Krankheitsmerkmale.

Kapitel 6 enthält eine allgemeine Schlussfolgerung, in der die wichtigsten Ergebnisse dieser Arbeit in breiteren Kontext gestellt werden. Zur nachhaltigen und wirksamen Bekämpfung der Anthraknose-Krankheit wird ein integrativer Ansatz empfohlen, der moderne Züchtungs-maßnahmen, Strategien zur Krankheitsvorbeugung und Mischkultur umfasst. Diese Arbeit bildet die Grundlage für die weitere Erforschung der Populationsdynamik von *C. lupini*, der Virulenz und der Wirtsspeziation, der Resistenzmechanismen der Weißen Lupine und nachhaltiger Möglichkeiten zur Bekämpfung der Anthraknosekrankheit, um den erfolgreichen Anbau der Weißen Lupine weiter zu erleichtern.

Samenvatting

Witte lupine (*Lupinus albus* L.) is een peulvrucht die bekend staat om zijn hoge eiwitgehalte, voedingskwaliteit, efficiënte stikstoffixatie en unieke vermogen om gespecialiseerde clusterwortels te vormen die fosfor vrijmaken. Ondanks dat aan het eind van de vorige eeuw de teelt bijna volledig verdwenen was, kent witte lupine de laatste jaren een heropleving om aan de vraag naar plantaardige eiwitten te voldoen en Europa's afhankelijkheid van ingevoerde soja te verminderen. Een groot probleem in de (witte) lupineteelt is anthracnose, veroorzaakt door de wereldwijd verspreide, door zaad en lucht overgedragen schimmelziekte *Colletotrichum lupini*. Dit proefschrift heeft als doel inzicht te verschaffen in hoe anthracnose ziekte bij witte lupine kan worden bestreden om de herintroductie van dit gewas in Europese teeltsystemen te ondersteunen. De thesis beschrijft (i) de genetische diversiteit en virulentie van *C. lupini*, (ii) de ontwikkeling van een snel en betrouwbaar fenotyperingsprotocol, (iii) een genetishce studie die kandidaat resistentie genen voor anthracnose identificeert en (iv) de zoektoch naar effectieve zaadbehandelingen.

Hoofdstuk 1 geeft een algemene inleiding over het onderwerp van dit proefschrift. Er wordt achtergrondinformatie gegeven over de toekomstige vraag naar eiwitten, de teelt van zaaddragende leguminosen in Europa, de geschiedenis van de lupineteelt en waarom witte lupine een goede aanvulling zou zijn op geïmporteerde sojabonen. Ook worden de verschillende ziekten beschreven die een probleem vormen voor de lupineteelt, hierbij wordt vooral gewezen op de relevantie van de zeer destructieve anthracnose ziekte, die momenteel de lupineteelt wereldwijd belemmert. Aan het eind worden de hoofdvraag en de doelstellingen van dit proefschrift uiteengezet.

Hoofdstuk 2 onderzoekt de diversiteit van 39 *C. lupini* isolaten die zijn verzameld van over de hele wereld. Multi-locus fylogenie en morfologische karakteriseringen tonen aan dat de diversiteit groter is dan eerder al was aangetoond, waarbij in totaal zes genetische groepen en tien verschillende morfotypen kunnen worden onderscheiden. De hoogste diversiteit werd gevonden in de Zuid-Amerikaanse Andes, wat aangeeft dat dit het oorsprongscentrum van *C. lupini* is. Dit hoofdstuk toont ook aan dat de huidige pandemie wordt veroorzaakt door schimmel varianten die behoren tot genetische groep II, die genetisch en morfologisch uniform zijn. Groep II isolaten bleken zeer virulent te zijn op geteste witte en Andes lupine lijnen. Isolaten die tot de andere vijf genetische groepen behoren werden meestal alleen plaatselijk aangetroffen en vertoonden verschillende virulentiepatronen op zowel witte als Andes lupine. Twee zeer virulente groep II isolaten uit Chili bleken de resistentie van geavanceerd wit lupine kweekmateriaal te breken, wat de noodzaak benadrukt om fytosanitaire protocollen voor internationaal zaadtransport op te stellen.

Hoofdstuk 3 beschrijft de ontwikkeling van een betrouwbaar fenotyperings protocol voor de identificatie van anthracnose resistentie in witte lupine. Fenotypering in een gecontroleerd mileu, waarbij steel-wond-inoculatie op zaailingen wordt uitgevoerd, bleek toepasbaar voor hoge

capaciteit en de ziektescores correleerden sterk met ziektescores van veldproeven (r = 0,95) en opbrengst (r = -0,64) in Zwitserland. Fenotypering van een gevarieerde set van 40 witte lupine lijnen, toonde acht lijnen met een verbeterde resistentie tegen anthracnose, die kunnen worden opgenomen in veredelingsprogramma's voor witte lupine. Dit hoofdstuk beschrijft ook de evaluatie van een genomisch voorspellingsmodel, gebaseerd op eerder gerapporteerde resistentie “quatitative trait loci” (QTL), op een onafhankelijke set van genotypes. De voorspelde resistentiewaarden bleken echter niet te correleren met waargenomen waarden onder gecontroleerde of veldomstandigheden.

Hoofdstuk 4 beschrijft een genoomwijde associatie studie (GWAS) om inzicht te krijgen in de genetische architectuur van anthracnose resistentie in witte lupine. Witte lupine lijnen, verzameld uit het centrum van domesticatie en traditionele teeltgebieden, werden gescreend op resistentie tegen anthracnose en gekarakteriseerd met genotypering-door-sequencing (GBS). Met GWAS konden twee significante SNPs, geassocieerd met resistentie tegen anthracnose, worden geïdentificeerd op gen Lalb_Chr05_g0216161 dat codeert voor een “RING zinc-finger E3 ubiquitin ligase” dat mogelijk betrokken is bij plant immuniteit. Verdere validatie-experimenten zijn nu nodig om de betrokkenheid bij plantenresistentie te bevestigen. Populatie-analyse toonde een opmerkelijk snel verval van “linkage disequilibrium” (LD), een zwakke populatiestructuur en groepering van commerciële variëteiten met landrassen, wat overeenkomt met de langzame domesticatiegeschiedenis en schaarse veredelingsinspanningen in witte lupine.

Hoofdstuk 5 toont het potentieel van zaadbehandelingen om het infectieniveau van *C. lupini* in witte lupine te verminderen. Een totaal van elf verschillende zaadbehandelingen werd getest in veldproeven in Zwitserland tussen 2018 en 2021. De behandelingen bestonden uit heet water, stoom, elektronen, langdurige opslag, azijn, plantenextracten en biologische bestrijdingsmiddelen (BCA's). De BCA's werden onder gecontroleerde omstandigheden getest op potentiële antagonistische activiteit tegen *C. lupini* tijdens witte lupine infectie voorafgaand aan veldproeven. De resultaten toonden aan dat langdurige opslag en azijnbehandelingen met succes de ziekte-incidentie kunnen verminderen en de opbrengst kunnen verhogen tot niveaus die vergelijkbaar zijn met die waargenomen voor gecertificeerd zaad. BCA-behandelingen vertoonden significante ziekteverminderingen onder gecontroleerde omstandigheden, maar niet in het veld.

Hoofdstuk 6 geeft een algemene conclusie die de belangrijkste resultaten van dit proefschrift in een bredere context plaatst. Om anthracnose ziekte bij witte lupine duurzaam en effectief te bestrijden wordt een integrale aanpak aanbevolen, inclusief moderne veredelingsinspanningen, ziektepreventiestrategieën en gemengde teeltsystemen. Dit proefschrift vormt de basis voor verder onderzoek naar de populatiedynamiek en virulentie van *C. lupini*, resistentiemechanismen van witte lupine en duurzame manieren om anthracnose te bestrijden.

Chapter 1

General Introduction

A rising global plant-based protein demand

In the past 50 years the demand for animal products increased by more than 300% (FAOSTAT 2021). As the world population is expected to grow and to become more prosperous, global demand is expected to increase even further in the coming decades (UNDESA 2019; Westhoek et al. 2011). To sustain those increasing production levels, high amounts of plant-based protein are required. Besides animal products, the increasing popularity of meat-replacements and the likelihood of large-scale cultured-meat production in the near future will yet further increase demand for plant-based protein (Ismail et al. 2020). Currently the most important source of plant-based protein is soybean (*Glycine max*), which is produced on a large-scale in the USA, Brazil and Argentina (FAOSTAT 2021). This large-scale soybean production has been accompanied by deforestation, increasing emissions and socio-economic disturbances (Leguizamón 2016; Oliveira 2018; Pendrill et al. 2019). The biggest soybean importer worldwide is China (69%; FAOSTAT 2021), but with two thirds of its total agricultural area in use for livestock production, Europe as well is strongly dependent on soybean imports (Westhoek et al. 2011). Large-scale livestock production is responsible for 30% of the total human-induced biodiversity loss and by far most of the total greenhouse gas emissions produced by agriculture (Westhoek et al. 2011). Additionally, high concentrations of livestock, depending on imported protein sources, cause global nutrient imbalances and nutrient pollution in coastal and inland waters (Grizzetti et al. 2021; Lu and Tian 2017). To reduce the impact of livestock production, a decrease in consumption in rich countries would be the most effective, but changing consumption patterns is a slow cultural process (Westhoek et al. 2011). Another option to reduce livestock impact and diversify agriculture would be to grow protein crops locally as efficiently as possible. Especially in Europe, which imports 75% of its plant-based protein, the call for locally and sustainably produced plant-based protein is growing.

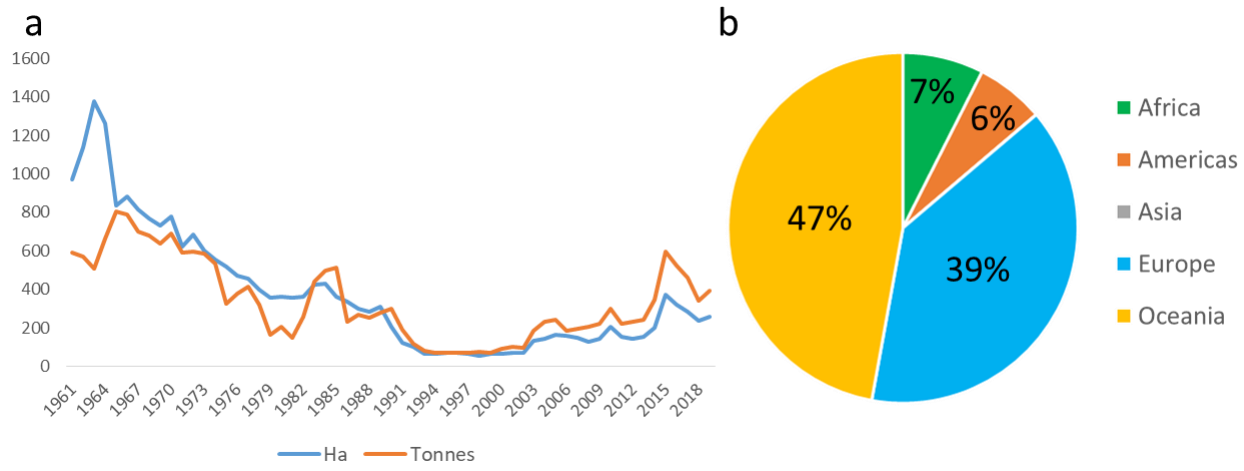

Figure 1. Lupin production. a: Lupin production in Europe from 1961 to 2019. With lupin cultivated area per 1000 ha (blue) and production in 1000 tonnes (orange), **b:** worldwide distribution of lupin production in 2019. Source: FAOSTAT (2021).

Grain legume cultivation in Europe

In the last decade the European Union has pushed to increase and diversify protein crop production to reduce its dependency on imported soybean and improve agricultural sustainability. Legumes offer great potential through their ability to fix nitrogen and improve soil fertility (Drevon et al. 2015). European cropping systems with legumes were shown to reduce nitrous oxide emissions by 18 and 33% and N fertilizer use by 24 and 38% in arable and forage systems, respectively, compared to systems without legumes (Reckling et al. 2016). Furthermore, legumes offer great potential to improve crop diversification, biodiversity and ecosystem services (Ditzler et al. 2021; Köpke and Nemecek 2010). Despite these advantages, grain legume cultivation in Europe has declined from 6.8% of arable land in 1961 to 3.5% in 2010 (FAOSTAT 2021). Reasons for this decline were low and variable yields (Cernay et al. 2015), volatile margins, underestimation of benefits and subsidies for competing crops (Zander et al. 2016). Recently, however, grain legume cultivation area recovered to almost 6% in 2019 (FAOSTAT 2021). This is mainly due to a recent surge in European soybean production which accounts for 53% of total grain legume cultivation area, followed by pea (*Pisum sativum*; 22%), faba bean (*Vicia faba*; 5%) and chickpea (*Cicer arietinum*; 5%). Soybean, however, requires appropriate day length to flower and sufficient growing degree-days to mature and is not suitable for more temperate regions. Further efforts are therefore necessary to further increase crop diversification and fully benefit from the advantages that legumes have to offer.

Lupins, the solution?

Lupins (*lupinus*) of the family *Leguminosae* (LPWG 2017), are grain-legumes with a high-quality protein content comparable to soybean (Annicchiarico 2008; Gulisano et al. 2019), have a high yield potential (Sellami et al. 2019), and are suitable as livestock feed (Abraham et al. 2019). Besides these beneficial characteristics, lupins were also shown to be suitable for aquaculture (Glencross et al. 2010; Molina-Poveda et al. 2013) and meat-replacement products (Santo et al. 2020), have potential health benefits (Lucas et al. 2015), positively influence endangered bumblebee species (Fijen et al. 2021) and are extraordinarily efficient atmospheric nitrogen fixers through their symbiosis with *Bradyrhizobium lupini* which is highly resistant to abiotic stresses (Fernández-Pascual et al. 2007; Kurlovich et al. 2000; Peix et al. 2015; Robinson et al. 2000). In contrast to other *Leguminosae* and most other plant species, lupins are non-host to arbuscular mycorrhizae (Akiyama et al. 2010; Oba et al. 2001). Instead, some lupin species form specialized root systems that secrete phosphate-mobilizing carboxylates to increase phosphorus availability (Lambers et al. 2013). This makes lupins highly suitable for cultivation under poor nutrient conditions and for increasing soil fertility. Lupins were widely cultivated in Europe in the beginning of the twentieth century, but production rapidly decreased after the 1960s due to cheap soybean imports, yield variability, low revenues and anthracnose disease outbreaks (Lucas et al. 2015; Talhinhas et al. 2016). Lupin production in Europe is currently making a comeback and accounts for 39% of the production worldwide (**Fig. 1**). To fulfill future demand, however, production needs to increase significantly.

The genus *Lupinus* consists of approximately 280 species worldwide (Drummond et al. 2012; Hughes and Eastwood 2006). The highest diversity is found in the Americas and emerged from two separate introductions from the hypothesized center of origin around the Mediterranean basin and Northern Africa (Drummond 2008; Hughes and Eastwood 2006). Only four species, Andean (*L. mutabilis* Sweet, 2n = 48), blue (*Lupinus angustifolius* L., 2n = 40), yellow (*L. luteus* L., 2n = 52) and white lupin (*L. albus* L., 2n = 50), are of agricultural importance (Gresta et al. 2017). Domestication of lupins dates back to at least 2000 BC and took place across the Mediterranean for blue, yellow and white lupin (Wolko et al. 2011) and in the highlands of Northern Peru for Andean lupin (Atchison et al. 2016). Andean lupin has been an important protein source for pre-Columbian societies and was widespread throughout the Incan empire, stretching from Colombia to Chile (Jacobsen and Mujica 2008). Up to today, highly diverse Andean lupin varieties are cultivated throughout the Andes and are served in soups and stews or in fishless "ceviche". The crop has received growing attention as it is characterized by an oil and protein content similar to soybean and is highly valued for its adaptability to colder climates and low input agriculture (Gulisano et al. 2019). Blue and yellow lupin, with their presumed origin in the Western Mediterranean (Berger et al. 2008; Mousavi-Derazmahalleh et al. 2018b), were used by Mediterranean societies for centuries but have only been fully domesticated in the early nineteenth century (Wolko et al. 2011). This recent and short domestication process involved severe bottleneck events (Berger et al. 2012), and resulted in a very narrow domesticated gene pool, lacking the genetic and adaptive diversity found in wild relatives (Berger and Ludwig 2014; Berger et al. 2013; Mousavi-Derazmahalleh et al. 2018a). Whereas yellow lupin is still a niche product, (sweet) blue lupin is being cultivated on a relatively large scale in Western Australia and Eastern Europe for mostly animal feed and aquaculture. In contrast to blue and yellow lupin, white lupin has been widely cultivated in ancient Egypt, Greece and throughout the Roman Empire (Wolko et al. 2011). Up to today, you can find white lupin being used as a snack throughout the Mediterranean region. Its center of domestication is believed to include the Balkans and the Aegean region where wild *graecus* types can still be found (Wolko et al. 2011). White lupin is characterized by a slow and extensive domestication process, resulting in low genetic differentiation between wild types, landraces and commercial cultivars; a weak population structure and an unusually low linkage disequilibrium decay (LD) of < 4 kb (Hufnagel et al. 2021). The limited breeding within white lupin and the availability of highly diverse germplasm offers great potential for further crop improvement (Annicchiarico et al. 2010; Wolko et al. 2011).

White lupin, a re-emerging protein crop

Compared to other lupin species and cool-season grain legumes, white lupin has a high protein content of approximately 39 % (Annicchiarico 2008). White lupin is recognized by its broad leaves with typical white hairs, its flowers can be white or blue and seeds are mostly white but can also show brown speckles (**Fig. 2**). While blue lupin is exclusively self-pollinating, white lupin is generally considered a cross-pollinated crop but high self-pollination rates of 50 to 85% are

common (Brebaum and Boland 1995). White lupin is considered highly nutritional because of its beneficial amino acid composition, good digestibility, outstanding fatty acid quality and other health benefits (Arnoldi et al. 2015; Monteiro et al. 2014; Sujak et al. 2006). Its seeds are appreciated for their transparent color and low starch content by the food industry (Arnoldi et al. 2015). Besides its symbiosis with the highly efficient nitrogen fixing *B. lupini*, (Fernández-Pascual et al. 2007; Peix et al. 2015), white lupin has the unique capability to form specialized cluster roots (**Fig. 2**), which increase phosphorus availability by secreting massive amounts of carboxylates into the soil and thereby significantly increasing soil fertility (Gallardo et al. 2019; Gallardo et al. 2020; Lambers et al. 2013). Since the development of sweet low alkaloid (0.05–0.02%) varieties (Kroc et al. 2017), white lupin has become increasingly interesting for the food and feed industry (Lucas et al. 2015). Cultivation area of white lupin, however, is still very limited. Reasons for this are the lack of domestication for key agronomic traits such as early flowering (Adhikari et al. 2013), cold tolerance (Annicchiarico and Iannucci 2007), tolerance to calcareous soils (Annicchiarico and Alami 2012), drought tolerance (Annicchiarico et al. 2018), and seed yield (Annicchiarico et al. 2019). Another important reason for the limited (white) lupin cultivation is its susceptibly to a wide range of diseases (White et al. 2008).

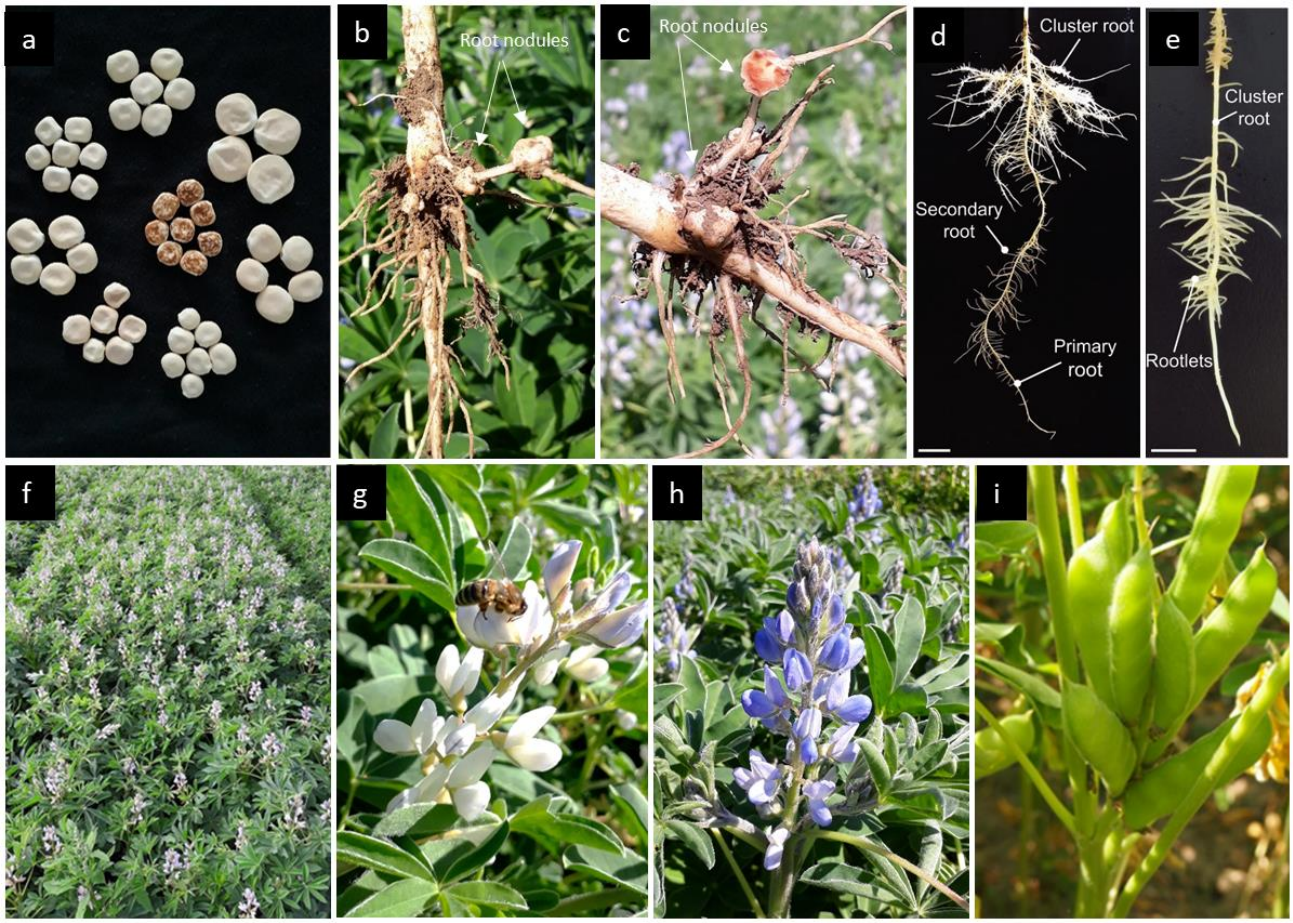

Figure 2. Impression of white lupin (*L. albus*) cultivation. a: Seeds, middle: R-6020 (Poland), from up clockwise: Blu-25 (Chile), Mollisse (France), La427 (Syria), Feodora (France), P27125 (Ethiopia), Egypte038 (Egypt), Dieta (Czech Republic). **b & c**: root nodules, **d & e**, roots with cluster roots, **f**, cultivation plot, **g**: white flower (cv. Feodora), **h**: blue flower (br: Blu-25), **i**: healthy pods (cv: Amiga). Pictures **b**, **c & g** by B Haug and a, **f, h & i** by JA Alkemade. Source **d & e**: Gallardo et al. (2019).

Major lupin diseases

Lupin cultivation worldwide suffers substantial yield losses from various diseases. Virus infections caused by yellow bean mosaic virus and cucumber mosaic virus, which are both seed-borne and aphid transmitted, cause problems worldwide (Jones et al. 2008; Jones and Mclean 1989; Wylie et al. 2008). Drippy Pod disease caused by *Brenneria quercina* pv. *lupinicola* is the most important bacterial disease in lupins and is recognized by a foamy excretion at the pods (Lu and Gross 2010). The disease can spread rapidly through transmission by phytophagous *Lygus* sp. insects and although the disease has often only a minor impact, complete yield losses have been reported. Fungal diseases are the most important in lupin cultivation. Major yield losses are caused by pleiochaeta root rot caused by the soil-borne fungus *Pleiochaeta setosa*, which also causes foliar brown spot disease, and mostly kills off young seedlings (Luckett et al. 2009). Another important soil-borne disease is Fusarium wilt caused by *Fusarium oxysporum* f. sp. *lupini.* The disease can be seed and soil transmitted and has been reported in lupin cultivation areas in Europe, Egypt, and South Africa (Abd El-Rahman et al. 2012; Lindbeck 2009). Fusarium wilt on white lupin might also be present in Argentina (I. Farina, personal communication, 21.10.2019). The broad host-range soil-borne fungus *Rhizoctonia solani* also has been reported to cause yield losses in lupin (Sweetingham 1989; Zhou et al. 2009). Phomopsis stem blight, caused by the fungus *Diaporthe toxica*, is another major concern in lupin cultivation. Phomopsis stem blight does not cause major yield losses but when infected plant material is fed to animals it can cause symptoms of lupinosis caused by mycotoxins produced by the fungus (Cowley et al. 2010). Lupinosis is a liver disease and can lead to severe acute disease, chronic liver dysfunction and even death. By far the biggest problem in lupin cultivation, however, is a lack of tolerance to anthracnose disease which causes high yield losses globally (Talhinhas et al. 2016).

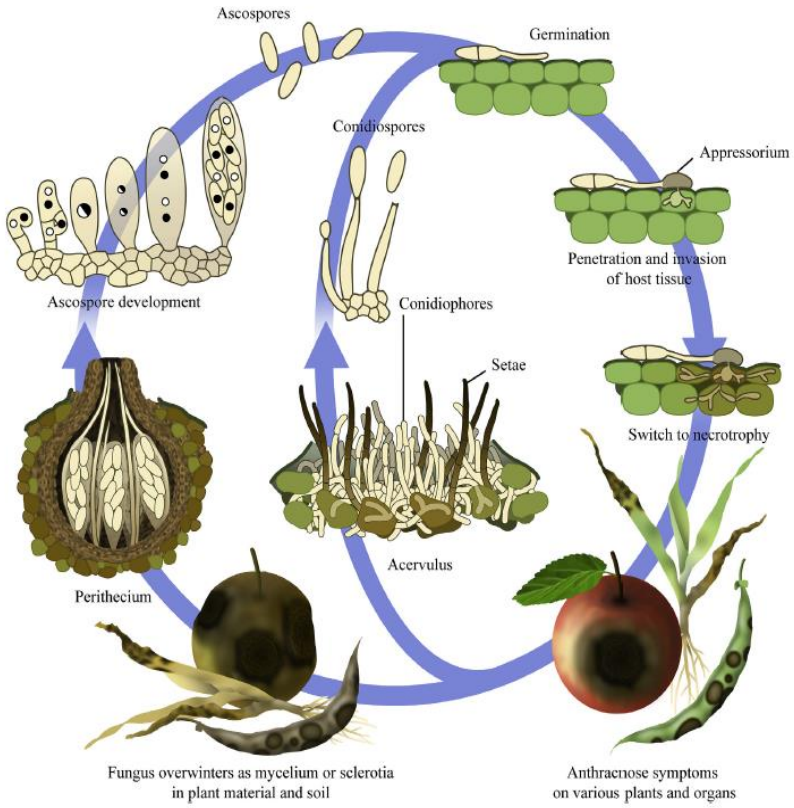

Figure 3. Life-cycle of hemibiotrophic *Colletotrichum* sp. Source: De Silva et al. (2017).

Anthracnose disease

Anthracnose disease is caused by the fungal pathogen *Colletotrichum lupini* and almost all lupin species are vulnerable, including all the above mentioned cultivated lupin species (blue, yellow, white and Andean lupin), but also ornamental lupins such as *L. polyphyllus* and *L. hartwegii* (Elmer et al. 2001; Rosskopf et al. 2014), and wild lupins such as *L. cosentinii* (Shea et al. 2008). The fungal genus *Colletotrichum*, consists of at least 15 species complexes (Cannon et al. 2012; Damm et al. 2019; Marin-Felix et al. 2017), harbors many important plant pathogenic species that cause anthracnose and other diseases in a wide variety of hosts (Lenné 2002; Shivas et al. 2016; Udayanga et al. 2013), and is listed in in the top 10 of the most important fungal plant pathogens worldwide (Dean et al. 2012). Besides being of economic importance, *Colletotrichum* spp. have been widely used as model species to study plant-fungus interactions because of the diversity of lifestyles and host-specialization events within this genus (Baroncelli et al. 2017; O'Connell et al. 2012; Perfect et al. 1999). The different life-styles can be broadly categorized as endophytic, quiescent, hemibiotrophic and necrotrophic, with most species being hemibiotrophic (**Fig. 3**; De Silva et al. 2017). The *Colletotrichum acutatum* species complex is especially notorious as it contains many plant pathogens (Bragança et al. 2016; Damm et al. 2012). Among hosts are important fruit, vegetable, and oil crops such as strawberry (Baroncelli et al. 2015), pepper (Than et al. 2008) and olive (Talhinhas et al. 2015). Multi-locus phylogeny revealed a high diversity within the *C. acutatum* species complex, showing at least 32 different species divided among five clades, with *C. lupini* grouped in clade 1. The most important morphological characteristic for members of this species complex are the cylindrical conidia with acute ends (Damm et al. 2012; **Fig. 4**). Although many species within the *C. acutatum* species complex have a broad host range, *C. lupini* appears to be highly host specific on lupins (Baroncelli et al. 2017).

Lupin anthracnose was first reported in 1912 in Brazil, and the fungal pathogen was described as *C. gloeosporioides* in 1939 (Talhinhas et al. 2016), followed by *C. acutatum* in 1994 (Sreenivasaprasad et al. 1994). In 2002, the pathogen was classified as a new species, *Colletotrichum lupini*, with two different variants *C. lupini* var. *lupini* and *setosum* (Nirenberg et al. 2002). This new species was confirmed by multi-locus analysis (Damm et al. 2012) and the two variants were referred to as group I (var. *lupini*) and II (var. *setosum*; **Fig. 4**; Dubrulle et al. 2020a). A first outbreak was reported in the 1940 - 50s mainly in North America and is believed to be caused by members of group I (Shivas et al. 1998). A second and more severe outbreak started around the 1970s and persists until today (Talhinhas et al. 2016). At first, mainly humid areas were affected, coinciding with a decrease in lupin production in Europe, but it quickly became a worldwide problem (**Fig. 5**), with reports from the USA (Rosskopf et al. 2014) to China (Zou et al. 2019), and even affecting the big lupin industry in Australia (Adhikari et al. 2009). The rapid global spread of the disease can be attributed to the trade of infected symptomless seeds (Semaskiene et al. 2008), and even low levels of initial seed infection (0.01-0.1 %) can cause high yield losses (Sreenivasaprasad and Talhinhas 2005; Thomas and Sweetingham 2004). Seeds are the main source of inoculum but there are also reports of *C. lupini* surviving in alternative hosts or infected

crop stubble (Talhinhas et al. 2016; White et al. 2008). Not much is known about its life-cycle and the interaction with its host, but a hemibiotrophic lifestyle is expected (Dubrulle et al. 2020b). Upon seed germination, *C. lupini* is expected to be dormant or growing endophytically and switches to a biotrophic followed by necrotrophic phase during plant development, causing the typical symptoms of stem twisting and bending and the formation of necrotic lesions with masses of orange conidia on stems and pods (**Fig. 6**). Conidia are rain-splashed or wind dispersed, causing secondary infections within the crop. Artificial inoculation showed appressoria formation and a switch from biotrophy to necrotrophy within 48 hours post inoculation (Dubrulle et al. 2020b). A high genetic diversity of *C. lupini* isolates was found in Chile (Riegel et al. 2010), and a distinct lupin infecting *C. acutatum* group was identified in Ecuador (Falconí et al. 2013). Members of clade 1 of the *C. acutatum* species complex are believed to originate from South America (Bragança et al. 2016; Damm et al. 2012) and the center of origin of *C. lupini* is hypothesized to be in South America. Further insights in *C. lupini* genetic diversity, phylogeography and host-pathogen interactions are crucial for developing successful disease management strategies and breeding programs.

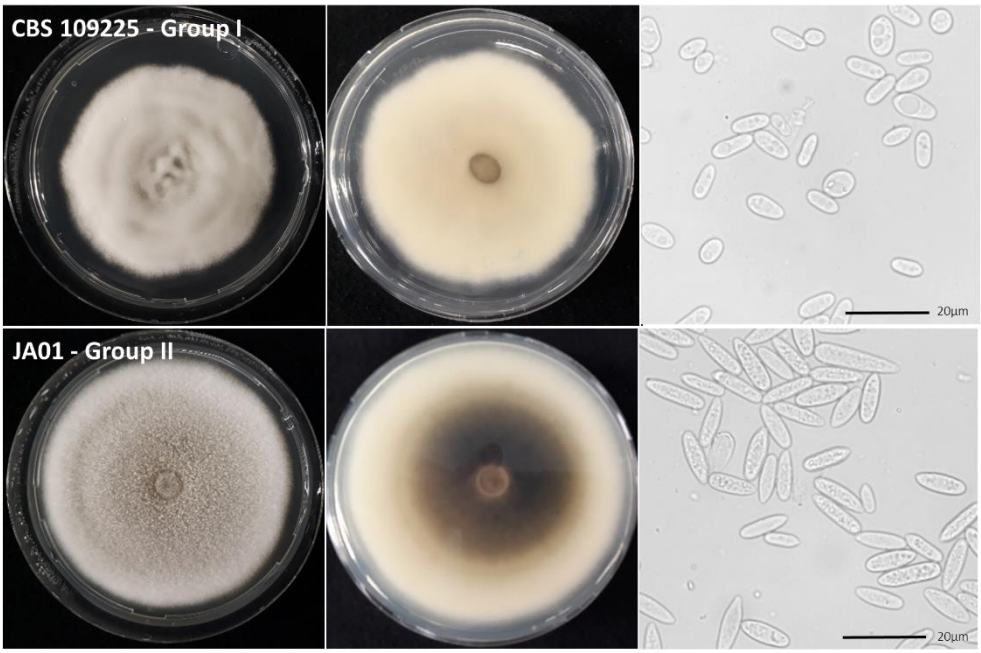

Figure 4: Colony and conidia morphology of *Colletotrichum lupini* group I and II. Plates show the front and reverse of 14 day old colonies on PDA. Scale bars indicate 20 µm. See also Chapter 2, Fig. 2.

Anthracnose disease control

Starting with disease-free seeds is crucial to control anthracnose disease and is, together with a good crop rotation and foliar fungicide treatments, the most common control measure (White et al. 2008). Disease-free seeds can be obtained when grown under strict phytosanitary control in environments unfavorable for the disease. A polymerase chain reaction (PCR) detection method has been developed to determine presence or absence of *C. lupini* in seeds (Pecchia et al. 2019), and a quantitative real time PCR (qPCR) method was developed to quantify levels of infection (Kamber et al. 2021). Fungicide applications were shown to successfully reduce inoculum viability

in seeds (Talhinhas et al. 2016; Thomas et al. 2008a). However, chemical control is not available for the organic sector and is considered problematic due to adverse environmental effects (Zubrod et al. 2019). Alternative seed treatments through dry-heat (Falconí and Yánez–Mendizábal 2016; Thomas and Adcock 2004), UV (Falconí and Yánez-Mendizábal 2018), or long term storage also showed promising results in reducing *C. lupini* inoculum in lupin seeds (Thomas and Sweetingham 1999). Seed treatments with hot water showed to reduce *Colletotrichum* incidence in *Eucalyptus* (Mangwende et al. 2020) and celery (Yamagishi et al. 2015). Seed inoculation with *Bacillus subtilis* was shown effective against anthracnose in Andean lupin (Yánez-Mendizábal and Falconí 2018), and might be promising for other lupin species as well. Biological control has also been effective in reducing *Colletotrichum* induced disease in numerous other crops and are worth further investigation (Chanchaichaovivat et al. 2007; Konsue et al. 2020; Pylak et al. 2019; Sharma and Gothalwal 2017; Verma et al. 2006). Although non-synthetic seed treatments are promising and could significantly contribute to a successful re-introduction of white lupin in Europe, the development of cultivars with durable resistances is deemed to be the most sustainable and profitable.

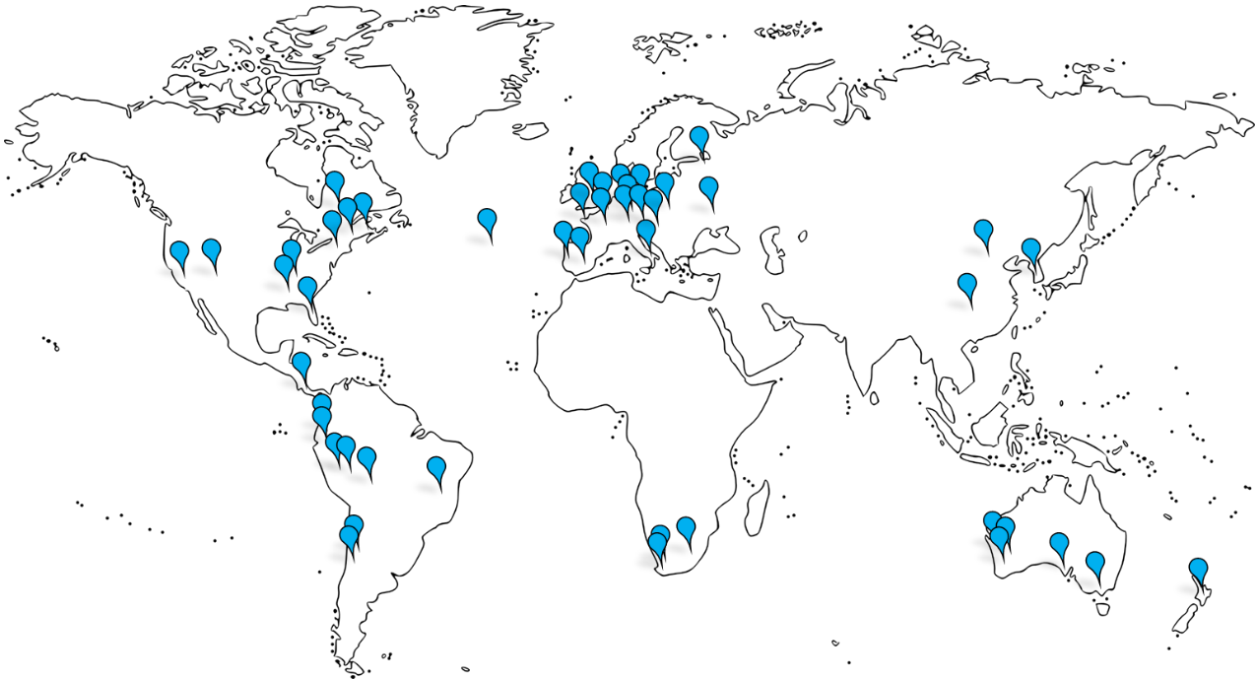

Figure 5. Global distribution of *C. lupini*. Sources: Damm et al. (2012); Dubrulle et al. (2020a); Elmer et al. (2001); Falconí et al. (2013); Frisullo et al. (2016); Han et al. (2014); Lotter and Berger (2005); Nirenberg et al. (2002); Riegel et al. (2010); Rosskopf et al. (2014); Shivas et al. (1998); Shivas et al. (2016); Yang and Sweetingham (1998); Zou et al. (2019) and Chapter 2.

Resistance breeding

In blue lupin, resistance is controlled by the single resistance genes *Lanr1* in cv. Tanjil (Yang et al. 2004)*, Anman* in cv. Mandelup (Yang et al. 2008) and *Lanrbo* in the breeding line Bo7212 (Fischer et al. 2015). Closely linked markers were developed to deploy marker assisted selection and a draft genome sequence is now available to support blue lupin breeding programs (Hane et al. 2017; Taylor et al. 2020). In yellow lupin, moderate resistance was identified in wild types and landraces from the Iberian Peninsula and Eastern Europe, but no complete resistance was found (Adhikari et al. 2011). In Andean lupin, multiple sources of resistance have been identified, but genetic architecture remains unknown (Falconi et al. 2015; Guilengue et al. 2020). In white lupin, resistance to anthracnose is considered a polygenic trait (Yang et al. 2010). Resistance screening in a Western Australian and New Zealand experiment identified resistance in Ethiopian landraces (Adhikari et al. 2009; Cowling et al. 1999), which form a distinct white lupin gene-pool (Raman et al. 2014). Quantitative trait locus (QTL) mapping, through a recombinant inbred line (RIL) population between the Ethiopian landrace P27174 and susceptible cultivar Kiev Mutant, revealed three major QTLs, antr04/05_1, antr04/05_2 and antr05_3, on chromosome 2, 4 and 10, respectively, that jointly explained up to 49% of the phenotypical variation observed under field conditions in 2004 and 2005 in Western Australia (Phan et al. 2007; Yang et al. 2010). The same QTLs, were identified with greater precision by Książkiewicz et al. (2017), using a large number of SNP markers identified by genotyping-by-sequencing (GBS). Based on this data, PCR-based markers and a genomic selection model were developed to support anthracnose breeding in white lupin (Rychel-Bielska et al. 2020). High synteny between blue and white lupin was observed but QTLs conferring anthracnose resistance did not match (Książkiewicz et al. 2017). Recently, a high quality white lupin reference genome was published (Hufnagel et al. 2020), opening the doors for modern breeding approaches and genomic studies.

Figure 6. Anthracnose disease in white lupin (*Lupinus albus* L.). a: Stem twisting **b & c:** Infected pods. **d**: completely infected plant. Pictures by JA Alkemade, see also Chapter 2, Fig. 1

Objectives

This PhD project aims to substantially improve our understanding on how to sustainably control anthracnose disease in white lupin cultivation and thereby contribute to a successful re-introduction into European cropping systems. The global dissemination and population structure of *C. lupini* is still little understood, hence there is a strong need to study its genetic diversity, phylogeography, and virulence to be able to develop appropriate disease management strategies and breeding programs. In order to identify sources of resistance in white lupin and study (white) lupin-*C. lupini* interactions, it is crucial to have a reliable phenotyping system that corresponds to field conditions under natural infection pressure. When *C. lupini* is mapped and a reliable phenotyping protocol is established, white lupin germplasm collected from the center of domestication and traditional cultivation areas needs to be phenotyped and genotyped to get insight in the genetic background of anthracnose resistance in white lupin. Application of sustainable non-synthetic seed treatments to reduce primary inoculum could further contribute to a successful control of anthracnose disease and re-introduction of white lupin. Hence the objectives of this study are:

i) Collect and characterize a globally representative collection of *C. lupini* isolates.
ii) Develop a controlled condition high-throughput phenotyping protocol that corresponds to field performance.
iii) Genotype and phenotype white lupin germplasm to dissect the genetic architecture of resistance to anthracnose disease.
iv) Screen seed treatments for efficacy against anthracnose disease in white lupin.

Chapter 2

Genetic diversity of *Colletotrichum lupini* and its virulence on white and Andean lupin

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Abstract

Lupin cultivation worldwide is threatened by anthracnose, a destructive disease caused by the seed- and air-borne fungal pathogen *Colletotrichum lupini*. In this study we explored the intraspecific diversity of 39 *C. lupini* isolates collected from different lupin cultivating regions around the world, and representative isolates were screened for their pathogenicity and virulence on white and Andean lupin. Multi-locus phylogeny and morphological characterizations showed intraspecific diversity to be greater than previously shown, distinguishing a total of six genetic groups and ten distinct morphotypes. Highest diversity was found across South America, indicating it as the center of origin of *C. lupini*. The isolates that correspond to the current pandemic belong to a genetically and morphologically uniform group, were globally widespread, and showed high virulence on tested white and Andean lupin accessions. Isolates belonging to the other five genetic groups were mostly found locally and showed distinct virulence patterns. Two highly virulent strains were shown to overcome resistance of advanced white lupin breeding material. This stresses the need to be careful with international seed transports in order to prevent spread of currently confined but potentially highly virulent strains. This study improves our understanding of the diversity, phylogeography and pathogenicity of a member of one of the world's top 10 plant pathogen genera, providing valuable information for breeding programs and future disease management.

Keywords

Colletotrichum, anthracnose, *Lupinus* sp.*,* phylogeny, morphology, pathogenicity, phylogeography, plant-microbe interactions

Introduction

The fungal genus *Colletotrichum* contains many important plant pathogenic species that cause anthracnose and other pre- and post-harvest diseases in a wide variety of hosts (Cannon et al. 2012; Lenné 2002; Shivas et al. 2016; Udayanga et al. 2013). Among potential hosts are important fruit, cereal and legume crops such as strawberry (Baroncelli et al. 2015), maize (Frey et al. 2011) and soybean (Boufleur et al. 2021; Rogério et al. 2020). Besides being of economic importance, *Colletotrichum* spp. have been widely used as model species to study plant-fungus interactions because of the diversity of lifestyles within this genus (Baroncelli et al. 2017; De Silva et al. 2017; Perfect et al. 1999; Yan et al. 2018). *Colletotrichum* is listed in the top 10 of most important fungal plant pathogens worldwide (Dean et al. 2012). Within the genus, members of the *Colletotrichum acutatum* species complex are notorious and cause disease in many important crops (Bragança et al. 2016; Damm et al. 2012). The most important morphological characteristic for members of this species complex are the acute ends of its conidia (Damm et al. 2012). Discrimination of *Colletotrichum* species solely based on morphological traits, however, is deemed unreliable due to the few and highly variable characteristics, the strong influence of environmental conditions and the high overlap between species (Cannon et al. 2000). Therefore, a polyphasic approach, combining morphological and genetic data is recommended (Cai et al. 2009; Johnston 2000). Multi-locus phylogeny revealed a high diversity within the *C. acutatum* species complex, showing at least 32 different species divided among five clades (Damm et al. 2012). Although many species within the *C. acutatum* species complex have a broad host range, *Colletotrichum lupini*, belonging to clade 1, appears to be highly host specific on lupins (*Lupinus*)(Lardner et al. 1999; Talhinhas et al. 2016).

Lupin anthracnose caused by *C. lupini* is the most important disease in lupin cultivation worldwide, affecting all economically important lupin species such as blue (*Lupinus angustifolius* L.), white (*L. albus* L.), Andean (*L. mutabilis* Sweet.), yellow (*L. luteus* L.) and ornamental lupin (*L. polyphyllus* Lindl.; Talhinhas et al. 2016). The disease was first reported in 1912 in Brazil (Bondar 1912), but the fungal pathogen was identified much later (Weimer 1943). A first outbreak was reported in the 1940 - 50s in North America and was followed by a more severe and globally widespread outbreak around the 1980s which is still persisting until this day (Talhinhas et al. 2016). The disease is mainly dispersed via seeds, facilitating rapid spread through international seed transports, and within the crop by rain splash during the growing season (White et al. 2008). Even low amounts of initial inoculum can cause total yield losses making this disease highly destructive (Shea et al. 2008; Thomas and Sweetingham 2004). Typical symptoms are stem twisting and necrotic lesions on stems and pods (**Fig. 1**; Alkemade et al. 2021b). Current disease management is focused on planting certified disease-free seed and chemical protection (Thomas et al. 2008a; White et al. 2008). However, crop resistance could offer a more sustainable alternative. In blue lupin, anthracnose resistance is controlled by single resistance genes (Fischer et al. 2015; Yang et al. 2004; Yang et al. 2008), whereas in white, Andean and yellow lupin no such single gene resistance is known and the observed quantitative resistance is considered to

be polygenic (Adhikari et al. 2009; Adhikari et al. 2011; Falconí 2012). The increasing demand for plant-based protein is renewing the interest for lupins as a high quality protein crop (Gulisano et al. 2019; Lucas et al. 2015; Van de Noort 2017), the current anthracnose pandemic, however, severely hampers cultivation.

The pathogen was first described as *Gloesporium lupinus*, followed by *C. gloeosporioides* and *C. acutatum* until it was fully described as *C. lupini* (Damm et al. 2012; Nirenberg et al. 2002; Shivas et al. 1998). Currently two genetic groups (I and II) are distinguished within *C. lupini* based on vegetative compatibility groups (VCG; Shivas et al. 1998), the ITS (internal transcribed spacer) region (Nirenberg et al. 2002) and multi-locus phylogeny of the ITS, GAPDH (glyceraldehyde-3-phosphate dehydrogenase), CHS-1 (chitin synthase), HIS3 (histone), ACT (actin), TUB2 (β-tubulin 2), HMG (HMG box region) and APN/MAT1 (Apn2-Mat1-2-1 intergenic) loci (Dubrulle et al. 2020a). The TUB2 and GAPDH loci were shown to be the most informative within the *C. acutatum* species complex and APN/MAT1 the most informative within *C. lupini*, whereas classification based on the ITS region can be problematic due to low resolution within the complex (Damm et al. 2012; Dubrulle et al. 2020a). Although only two groups within *C. lupini* have been distinguished, with most of the reported strains belonging to group II (Dubrulle et al. 2020a), intraspecific diversity is thought to be greater as a high diversity was found in a Chilean *C. lupini* collection using random amplified polymorphic DNA (RAPD) markers (Riegel et al. 2010) and a distinct lupin infecting *C. acutatum* group was identified in Ecuador based on the ITS region (Falconí et al. 2013). This suggests that highest intraspecific diversity is found in South America, which is believed to be the center of origin of members belonging to clade 1 of the *C. acutatum* species complex (Baroncelli et al. 2017; Bragança et al. 2016).

The overall aim of this study was to assess a worldwide collection of lupin-infecting *Colletotrichum* isolates through (i) multi-locus phylogeny, (ii) morphology and (iii) virulence on white and Andean lupin. Insights into *C. lupini* diversity, phylogeography and plant-*C. lupini* interactions will improve our understanding of the current lupin anthracnose pandemic and support future disease management strategies and lupin breeding programs.

Materials and Methods

Fungal and plant material

A diverse collection of 39 *Colletotrichum lupini* and 11 closely related *Colletotrichum* spp. isolates, originating from Europe, Australia, Southern Africa and South and North America, was analyzed (**Table 1**). Nine isolates were collected from symptomatic lupin plants in this study, whereas the rest of the isolates was already identified as *C. lupini* or as other members of the *C. acutatum* species complex representing clades 1, 2 and 4. The *C. lupini* strains CBS 109225 (Ukraine), CBS 509.97 (France) and CBS 109226 (Canada) were chosen as reference for genetic group I, strains CBS 109221 (Germany), IMI 375715 (Australia) and RB221 (France) served as reference for genetic group II and the *C. acutatum* strains CBS 369.73 and CBS 370.73 were used as outgroup in the phylogenetic analysis. Inoculations were performed on two white lupin (*Lupinus albus* L.)

accessions: Feodora (susceptible; breeder: Jouffrai Drillaud, France) and Blu-25 (tolerant; breeder: Semillas Baer, Chile), and two Andean lupin (*L. mutabilis*) accessions: LUP 17 and LUP 100 (genebank: IPK, Germany). Plant material can be requested at mentioned breeders and genebanks, who performed formal identification and gave permission to use the material for research purposes. The experimental research of the plant material used in this study complies with relevant institutional, national, and international guidelines and legislation.

Fungal isolation and culture conditions

Symptomatic (dried) lupin stem or pod tissue (**Fig. 1**) of 1-3 cm was surface sterilized (after rehydration in sterile ddH_2O for dried samples) for 5 seconds with 0.25% sodium hypochlorite solution and rinsed thrice for 5 seconds in sterile ddH_2O. Thin slices of 1 mm were cut and placed on PDA (potato dextrose agar, Carl Roth, Karlsruhe, Germany) amended with Tetracycline (0.02 gr/l, Carl Roth) for 3 to 4 days at 22°C in the dark. Single cultures were selected and grown on fresh PDA plates amended with Tetracycline for 4 to 6 days at 22°C in the dark and suspected *Colletotrichum* species were sub-cultured. Single spore cultures were obtained and transferred to PDA and maintained at 22°C in the dark as working cultures and stored at -80°C in 25% glycerol for long-term storage.

Morphology

A globally representative subset of 28 *C. lupini* isolates was characterized based on colony morphology (form, aerial mycelium, margin type and color of the reverse side). From those, a subset of 18 isolates was further characterized for growth rate (mm/day), and conidial shape and size (Lardner et al. 1999). Isolates were sub-cultured by placing a droplet of 5 µl spore suspension in the middle of three PDA plates and grown for 14 days at 22°C in the dark. Culture diameter was recorded every 3 days. Photographs were taken from the front and reverse sides of the PDA plates after 14 days of incubation. Conidia were collected with a sterile spreader after flooding the Petri plate with 2 ml sterile ddH_20, the spore suspension was filtered with sterile cheese cloth and microscopic slides were prepared with sterile ddH_2O. Conidia morphology was observed using light microscopy (DM2000-LED, Leica Microsystems, Wetzlar, Germany) equipped with a high definition camera (Gryphax Subra, Jenoptik AG, Jena, Germany). A minimum of at least 50 measurements were performed to determine conidia length and width. A principal component analysis (PCA) was performed on a subset of 17 representative *C. lupini* isolates, based on average conidia length and width, length width ratio, colony growth rate, form (circular = 1, most irregular = 4), aerial mycelia (no aerial mycelia = 1, most aerial mycelia = 4), color (palest = 1, darkest = 4) and filiform margin (yes = 1, no = 0), using R 4.0.3 (R Core Team 2020) and the *FactoMineR* package (Lê et al. 2008).

DNA extraction, PCR amplification and sequencing

Mycelium from single-spore cultures was collected after 7-10 days on PDA at 22°C with a sterile spreader after flooding the Petri dish with 2 ml sterile ddH_20. Genomic DNA was isolated with a CTAB extraction protocol (Minas et al. 2011). Partial gene sequences were determined for the internal transcribed spacer (ITS) region using primers ITS5 and ITS4 (White et al. 1990), the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene using primers GDF1 and GDR1 (Guerber et al. 2003), the β-tubulin 2 (TUB2) gene using primers Btub2Fd and Btub4Rd (Woudenberg et al. 2009) and the Apn2-Mat1-2-1 intergenic (APN/MAT1) spacer and partial mating type gene using Apnmat1F and Apnmat1R (Dubrulle et al. 2020a). PCR was performed in a S1000 Thermal Cycler (Bio-Rad Laboratories, Inc., Hercules, CA, USA) according to conditions described in Dubrulle et al. (2020a). PCR products were verified by gel electrophoresis, purified using the QIAquick PCR Purification Kit (Qiagen, Hilden, Germany), quantified with a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) and sent to Eurofins Genomics (Ebersberg, Germany) for sequencing. The obtained DNA sequences were analyzed and consensus sequences were generated using BioEdit v. 7.2.5 (Hall 1999).

Figure 1. Symptoms on lupin tissue associated with *Collletotrichum lupini*. **A**, typical stem twisting (*Lupinus mutabilis*); **B**, on the leaves (*L. albus*); **C**, on the main stem (*L. albus*); **D** - **F**, on the pods (*L. albus*). Pictures by Alkemade JA.

Table 1. Isolation details and GenBank accessions of *Colletotrichum* strains used in this study.

Strain[a]	Alternative code(s)	Species	Host	Origin	Year	GenBank no.[b]			
						ITS	GAPDH	TUB2	APN/MAT1
JA01		*Colletotrichum lupini*	*Lupinus albus*	Switzerland, Melikon	2018	MT741840	**MW342515**	**MW342537**	**MW342559**
JA02		*C. lupini*	*L. albus*	Switzerland, Feldbach	2019	**MW342494**	**MW342516**	**MW342538**	**MW342560**
JA03		*C. lupini*	*L. albus*	Germany, Hattenhofen	2019	**MW342495**	**MW342517**	**MW342539**	**MW342561**
JA04		*C. lupini*	*L. albus*	Germany, Witzenhausen	2018	**MW342496**	**MW342518**	**MW342540**	**MW342562**
JA05		*C. lupini*	*L. albus*	Germany, Westerau	2018	**MW342497**	**MW342519**	**MW342541**	**MW342563**
JA06		*C. lupini*	*L. albus*	Russia, Saint Petersburg	2018	**MW342498**	**MW342520**	**MW342542**	**MW342564**
JA07	BRIP 63850, WAC 12994	*C. lupini*	*L. angustifolius*	Australia, WA, Dongara	2004	**MW342499**	**MW342521**	**MW342543**	**MW342565**
JA08	BRIP 63851, WAC 12995	*C. lupini*	*L. luteus*	Australia, WA, Mingenew	2004	**MW342500**	**MW342522**	**MW342544**	**MW342566**
JA09	BRIP 63857, WAC 13001	*C. lupini*	*L. albus*	Australia, WA, Yandanooka	2004	**MW342501**	**MW342523**	**MW342545**	**MW342567**
JA10	CMW 9930, SHK 788	*C. lupini*	*L. albus*	South Africa, Bethlehem	1994	**MW342502**	**MW342524**	**MW342546**	**MW342568**
JA11	CMW 9931, SHK 1033	*C. lupini*	*L. albus*	South Africa, Stellenbosch	1995	**MW342503**	**MW342525**	**MW342547**	**MW342569**
JA12	CMW 9933, SHK 2148	*C. lupini*	*L. albus*	South Africa, Malmesbury	1999	**MW342504**	**MW342526**	**MW342548**	**MW342570**
JA13		*C. lupini*	*L. mutabilis*	USA, Florida, Martin County	2013	**MW342505**	**MW342527**	**MW342549**	**MW342571**
JA14		*C. lupini*	*L. hartwegii*	USA, Florida, Martin County	2013	**MW342506**	**MW342528**	**MW342550**	**MW342572**
JA15	A-02	*C. lupini*	*L. albus*	Chile, Cajón	2009	**MW342507**	**MW342529**	**MW342551**	**MW342573**
JA16	A-10	*C. lupini*	*L. angustifolius*	Chile, Cajón	2009	**MW342508**	**MW342530**	**MW342552**	**MW342574**
JA17	A-24	*C. lupini*	*L. albus*	Chile, Temuco	2015	**MW342509**	**MW342531**	**MW342553**	**MW342575**
JA18	Lup1	*C. lupini*	*L. mutabilis*	Ecuador, Juan Montalvo	2007	**MW342510**	**MW342532**	**MW342554**	**MW342576**
JA19	Lup18	*C. lupini*	*L. mutabilis*	Ecuador, Pujili	2007	**MW342511**	**MW342533**	**MW342555**	**MW342577**
JA20		*C. lupini*	*L. mutabilis*	Peru, Carhuaz	2019	**MW342512**	**MW342534**	**MW342556**	**MW342578**
JA21		*C. lupini*	*L. mutabilis*	Peru, Carhuaz	2019	**MW342513**	**MW342535**	**MW342557**	**MW342579**
JA22		*C. lupini*	*L. mutabilis*	Peru, Carhuaz	2019	**MW342514**	**MW342536**	**MW342558**	**MW342580**
CBS 109216	BBA 63879	*C. lupini*	*L. mutabilis*	Bolivia		JQ948156	JQ948486	JQ949807	**MW342581**
CBS 109221	BBA 70352, RB172	*C. lupini*	*L. albus*	Germany		JQ948169	JQ948499	JQ949820	MK478328
CBS 109225	BBA 70884	*C. Lupini*	*L. albus*	Ukraine		JQ948155	JQ948485	JQ949806	MK478329
CBS 109226	RB121, IMI 504884, HY09, BBA 71249	*C. lupini*	*L. albus*	Canada, Nova Scotia		JQ948158	JQ948488	MK478189	MK478316
CBS 509.97	RB235, LARS 178	*C. lupini*	*L. albus*	France		JQ948159	JQ948489	JQ949810	MK478355
IMI 375715	96A4	*C. lupini*	*L. albus*	Australia, WA, Perth	1997	JQ948161	JQ948491	JQ949812	MK478341
RB020	PT30	*C. lupini*	*L. albus*	Portugal, Azores	1999	MK463722	KM252117	MK478186	MK478308

RB042	CBS 129944, CMG12	*C. lupini*	*Cinnamonium zeylanicum*	Portugal, Lisbon	1996	MH865693	JQ948508	JQ949829	MK478310
RB116	CSL 1294	*C. lupini*	*L. polyphyllus*	UK, York		MK463723	KM252194	KM251944	MK478313
RB122	BBA 71310, C3	*C. lupini*	*L. luteus*	Poland		MK463726	MK463750	MK478190	MK478317
RB123	IMI 504885, SHK788	*C. lupini*	*L. albus*	South Africa, Bethlehem	1994	MK463727	MK463751	MK478191	MK478318
RB124	BBA 70555	*C. lupini*	*L. albus*	Chile		MK463728	MK463752	MK478192	MK478319
RB125	CBS 109224, BBA 70399	*C. lupini*	*L. albus*	Austria		JQ948172	JQ948502	JQ949823	MK478320
RB127	PT702	*C. lupini*	*Olea europaea*	Spain		MK463729	MK463753	MK478193	MK478321
RB147	IMI 350308	*C. lupini*	*Lupinus* sp.	UK, Kent	1991	MK463730	KM252203	KM251951	MK478322
RB221	IMI 504893	*C. lupini*	*Lupinus* sp.	France, Brittany	2016	MK463733	MK463756	MK478196	MK478345
RB226		*C. lupini*	*Lupinus* sp.	France, Brittany	2016	MK463738	MK463761	MK478201	MK478350
CBS 129814	T.A6	*C. tamarilloi*	*Solanum betaceum*	Colombia, Gundinamarca	2012	JQ948184	JQ948514	JQ949835	**MW342584**
CBS 129955	RB018, Tom-12	*C. tamarilloi*	*Solanum betaceum*	Colombia, Antioquia, Santa Rosa	1998	JQ948189	JQ948519	JQ949840	MK478307
CBS 211.78	IMI 309622, RB184	*C. costaricensis*	*Coffea* sp.	Costa Rica, Turrialba		JQ948181	JQ948511	JQ949832	MK478333
CBS 134730	RB237	*C. melonis*	*Malus domestica*	Brazil, Rio Grande do Brazil		KC204997	KC205031	KC205065	MK478357
IMI 304802	RB216	*C. cuscutae*	*Cuscuta* sp.	Dominica		JQ948195	JQ948525	JQ949846	MK478340
IMI 384185	CPC 18937, RB218	*C. paranaense*	*Caryocar brasiliense*	Brazil		JQ948191	JQ948521	JQ949842	MK478342
CBS 130239	Frag NL-1	*C. nymphaeae*	*Fragaria x ananassa*	The Netherlands	2011	JQ948250	JQ948580	JQ949901	**MW342583**
IMI 360928	CPC 18926, RB163	*C. nymphaeae*	*Fragaria x ananassa*	Switzerland, Zürich	1993	JQ948243	JQ948573	JQ949894	MK478326
CBS 122122	BRIP28519, RB179	*C. simmondsii*	Carica papaya	Australia	1987	JQ948276	JQ948606	JQ949927	MK478332
CBS 369.73	NRCC 10081	*C. acutatum*	*L. angustifolius*	New Zealand, Kumeu	1968	JQ948350	JQ948681	JQ950001	**MW342582**
CBS 370.73	NRCC 10088, RB187	*C. acutatum*	*Pinus Aridata*	New Zealand, Tokoroa	1965	JQ948351	JQ948682	JQ950002	MK478335

[a] JA: Strains from the FiBL culture collection characterized in this study. RB: Personal collection of Riccardo Baroncelli described in Dubrulle et al. (2020a). CBS: collection of the Westerdijk Fungal Biodiversity Institute, Utrecht, The Netherlands; IMI: Culture collection of CABI Europe UK Centre, Egham, UK. Codes in bold were used for morphology analysis in this study.

[b] ITS: internal transcribed spacers 1 and 2 together with 5.8S nrDNA; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; TUB2: β-tubulin 2, APN/MAT1: Apn2-Mat1-2-1 intergenic. Accession numbers in bold are newly determined in this study

Phylogenetic analyses

Alignments for each of the four loci, including sequences obtained in this study and downloaded from GenBank (**Table 1**), were performed with ClustalW using MEGA X (Kumar et al. 2018). Obtained multiple alignments where manually corrected and trimmed to obtain comparable sequences. Best-fit substitution models were determined for each locus separately and for the concatenated multi-locus alignment (ITS, TUB2, GAPDH and APN/MAT1). Phylogenetic analyses of the multi-locus alignment were based on Maximum Likelihood (ML) and Bayesian Inference (BI). The ML analysis was performed using RAxML v. 8 (Stamatakis 2014), through the CIPRES science gateway portal (Miller et al. 2011), using default parameters and 1,000 bootstrap iterations. The BI analysis was performed with MrBayes v. 3.2.7 (Ronquist et al. 2012), using a Markov Chain Monte Carlo (MCMC) algorithm using four chains and starting from a random tree topology. Substitution models for each locus were included for each partition. The analysis ran for 500,000 generations with trees sampled every 1000 generations to reach average standard deviations of split frequencies below 0.01. The first 25% of saved trees were discarded at the 'burn-in' phase and the 50% consensus trees and posterior probabilities (PP) were determined from the remaining trees. Bootstrap support values (BS) from the ML analysis were plotted on the Bayesian phylogeny. Further phylogenetic analyses were performed with the unweighted pair group method with arithmetic mean (UPGMA) with 10,000 replicates in Mega X. All generated sequences were deposited in GenBank (**Table 1**) and alignments and trees in TreeBASE.

Virulence assays

Virulence tests were performed on white and Andean lupin with representative *C. lupini* strains (**Fig. 3**), *C. tamarilloi* strain CBS 129814 and *C. acutatum* strain CBS 369.73 through stem-wound inoculation as described by Alkemade et al. (2021a), which was shown to highly correspond to field performance in Switzerland (r = 0.95). Disease scores ranging from 1 (non-pathogenic), 2 (low virulence) to 9 (highly virulent) were taken 4, 7 and 10 days post inoculation (dpi) and the standardized area under the disease progress curve was calculated (sAUDPC; Alkemade et al. 2021a). All inoculations were performed in a growth chamber (25 ± 2°C, 16 h light and ~70% relative humidity) in a completely randomized block design with a minimum of six replicates per experiment.

Statistical analysis

Statistical analyses were performed with R 4.0.3 using the packages *lme4* (Bates et al. 2015), *lmerTest* (Kuznetsova et al. 2017) and *emmeans* (Lenth et al. 2019), following a mixed model with factors of interest (i.e. strain, lupin species, lupin accession) as fixed and replicated block nested in experiment as random factor. Datasets that did not follow assumptions of normality of residuals and homogeneity of variance were log10 transformed. Data are presented as estimated least-squares means using the aforementioned mixed model. A Tukey-HSD test ($p \leq 0.05$) was

applied for pairwise mean comparison of the different *Colletotrichum* strains within each lupin accession.

Results

Colletotrichum lupini comprises of six genetic groups supported by morphology

From the 50 sequenced isolates, 39 belonged to *C. lupini* (**Table 1**). A globally representative subset of 28 *C. lupini* isolates was characterized based on colony morphology (form, aerial mycelium, margin type and color of the reverse side) and 18 of those were further characterized for growth rate and conidial shape and size, revealing ten distinct morphotypes (A-J; **Fig. 2**, **Table 2** and **Fig. S1** and **S2**). Despite certain variability, all observed conidia shared features typical for *C. lupini* (hyaline, smooth-walled, aseptate, straight and with one acute end) as described by Damm et al. (2012). Morphotype A was the most common and was observed for isolates from across the world (Europe, Australia, North- and South America), all belonging to genetic group II. Morphotypes B, C and G were observed for isolates from South Africa and morphotypes D, E, G, I and J were observed for isolates from South America.

Multi-locus phylogenetic analyses of 50 *Colletotrichum* isolates identified six distinct genetic groups within *C. lupini* (I-VI; **Fig. 3** and **S3**). The combined sequence dataset contained 2,251 characters (ITS: 1-496, GAPDH: 497-745, TUB2: 746-1,200, APN/MAT1: 1,201-2,251) including alignment gaps. The APN/MAT1 locus showed the highest variability across the nucleotide data set, with 75.8% conserved sites for the whole data set (including out-groups) and 97.4% within *C. lupini* (**Table S1**). The TUB2 and GAPDH loci showed 89.9% and 81.1% identical sites for the entire dataset and 97.8% and 98.4% identity within *C. lupini*, respectively. The ITS region showed the lowest variability with 97% identical sites across the whole dataset and 99.2% within *C. lupini.* As shown in **Fig. 3**, most *C. lupini* strains clustered with a high bootstrap support (BS) value of 79 and posterior probability (PP) of 1 with reference strains representing genetic group II (CBS 109221, IMI 375715 and RB221). Strains within group II showed a high identity among each other (>99.9%) and showed morphotype A, except for Chilean strain JA15 showing morphotype D (**Fig. 2**). South African strain JA10 and Peruvian strain JA20, with morphotypes G and F, respectively, clustered together with a BS of 84 and PP of 1, forming a highly supported group (III). South African strains JA11 and JA12, with morphotypes C and B, respectively, clustered together with a BS of 98 and PP of 1, forming a highly supported group (IV). Ecuadorian strains JA18 and JA19 with distinct morphotypes I and J, respectively, showed 99.7% identity with reference strains of group II and clustered together with a BS of 60 (**Fig. 3** and **S3**) and a PP of 1 in (**Fig. 3**), forming a distinct group (V). The reference strains for group I (CBS 109225 with morphotype H, CBS 109226 and CBS 509.97) are clustered together with a BS of 99 and PP of 1 and show 100% identity with each other and 99.6% identity with reference strains of group II. South American strains JA21, JA22 and CBS 109216, with morphotype E, cluster together with a BS of 98 and PP of 1 (**Fig. 3**) and a BS of 54 (**Fig. S3**) forming a highly supported group (VI). JA21 and JA22 showed 99.8% and

CBS 109216 showed 99.7% identity with reference strains of group I and 99.4% and 99.2% identity with references strains of group II, respectively.

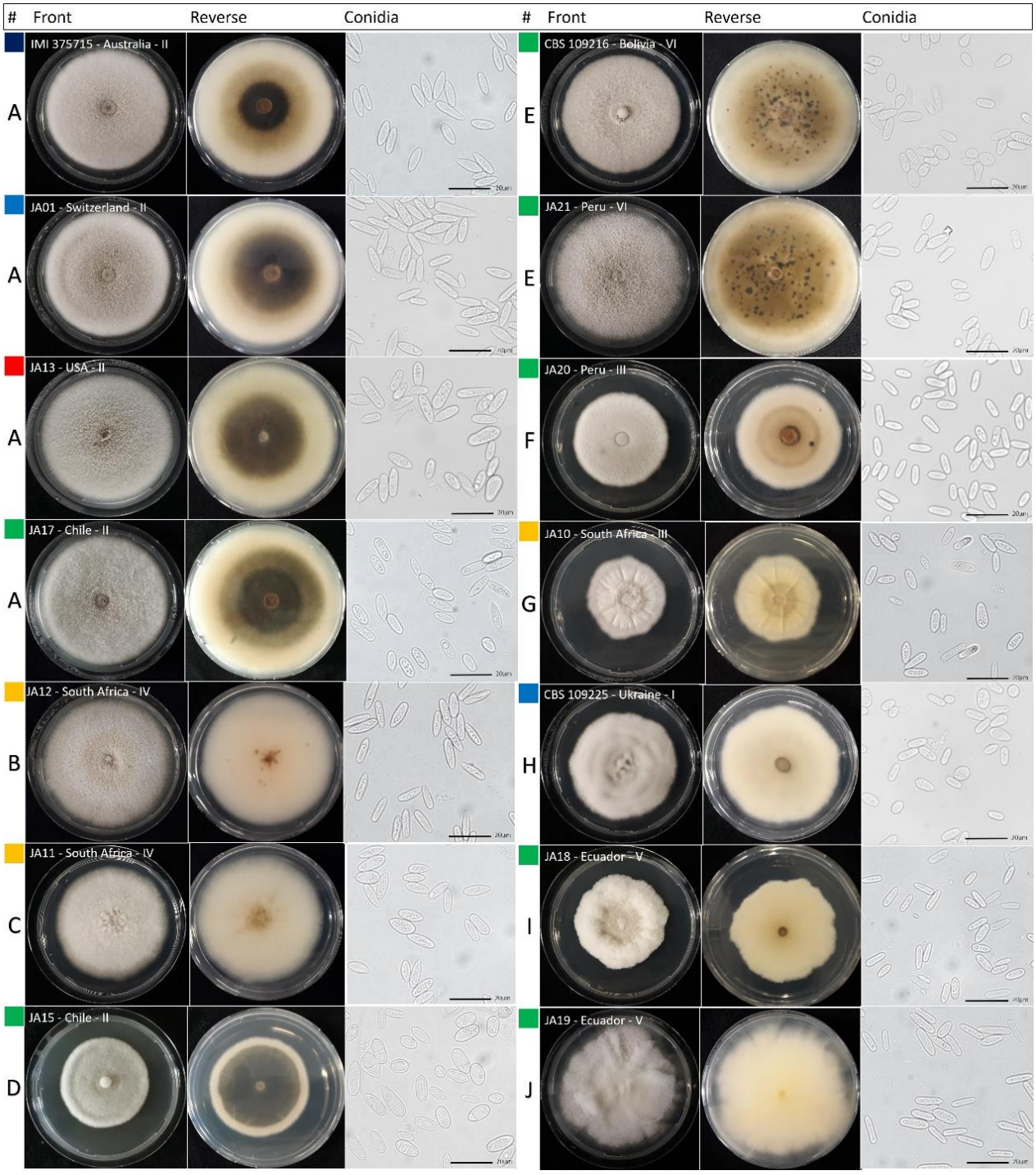

Figure 2. *Colletotrichum lupini* morphology. Capital letters (A-J) indicate the different morphology types based on conidia shape and size and colony growth rate and morphology (see **Table 2**). Strain codes are followed by country of origin and roman numbers (I-VI) indicate genetic groups. Plates show the front and reverse of 14 day old colonies on PDA. Scale bars indicate 20 µm. Colors indicate strain origin: blue = Europe, green = South America, red = North America, orange = Southern Africa, dark blue = Australia.

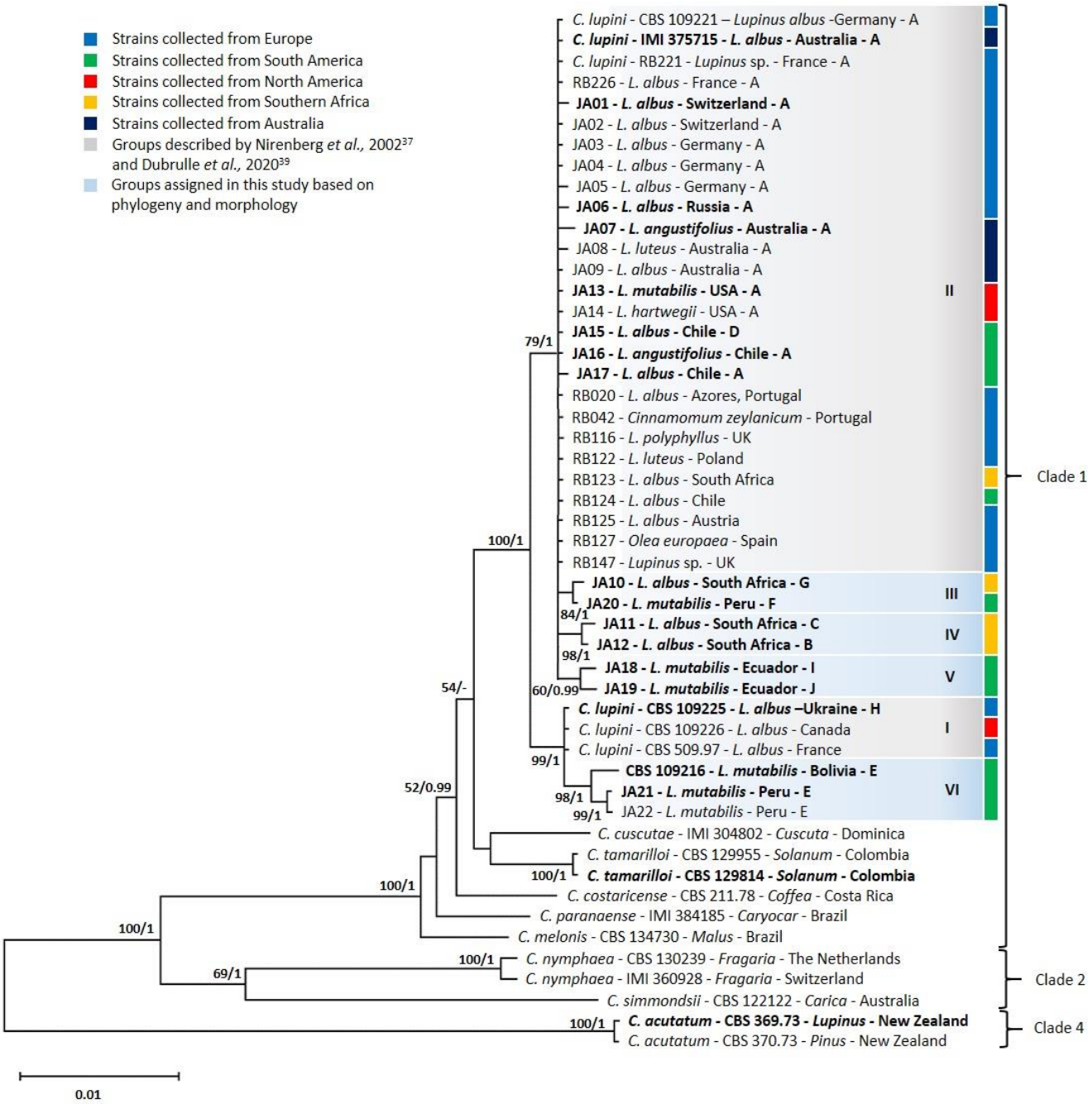

Figure 3. Multi-locus phylogeny of *Colletotrichum lupini*. Bayesian analysis tree inferred from the combined ITS, TUB2, GAPDH and APN/MAT1 sequence datasets of 50 *Colletotrichum* strains used in this study. Bootstrap support values (>50) and Bayesian posterior probabilities (>0.95) are given at each node. The tree is rooted to *C. acutatum* (CBS 369.73 and CBS 370.73). Strain codes are followed by host, country of origin and morphology (A-J). Grouping (I-VI) is based on phylogeny and morphology. Strains used for virulence assays are highlighted in bold. Clades indicate the different clades within the *C. acutatum* species complex.

Table 2. Growth rate, conidial size and shape, and colony morphology for the different morphotypes observed within *Colletotrichum lupini.*

Strain	Morpho type	Genetic group	Growth rate (mm/day)[a]	Conidia L x W (µm)[ab]	Conidia shape[cd]	Colony morphology[d]
IMI 375715, JA01, -06, -07, -13, -16, -17	A	II	6.2 ± 0.1	12 ± 2.1 x 4 ± 0.7	Cylindrical to elliptical, occasionally clavate	Flat, circular, with entire margins, white-greyish cottony aerial mycelium, pale to orange on reverse, dark in center
JA12	B	IV	5.6 ± 0.1	13.3 ± 1.4 x 3.4 ± 0.5	Cylindrical to elliptical, occasionally clavate	Flat, circular, with entire margins, white-brownish cottony aerial mycelium, pale on reverse
JA11	C	IV	5.5 ± 0.1	12 ± 1.7 x 4.5 ± 0.7	Cylindrical to elliptical, occasionally clavate	Flat, circular, slightly filiform margins, white-greyish cottony aerial mycelium, pale on reverse, orange in center
JA15	D	II	5 ± 0	9.7 ± 2.4 x 4.2 ± 1.0	Cylindrical, occasionally roundish	Flat, circular, with entire margins, white-greyish cottony aerial mycelium, dark on reverse, pale at margins
CBS 109216, JA21, -22	E	VI	5.4 ± 0.3	8.5 ± 2.1 x 3.5 ± 0.7	Cylindrical to clavate	Flat, circular, with entire margins, white-greyish cottony aerial mycelium, pale orange on reverse with black dots
JA20	F	III	4.2 ± 0.3	8.7 ± 1.1 x 3.2 ± 0.6	Cylindrical, occasionally clavate	Flat, circular, with entire margins , sparse white-greyish aerial mycelium, pale on reverse
JA10	G	III	4.9 ± 0.2	9.2 ± 1.7 x 3.6 ± 0.7	Cylindrical to elliptical, occasionally clavate	Irregular and radially sulcate with aerial mycelia growth in the center, pale on reverse
CBS 109225	H	I	5.2 ± 0.1	8.5 ± 1.7 x 3.8 ± 0.8	Cylindrical to clavate	Slightly irregular and thickly covered with wooly white-greyish aerial mycelia, pale on reverse
JA18	I	V	4.1 ± 0	10 ± 1.8 x 2.9 ± 0.7	Cylindrical	Irregular, wooly white areal mycelia on the margins, pale on reverse
JA19	J	V	6 ± 0.2	12.1 ± 1.8 x 2.4 ± 0.7	Cylindrical	Irregular, white-greyish wooly aerial mycelium, pale on reverse with occasional black/orange dots

[a] mean ± SD, see also **Fig. S2**.
[b] L = Length and W = Width.
[c] observed conidia were rather variable in shape and size, but all conidia were hyaline, smooth-walled, aseptate, straight, with one end round and one end acute as described for *Colletotrichum lupini* in Damm et al. (2012).
[d] see also **Fig. 2.**

Distinct virulence patterns on white and Andean lupin.

Virulence assays performed on two white lupin (*L. albus* L.) accessions (Feodora and Blu-25) and two Andean lupin (*L. mutabilis* Sweet.) accessions (LUP 17 and LUP 100) with strains representing the different morphotypes and genetic groups indicated in **Fig. 3**, revealed strong strain ($P < 0.001$), lupin species ($P < 0.001$) and strain x lupin species interaction effects ($P < 0.001$). A strong accession effect was found within white lupin ($P < 0.001$), whereas for Andean lupin there was no significant accession effect ($P = 0.43$). Strain ($P < 0.001$) and strain x accession ($P < 0.001$) interaction effects were found for both species. Strains belonging to genetic group II with morphotype A, caused severe disease on white lupin accession Feodora and both Andean lupin accessions (**Fig. S4**), showing standardized area under the disease progress curve (sAUDPC) means ranging from 3.95 to 5 (**Fig. 4**). On the more tolerant white lupin accession Blu-25, sAUDPC

means for strains of group II with morphology A were more variable, with JA01 and IMI 375715 showing moderate (2.7 - 2.9) and Chilean strains JA16 and 17 showing high (3.8 - 4.1) virulence. Chilean strain JA15, also belonging to genetic group II but with a different morphology (D), caused low disease on LUP 100 and Blu-25 (1.9), showing a different virulence spectrum compared to the other tested strains of genetic group II. South African strains JA11 and JA12, belonging to genetic group IV with morphotypes C and B, respectively, showed a similar virulence spectrum on white lupin as strains of group II. JA10 and JA20, representing group III and morphotype G and F, respectively, were overall avirulent (<2), with the exception of JA10 on Feodora, showing moderate virulence (2.95). Peruvian strain JA21, representing genetic group VI and morphotype E, caused low disease on white lupin (1.4 - 1.8), but severe disease on Andean lupin (4.25 - 5). A similar observation was found for the two Ecuadorian strains JA18 and JA19 of genetic group V and morphotypes I and J, respectively. These two strains caused low disease on white lupin and high disease on Andean lupin LUP 100. On Andean lupin LUP 17, however, a severe disease phenotype was only found for JA18 (3.6), whereas JA19 barely caused any disease symptoms (1.25). Similar to the observations for JA19, the Ukrainian strain CBS 109225 (genetic group I, morphotype H) caused severe disease on Andean lupin LUP 100 (3.36) and low disease on Andean lupin LUP 17 and white lupin (1.2 - 2). The *C. tamarilloi* and *C. acutatum* strains were avirulent across the lupin accessions (<1.26).

Discussion

This study compared 39 *C. lupini* and 11 *Colletotrichum* spp. isolates collected from across the world to explore intraspecific diversity of *C. lupini* and to better understand the dynamics of the current lupin anthracnose pandemic and potential implications of further migrations of distinct pathogenic strains. Based on multi-locus phylogeny supported by isolate morphology, we identified four distinct genetic groups additional to previously described genetic groups I and II. Highest intraspecific diversity was identified among *C. lupini* isolates collected from across the South American Andes region. This is in line with reports of Falconí et al. (2013) and Riegel et al. (2010) showing high diversity in Ecuador and Chile, respectively. In those regions, Andean lupin has been cultivated for more than 2,000 years (Atchison et al. 2016), growing alongside numerous wild lupin species (Nevado et al. 2016). Isolates collected in South Africa showed a distinct morphology and virulence spectrum, indicating higher diversity than previously shown (Lotter and Berger 2005). Although lupins form a significant part of the local agriculture and have been researched there since at least 1897 (Van Der Mey 1996), they are not native to South Africa and lupin anthracnose was not reported in South Africa until 1993 (Ghebremariam et al. 2002). Taking into account the relatively recent reports of anthracnose in South Africa, the low diversity in Europe and Australia and the center of origin for species within clade 1 of the *C. acutatum* species complex being in South America (Baroncelli et al. 2017; Bragança et al. 2016), we consider the South American Andes to be the center of origin of *C. lupini*.

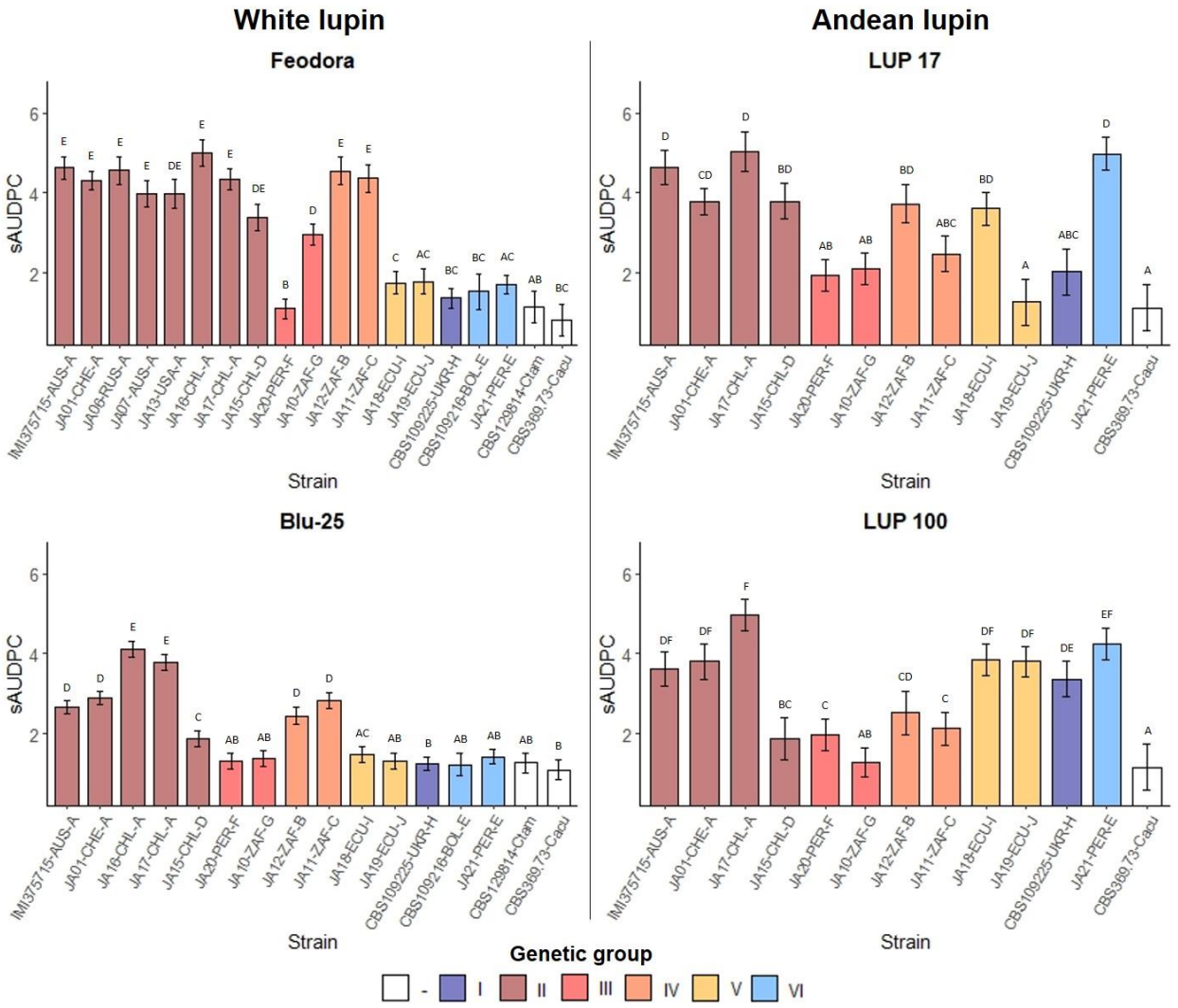

Figure 4. Virulence of *Colletotrichum lupini* strains on white (*Lupinus albus)* and Andean lupin (*L. mutabilis*). Anthracnose severity is expressed in standardized area under the disease progress curve (sAUDPC) and estimated means are shown. Strain codes are followed by abbreviated country of origin and morphotype (A-J). Different capital letters above bars indicate significant differences between strains (Tuckey-HSD, $P < 0.05$). Error bars indicate the standard error of the estimated

The majority of the *C. lupini* isolates (26 out of 39) belong to the highly virulent genetic group II, showing morphotype A, and were collected in Europe, Australia, South Africa, the USA and Chile. This result confirms previous reports classifying most *C. lupini* strains from across the world in the same genetic group (Damm et al. 2012; Dubrulle et al. 2020a; Elmer et al. 2001; Talhinhas et al. 2002; Yang and Sweetingham 1998). The low genetic diversity among strains of group II, the uniform morphology and non-observed sexual morph (Damm et al. 2012), indicates clonality as suggested by Talhinhas et al. (2016). Pathogenicity of group II strains has also been shown on blue (Yang et al. 2004), yellow (Adhikari et al. 2011) and various other lupin species across the world (Talhinhas et al. 2016), indicating a broad host range within the genus *Lupinus*. Reports from South Korea and China indicate that group II strains also cause disease in those regions (Han

et al. 2014; Zou et al. 2019), highlighting that these strains are globally widespread and are the cause of the current anthracnose pandemic in lupin. The group II strain RB221 can be used as reference, as it is now fully sequenced (Baroncelli et al. 2021) and tested on both Andean and white lupin (Guilengue et al. 2020).

The stem-wound inoculation assay used in this study was previously described to be highly reproducible and strongly correlated to field performance under natural infection pressure (Alkemade et al. 2021a). In the present study, virulence assays based on stem-wounding showed strong strain x accession interaction effects for white and Andean lupin, suggesting a strain-dependent host spectrum and the existence of different physiological races within *C. lupini*. Similar observations were described by Falconí et al. (2013), showing a *C. lupini* strain x Andean lupin accession interaction effect. The existence of physiological races has been observed for various *Colletotrichum* species, such as for *C. lindemuthianum* on common bean (Falleiros et al. 2018), *C. sublineola* on sorghum (Xavier et al. 2018) and *C. truncatum* on lentil (Armstrong-Cho et al. 2012), but, in general, this is not common within the genus *Colletotrichum*. The similar virulence levels of isolates belonging to group II observed on Andean and white lupin accessions are in line with Alkemade et al. (2021a), in which equal virulence was observed for IMI 375715 (Australia) and JA01 (Switzerland) when inoculated on six different white lupin accessions. However, an exception within group II is Chilean strain JA15, which, besides having a distinct morphology, was less virulent on Andean lupin LUP 100 and white lupin Blu-25. Further, Chilean strains JA16 and JA17 (also group II) overcame resistance of the resistant advanced breeding line Blu-25, which has been specifically bred for anthracnose resistance in Chile and was shown resistant under Swiss field conditions (Alkemade et al. 2021a). These results indicate that new introductions of highly virulent foreign strains can have severe consequences as seen for many other crops (Godfray et al. 2016; Lidwell-Durnin and Lapthorn 2020; Ordonez et al. 2015), and it should be investigated if this high virulence is also affecting other resistant (white) lupin material (Adhikari et al. 2009; Alkemade et al. 2021a; Jacob et al. 2017). Although disease development after stem-wounding of seedlings correlated strongly to field disease scores of mature plants (Alkemade et al. 2021a), we cannot exclude the possibility that conclusions drawn on virulence level might differ for secondary infection processes (e.g. via rain splash).

This study provides first solid evidence that, based on multi-locus phylogeny and morphology, genetic diversity within *C. lupini* is higher than previously shown. High-resolution genome-wide sequencing and an increased sampling density from especially the South American Andes region are now necessary to increase genetic resolution and to better understand *C. lupini* phylogeny and phylogeography. This could provide the basis for in-depth comparative genomic studies to identify effector gene clusters within the *C. lupini* genome. This study confirms that the current lupin anthracnose pandemic is caused by a genetically uniform group of highly virulent strains. The identification of strains with an increased virulence on tolerant white lupin breeding material and the observation of strain-specific virulence patterns should be taken into account in lupin resistance breeding programs. Due to its seed-borne nature, caution should be taken when

importing seeds, especially from South America, to prevent further introductions of potentially virulent strains across the world.

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Author contribution

JAA, PH and MMM conceived the original idea for this study. JAA conducted the experiments and took the lead in manuscript writing. JAA analyzed the data with contributions from PH and MMM. All authors significantly contributed to data interpretation and provided critical feedback that shaped the final version. JAA designed the figures and tables with input from PH, MMM, RTV and MRF.

Data availability

The datasets generated during the current study are available at: https://doi.org/10.5281/zenodo.5235052. Acuired sequences are openly available in Genbank at https://www.ncbi.nlm.nih.gov/genbank/ (for reference numbers see Table 1), and sequence alignments and phylogenetic trees are available in Treebase at: http://purl.org/phylo/treebase/phylows/study/TB2:S27356?x-access-code=260136f8e6416a0614b93528ddbfe0ef&format=html.

Supplementary

Table S1: Identical sites (%) across the nucleotide dataset per locus. CaSC indicates variability within *Colletotrichum acutatum* species complex. *C. lupini* indicates variability within *C. lupini*.

Locus	CaSC	*C. lupini*
ITS	97	99.2
GAPDH	81.1	98.4
TUB2	89.9	97.8
APN/MAT1	75.8	97.4

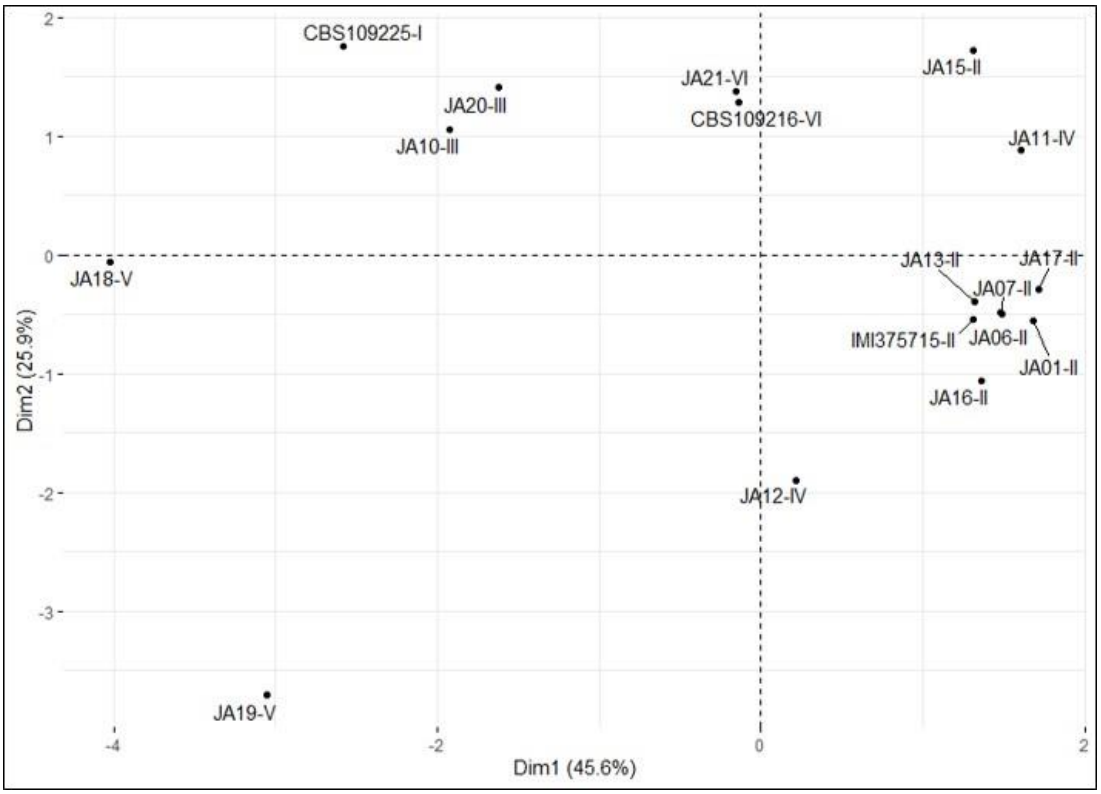

Figure S1. Principal component analysis (PCA) on strain morphology. Based on conidial length, width and length width ratio, growth rate on PDA, colony form (circular = 1, most irregular = 4), aerial mycelia (no aerial mycelia = 1, most aerial mycelia = 4), color (palest = 1, darkest = 4) and filiform (yes = 1, no = 0) of 17 *Colletotrichum lupini* strains. Strain codes are followed by genetic group.

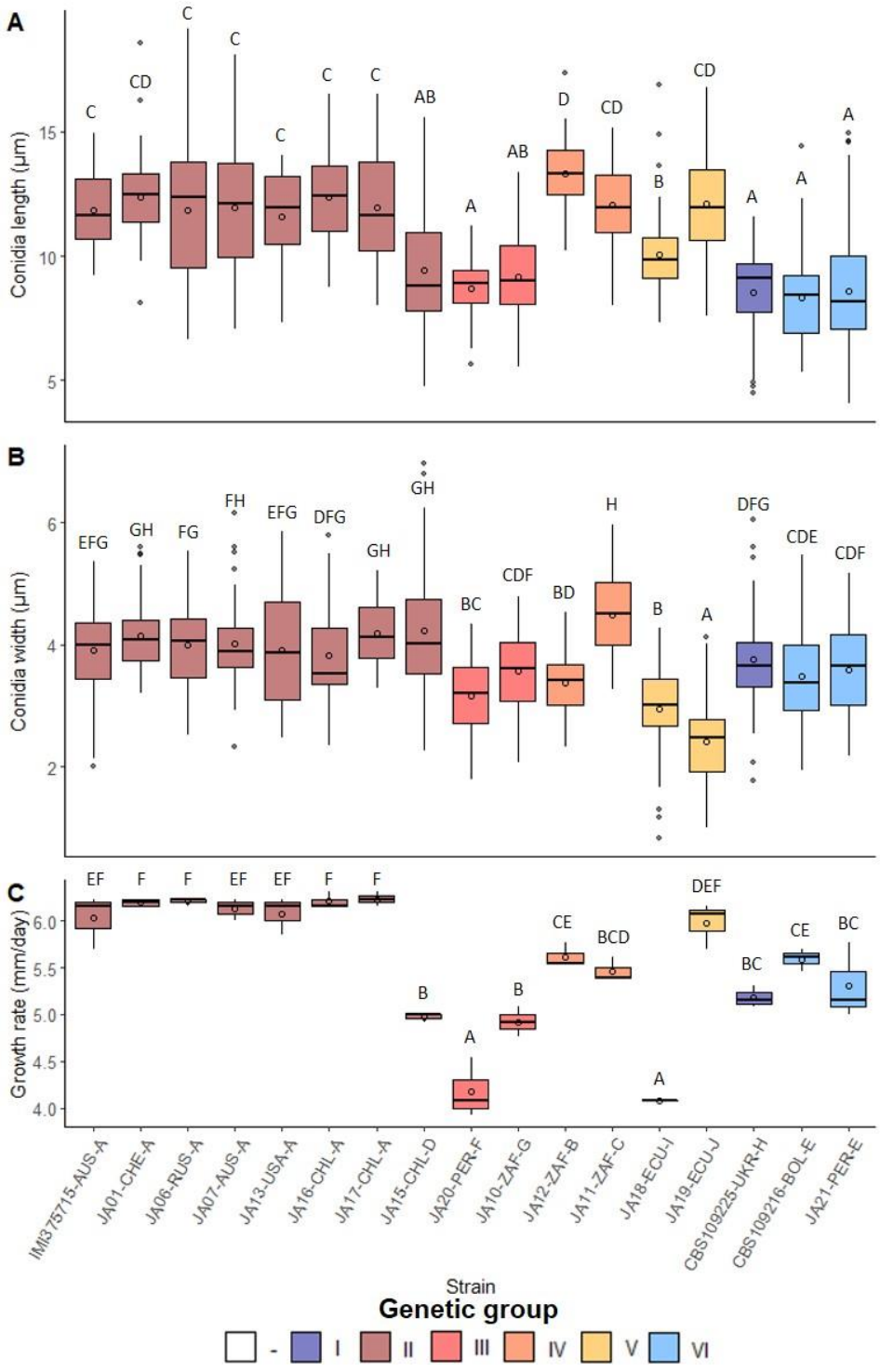

Figure S2. Boxplots of conidia length (A), width (B) and colony growth rate (C). Circles within boxplot indicate mean. Strain codes are followed by abbreviated country of origin and capital letters (A-J) indicating strain morphotype. Capital letters above boxplots indicate significant differences between strains (Tuckey-HSD, $P < 0.05$).

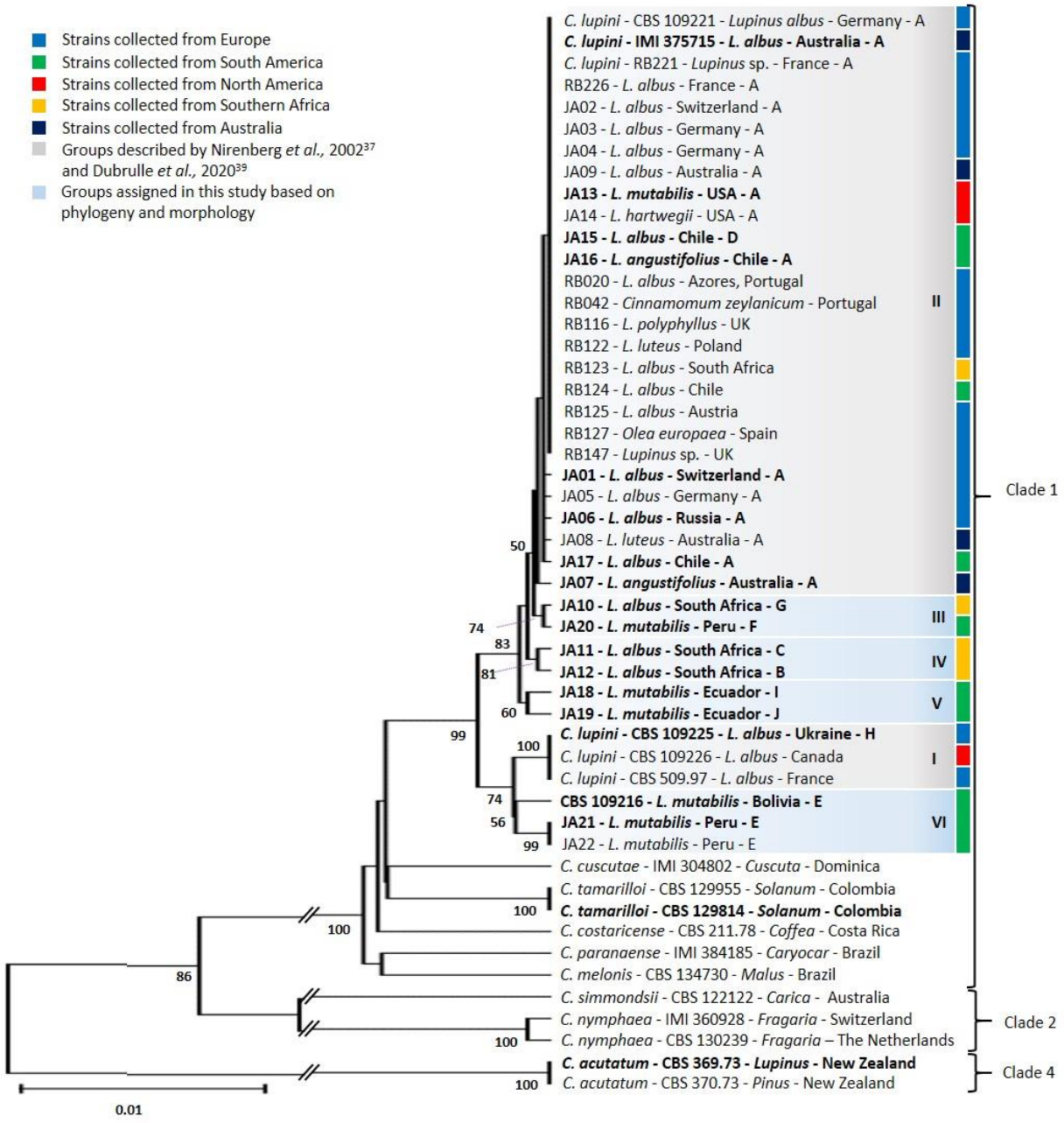

Figure S3. Multi-locus phylogeny of *Colletotrichum lupini*. UPGMA tree inferred from the combined ITS, TUB2, GAPDH and APN/MAT1 sequence datasets of 50 *Colletotrichum* strains used in this study. Bootstrap support values (>50%) are given at each node. The tree is rooted to *C. acutatum* (CBS 369.73 and CBS 370.73). Strain codes are followed by host, country of origin and morphology (A-J). Grouping is based phylogeny and morphology. Strains used for virulence assays are highlighted in bold.

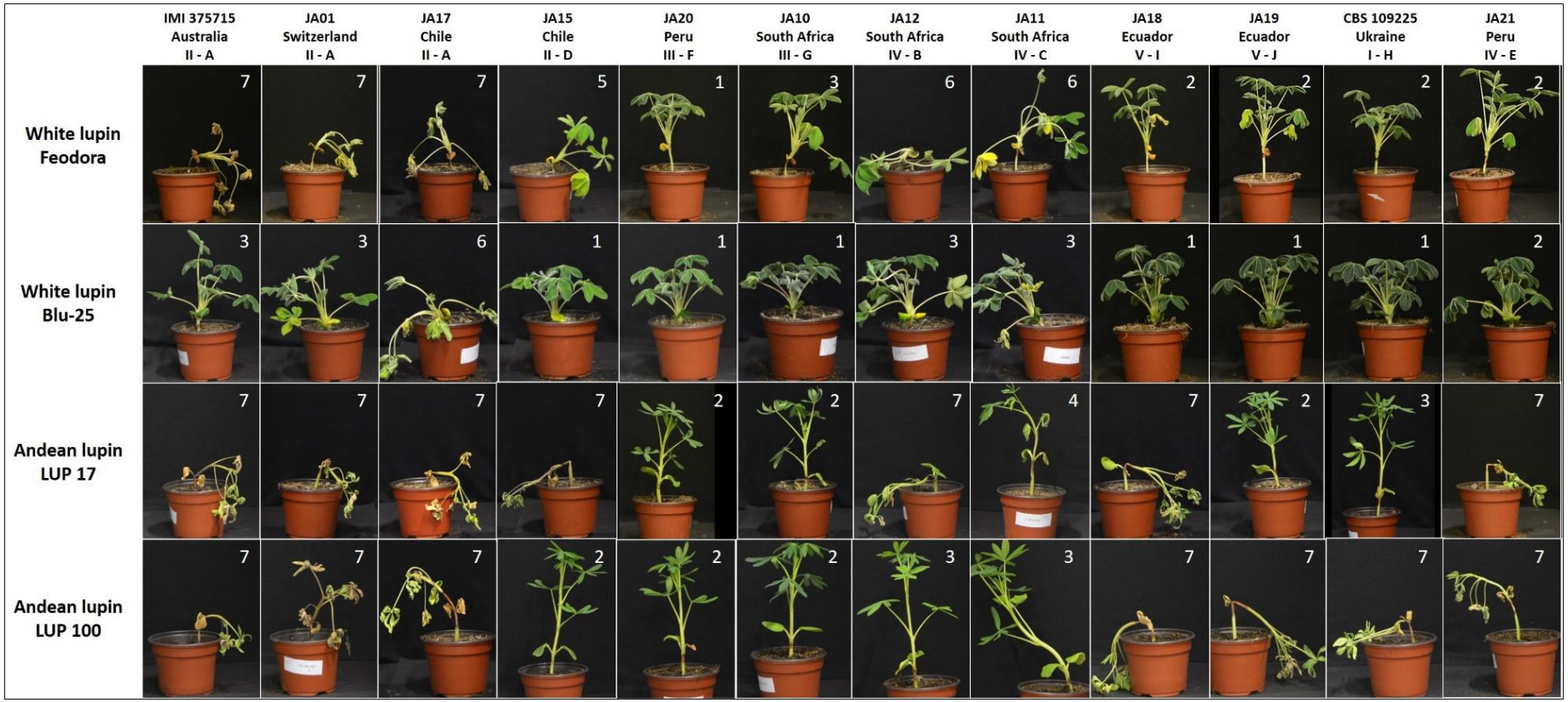

Figure S4. Representative plants showing disease symptoms of *Colletotrichum lupini* strains on white (*Lupinus albus)* and Andean lupin (*L. mutabilis*). Lupin seedlings of the different species and accessions (Feodora, Blu-25, LUP 17, LUP 100) were stem inoculated with different *C. lupini* strains 14 days after sowing. Photos and disease score indices (DSI; top right corners) were taken 10 days post-inoculation. Strain codes are followed by country of origin, genetic group and morphotype.

Chapter 3

A high-throughput phenotyping tool to identify field-relevant anthracnose resistance in white lupin

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Abstract

The seed- and air-borne pathogen *Colletotrichum lupini*, the causal agent of lupin anthracnose, is the most important disease in white lupin (*Lupinus albus*) worldwide and can cause total yield loss. The aims of this study were to establish a reliable high-throughput phenotyping tool to identify anthracnose resistance in white lupin germplasm and to evaluate a genomic prediction model, accounting previously reported resistance QTLs, on a set of independent lupin genotypes. Phenotyping under controlled conditions, performing stem inoculation on seedlings, showed to be applicable for high-throughput and its disease score strongly correlated with field plot disease assessments (r = 0.95, P < 0.0001) and yield (r = -0.64, P = 0.035). Traditional 1-row field disease phenotyping showed no significant correlation with field plot disease assessments (r = 0.31, P = 0.34) and yield (r = -0.45, P = 0.17). Genomically-predicted resistance values showed no correlation with values observed under controlled or field conditions, and the parental lines of the RIL population used for constructing the prediction model exhibited a resistance pattern opposite to that displayed in the original (Australian) environment used for model construction. Differing environmental conditions, inoculation procedures or population structure may account for this result. Phenotyping a diverse set of 40 white lupin accessions under controlled conditions revealed eight accessions with improved resistance to anthracnose. The standardized area under the disease progress curves (sAUDPC) ranged from 2.1 to 2.8 compared to the susceptible reference accession with a sAUDPC of 3.85. These accessions can be incorporated into white lupin breeding programs. In conclusion, our data supports stem inoculation-based disease phenotyping under controlled conditions as a time-effective approach to identify field-relevant resistance which can now be applied to further identify sources of resistance and their underlying genetics.

Keywords

Lupinus albus, *Colletotrichum lupini*, wound inoculation, field resistance, breeding, genomic prediction

Introduction

The current demand for plant-based protein is high and is expected to increase even further with the growing world population (Henchion et al. 2017). Most of the global demand is met by soybean produced on a large-scale in the USA and Brazil (Pagano and Miransari 2016). As lupins have a similar protein content and better digestibility (Monteiro et al. 2014), they offer great potential to complement soybean as an alternative protein source (Lucas et al. 2015). The genus *Lupinus*, belonging to the family Fabaceae, consists of about 170 species worldwide, with blue (*Lupinus angustifolius* L.), Andean (*L. mutabilis*), yellow (*L. luteus* L.) and white lupin (*L. albus* L.) being of agricultural importance (Gresta et al. 2017). Their unique symbiosis with *Bradyrhizobium* sp. (*lupini*) makes lupins highly efficient atmospheric nitrogen fixers (Fernández-Pascual et al. 2007; Peix et al. 2015). Additionally, white lupin is capable of forming specialized cluster roots which release carboxylates to mobilize poorly available phosphorus sources (Gallardo et al. 2019; Lambers et al. 2013). White lupin is recognized for its high protein content (36 to 38%), starch-free white seeds, outstanding fatty acid quality and additional health benefits, making its grains ideal for the food and feed industry (Arnoldi et al. 2015; Boschin et al. 2008; Monteiro et al. 2014).

Despite its potential for low-input agriculture as highlighted above, the cultivation of white lupin has been severely limited by anthracnose disease caused by the seed- and air-borne ascomycete *Colletotrichum lupini*, which is a member of the *Colletotrichum acutatum* species complex (Damm et al. 2012; Nirenberg et al. 2002). Contrary to the broad host range seen for most members of the *C. acutatum* species complex, *C. lupini* is host-specific to members of the *Lupinus* genus (Talhinhas et al. 2016). Infected seeds are the primary source of inoculum and small amounts of infected seeds (0.1%) can cause severe yield loss (Thomas and Sweetingham 2004). Little is known about the disease cycle and interaction between *C. lupini* and its host but gene expression and protein synthesis during white lupin infection shared similarities to those of hemibiotrophic pathogens (Dubrulle et al. 2020b), which is the most common lifestyle within the *C. acutatum* species complex (De Silva et al. 2017; Peres et al. 2005). At the start of the growing season, C. lupini is believed to colonize the host endophytically, causing no to minor disease symptoms. Upon flowering, disease incidence rapidly increases, causing the typical disease symptoms of stem elongation and pod twisting, followed by the formation of necrotic lesions containing orange masses of conidia which are rain-splash dispersed within the crop (Thomas and Sweetingham 2004; White et al. 2008). At first, the disease occurred predominantly in humid areas but it has quickly spread around the globe, making anthracnose the most notorious disease for lupin cultivation worldwide (Talhinhas et al. 2016). The current disease epidemic in Europe started in the 1970's, coinciding with a strong decline in lupin cultivated area (FAOSTAT 2017). Currently, disease management of white lupin is focused on planting pathogen-free seed and chemical control (Thomas et al. 2008a; White et al. 2008).

Breeding efforts for anthracnose resistance mainly took place under field conditions in New Zealand, Australia, Chile and Germany (Adhikari et al. 2009; Cowling et al. 1999; Jacob et al. 2017;

von Baer et al. 2009). These efforts have led to the discovery of resistance in the Ethiopian landraces P27174 and P27175 (Adhikari et al. 2009; Cowling et al. 1999), which form a distinct genetic group within white lupin (Raman et al. 2014). Analysis of a recombinant inbred line (RIL) population of P27174 and the susceptible cultivar Kiev Mutant revealed that the resistance was under polygenic control (Yang et al. 2010), which is also the case for yellow lupin (Adhikari et al. 2011), and unlike blue lupin, where single dominant genes, *Lanr1*, *AnMan* or *LanrBo*, control anthracnose resistance (Fischer et al. 2015; Yang et al. 2004; Yang et al. 2008). Quantitative trait locus (QTL) mapping of the RIL population between P27174 and Kiev Mutant revealed three major anthracnose resistance QTLs, antr04/05_1, antr04/05_2 and antr05_3, on linkage groups ALB02 (LG4), ALB04 (LG17) and ALB10 (LG2), respectively, and jointly explained up to 49% of the phenotyping variation observed under field conditions in 2004 and 2005 in Western Australia (Phan et al. 2007; Yang et al. 2010). The same QTLs, were recently identified with greater precision by Książkiewicz et al. (2017) using a large number of SNP markers identified from genotyping-by-sequencing (GBS) (Elshire et al. 2011). Despite the high synteny between blue and white lupin, QTLs conferring anthracnose resistance in white lupin did not correspond to those found in the latter species (Książkiewicz et al. 2017). The high number of markers made available by GBS not only facilitated QTL mapping but also opened the way for genomic selection, by which a statistical model is constructed from phenotypic and genotypic data of a representative population to predict breeding values for a target (polygenic) trait of inbred lines or genetic resources (Heffner et al. 2009). A genomic prediction model, proposed to aid in marker assisted selection based on the RIL population used to identify the major QTLs, showed a predictive ability of 0.56 for anthracnose resistance and also took into account variation not explained by the observed QTLs (Rychel-Bielska et al. 2020).

Disease phenotyping under field conditions is subject to unpredictable environmental conditions. Therefore, reliable screening tools for anthracnose resistance are essential to further exploit genetic diversity within white lupin. This study describes a reproducible and time-effective phenotyping tool for anthracnose resistance breeding, performing stem inoculation on seedlings under controlled conditions. For this, a diverse collection of white lupin landraces that was shown to include outstanding material for key traits such as grain yield in different climatic zones (Annicchiarico et al. 2010) and drought tolerance (Annicchiarico et al. 2018), which likely holds new sources of anthracnose resistance, was used. The objectives of this study were to (i) compare the developed high-throughput phenotyping tool and traditional 1-row field assessments with field plot assessments obtained over two years at two sites, (ii) identify anthracnose resistance in a collection of 40 white lupin accessions and (iii) evaluate the consistency of resistance values predicted according to the Rychel-Bielska et al. (2020) genomic prediction model with resistance values observed under controlled and field conditions for an unrelated germplasm set.

Materials and Methods

Colletotrichum lupini strain characterization

C. lupini strain JA01, used as inoculum in this study, was isolated from pod tissue of an infected white lupin plant in 2018 in Mellikon, Switzerland (47°34'05.7"N 8°21'18.7"E). Mycelium from a pure JA01 culture, grown for 10 days on PDA (potato dextrose agar, Carl Roth®, Karlsruhe, Germany) at 22°C in the dark, was harvested with a sterile spreader after flooding the Petri dish with 2 ml sterile ddH_20. Genomic DNA was isolated with a cetyltrimethylammonium bromide (CTAB) extraction protocol as described by Minas et al. (2011). The 5.8S nuclear ribosomal gene with the two flanking internal transcribed spacers (ITS) was amplified by performing polymerase chain reaction (PCR) in a S1000 Thermal Cycler (Bio-Rad, CA, USA) using the ITS5 "GGAAGTAAAAGTCGTAACAAG" and ITS4 "TCCTCCGCTTATTGATATGC" (White et al. 1990) primers as described by Dubrulle et al. (2020a). PCR products were purified using the QIAquick® PCR Purification Kit (Qiagen, Hilden, Germany) and sent for sequencing to Eurofins Genomics (Ebersberg, Germany). The consensus sequence was assembled using BioEdit v. 7.2.5 (Hall 1999) and identified as *C. lupini* through BLAST analysis and deposited at GenBank under accession number MT741840. Australian *C. lupini* strain IMI 375715 (genetic group II) (Dubrulle et al. 2020a) was used in one experiment to compare with strain JA01 for virulence on white lupin.

Genomic prediction

The genomic prediction model was based on results published by Rychel-Bielska et al. (2020). The germplasm used for genomic prediction modelling included 191 F_8 individuals issued from the RIL population of Kiev Mutant x P27174. Phenotypic data was collected in Perth (Western Australia) from two experiments in 2004 and 2005 (three replicates), under field conditions in a 1-row setup, using spray-inoculated (*C. lupini* strain IMI 375715) disease spreader rows (Adhikari et al. 2009; Yang et al. 2010). The same material underwent genotyping-by-sequencing (GBS) characterization according to Elshire et al. (2011) method as described in Książkiewicz et al. (2017). A genomic prediction model was set up on the basis of intra-population predictions estimated via cross-validations with 10% validation fraction, averaged across 20 repetitions outputted by two possible models, rrBLUP and Bayesian Lasso, using the R package GROAN (Nazzicari and Biscarini 2017). The best-predictive model, as correlation between predicted and observed values based on cross-validation for the RIL population, was applied to an independent set of 384 landrace accessions originating from across the Mediterranean, East Africa, Madeira and the Canaries islands, selected from a diverse collection described in Annicchiarico et al. (2010). SNP-generated markers were obtained for this material using the same procedure described for the model training set. Concurrently, we used Książkiewicz et al.'s (2017) information on SNP markers closely linked to the three QTLs for anthracnose resistance, namely, marker TP222136 for antr04/05_1 on linkage group ALB02, markers TP61122 and TP26007 for antr04/05_2 on linkage group ALB04, and markers TP251482, TP252015 and TP1928 for antr05_3 on linkage group ALB10, to infer the allelic value of these QTLs in the landrace genotypes.

Plant material

A total of 40 white lupin (*L. albus*) cultivars, breeding lines and landraces were collected from different sources and will be referred to as accessions throughout this publication (**Table 1**). Fifteen accessions were selected based on their contrasting predicted disease scores and because they represented different allelic combinations with three useful QTLs (two accessions), two useful QTLs (antr04/05_1 + antr05_3 for three accessions; antr04/05_1 + antr04/05_2 for one accession), one useful QTL (antr04/05_1 for three accessions; antr05_3 for three accessions), or no useful QTL (three accessions). The rest of the accessions was selected based on their field performance in 2015 to 2017 (C. Arncken, personal communication). All accessions were phenotyped for anthracnose resistance under controlled conditions and 26 accessions were phenotyped under field conditions in a 1-row setup, from which eleven accessions were also phenotyped in field plots. The commercial cultivar Feodora served as reference throughout all phenotyping assays.

Controlled condition experiments

For high-throughput resistance phenotyping under controlled conditions, one seed per pot was sown in 0.2 liter pots with a diameter of 6 cm, containing non-sterilized peat soil (Einheitserde® Classic, Einheitserdewerke Werkverband e.V., Sinntal-Altengronau, Germany) and vermiculite (ISOLA Vermiculite AG, Bözen, Switzerland) in a ratio of 5 to 1. Pots were placed in a growth chamber (25 ± 2°C, 16 h light and ~70% relative humidity). *C. lupini* JA01 (or IMI 375715) was grown on PDA for 6 to 8 days at 22°C in the dark. Spores were harvested with a sterile spreader after flooding the Petri dish with 2 ml sterile ddH_20. The concentration was determined using a haemocytometer (0.1 mm; Marienfeld, Lauda-Königshofen, Germany) and adjusted to 10^5 spores/ml. Stem inoculation, inspired by protocols described by Falconi et al. (2015) and Dubrulle et al. (2020a), was performed on 14 day old seedlings (3-4 leaf stage) by carefully puncturing the apical main stem with a sterile syringe needle followed by application of 5 µl spore suspension. Control plants were inoculated with 5 µl sterile ddH_20. After inoculation, plants were incubated for 48 hours at 100% relative humidity (16 h light, 22°C) before being returned to the growth chamber. Plants were watered every three days with 30 ml per plant, and fertilized twice with 100 ml Universal-Flüssigdünger (Gebr. Mayer Produktions- und Vertriebs. GmbH, Wahrenholz, Germany) at two and eight days post inoculation (dpi). The experiments were performed in a randomized complete block design with a minimum of five replicates per experiment.

Field experiments

Field experiments were performed in Switzerland at site F (Feldbach, 47°14'20.0"N 8°47'18.8"E) and site M (Mellikon) in 2018 and 2019 within 6-row plots. Plot sizes in Mellikon were 1.5 x 5 m and in Feldbach 1.5 x 2.7 m with a seed density of 65 seeds/m^2. Total field trial size in Mellikon 2019 was 608 m^2, in 2018 450 m^2, in Feldbach 2019 146 m^2 and in 2018 243 m^2. Plot experiments relied on natural infection and were performed in a randomized complete block design with three replicates in 2019 and eight replicates in 2018. In addition, a high-throughput 1-row setup was

used at both sites in 2019 with two replicates in a randomized complete block design according to a protocol described by Adhikari et al. (2009). Accessions were sown in 1 m rows containing eight plants, with every second row sown with the susceptible cultivar Amiga containing natural seed infection as disease spreader rows. Seeds were sown at the end of March and harvested in mid-August. Before sowing, seeds were inoculated with *Bradyrhizobium* sp. *lupini* (HiStick©L, BASF, UK) and no fertilizer was applied during the growing season.

Table 1. Origins and characteristics of white lupin (*Lupinus albus*) accessions phenotyped for anthracnose (*Colletotrichum lupini*) resistance in this study[a].

Accession	Origin	GenBank code	Institution	Type	GP	QTL[b]	n
Mut28 [c]	-	LUP 6445	IPK (Germany)	Genetic Resource	-	-	10
Cro01[c]	Yugoslavia	LUP 237	IPK (Germany)	Genetic Resource	-	-	15
Eth01[c]	Ethiopia	LUP 2078	IPK (Germany)	Genetic Resource	-	-	10
Frieda[c]	Germany	Frieda	DSV (Germany)	Cultivar	-	-	19
Sulimo*[c]	France	Sulimo	Jouffrai-Drillaud (France)	Cultivar	-	-	11
Blu-25*[c]	Chile	Blu-25	Semillas Baer (Chile)	Breeding line	-	-	26
Feodora*[c]	France	Feodora	Jouffrai-Drillaud (France)	Cultivar	-	-	37
Amiga*[c]	France	Amiga	Florimond-Desprez (France)	Cultivar	-	-	20
Figaro*[c]	France	Figaro	Jouffrai-Drillaud (France)	Cultivar	-	-	8
Zulika*[c]	Czech Republic	Zulika	Oseva s.r.o. (Czech Republic)	Cultivar	-	-	7
Dieta*[c]	UK	Dieta	Soya UK (UK)	Cultivar	-	-	8
KievMutant	Ukrain	95413	Polish Genebank (Poland)	Cultivar	-	-	23
P27175	Ethiopia	P27175	DPIRD (Australia)	Landrace	-	-	18
P27174	Ethiopia	P27174	DPIRD (Australia)	Landrace	-	-	15
Alg01	Algeria	La 572	CREA (Italy)	Genetic Resource	-	-	10
Eth02	Ethiopia	LUP 258	IPK (Germany)	Genetic Resource	-	-	11
Eth03	Ethiopia	LUP 2076	IPK (Germany)	Genetic Resource	-	-	6
NL01 [d]	The Netherlands[c]	LUP 2079	IPK (Germany)	Genetic Resource	-	-	10
Egy01	Egypt	503	Vavilov Research Institute (Russia)	Genetic Resource	-	-	6
MuPl	-	LUP 2104	IPK (Germany)	Genetic Resource	-	-	6
EdwBra	The Netherlands	V11-12-6	Louis Bolk Institute (NL)	Breeding line	-	-	6
Bianca	Italy	LUP 238	IPK (Germany)	Cultivar	-	-	6
Andro	Australia	Andromeda	DPIRD (Australia)	Cultivar	-	-	10
Hetman	-	LUP 6449	IPK (Germany)	Cultivar	-	-	12
Spn01	Spain	-	Local market (Spain)	-	-	-	6
Eth04[c]	Ethiopia	LAP0084	CREA (Italy)	Genetic Resource	2.17	1,1,1	7
Isr01[c]	Israel	LAP0071	CREA (Italy)	Genetic Resource	3.5	1,1,1	7

Egy02[c]	Egypt	LAP0093	CREA (Italy)	Genetic Resource	3.31	1,0,1	7
Can01[c]	Canaries	LAP0063	CREA (Italy)	Genetic Resource	3.46	1,0,1	8
Leb01[c]	Lebanon	LAP0070	CREA (Italy)	Genetic Resource	3.56	1,0,1	8
Ita01[c]	Italy	LAP0098	CREA (Italy)	Genetic Resource	2.69	1,1,0	8
Ita02[c]	Italy	LAP0101	CREA (Italy)	Genetic Resource	3.51	1,0,0	6
Ita03[c]	Italy	LAP0099	CREA (Italy)	Genetic Resource	3.69	1,0,0	6
Ita04[c]	Italy	LAP0107	CREA (Italy)	Genetic Resource	3.17	1,0,0	8
Sud01[c]	Sudan	LAP0082	CREA (Italy)	Genetic Resource	3.57	0,0,1	7
Mor01[c]	Morocco	LAP0055	CREA (Italy)	Genetic Resource	3.86	0,0,1	8
Ares[c]	France	Ares	CREA (Italy)	Cultivar	4.6	0,0,1	7
Ita05[c]	Italy	LAP0097	CREA (Italy)	Genetic Resource	3.79	0,0,0	8
Leb02[c]	Lebanon	LAP0069	CREA (Italy)	Genetic Resource	4.08	0,0,0	8
Ludet[c]	France	Ludet	CREA (Italy)	Cultivar	3.59	0,0,0	7

[a] GP = Genomic prediction; QTL = quantitative trait locus; and n = total observations obtained with stem inoculation under controlled conditions. Accession names highlighted in bold were tested in field plots in 2019 and accession names followed by an * were also tested in in 2018.

[b] Presence/absence (1/0) of resistance QTL antr04/05_1 (ALB02), antr04/05 _2 (ALB04) and antr05_3 (ALB10).

[c] Phenotyped with 1-rows under field conditions in 2019.

[d] Accession NL01 might have an Ethiopian origin based on plant morphology in the field.

Disease assessments

Under controlled conditions, disease phenotypes were assessed at 4, 8 and 12 dpi (18, 22 and 26 days after sowing) using a 1 to 9 disease score index (DSI) based on a protocol described by Thomas et al. (2008b): 1 = healthy plant, lesion size is 0 to 5% of stem length; 2 = minor disease symptoms, minor petiole elongation and slight yellowing of older leaves, lesion size is 5 to 15% of stem length; 3 = minor disease symptoms, increased petiole elongation and yellowing of older leaves, lesion size is 15 to 30% of stem length; 4 = moderate disease symptoms, main stem and petiole elongation, minor leaf and stem twisting, leaves are yellowing, lesion size is 30 to 45% of stem length; 5 = moderate disease symptoms, whole plant starts to wilt, leaves and stem start twisting, main stem on the verge of collapsing, lesion is starting to sporulate and its size is 45 to 60% of stem length; 6 = severe disease symptoms, plant has collapsed, severe leaf wilting but plant still has a few green leaves, lesion is sporulating and the size is 60 to 70% of stem length; 7 = severe disease symptoms, plant has completely wilted and only the youngest leaves have not dried out, the lesion is sporulating and the size is 70 to 80% of stem length; 8 = very severe disease symptoms, plant has completely wilted and dried out, lesion shows abundant sporulation and its size is 80 to 90% of stem length and 9 = complete plant decay, lesion size is 90 to 100% of stem length (illustrated in **Fig. 1A**). The controlled conditions standardized area under the disease progress curve ($sAUDPC_{CC}$) was calculated to assess and compare disease progression (Jeger and Viljanen-Rollinson 2001; Madden et al. 2007). Although disease scores obtained at single time points gave similar results as sAUDPC (**Table S1**), the sAUDPC was preferred to account for potential biases. At 12 dpi, lesion size, stem length and shoot dry weight were determined. Lesion

size is expressed relative to overall stem length ($Lesion_{rel}$) and shoot dry weight is expressed relative to control (SDW_{rel}).

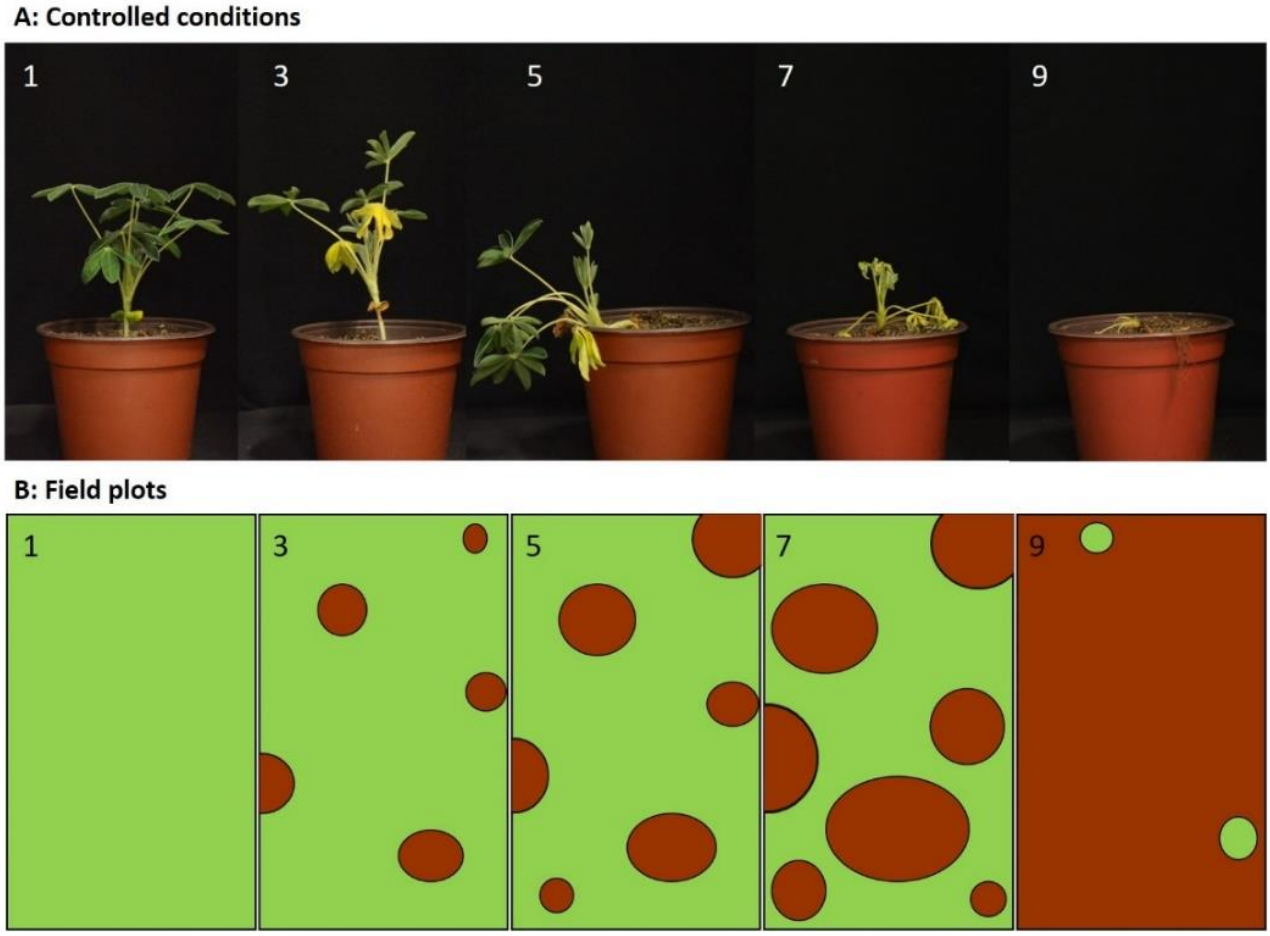

Figure 1. Disease assessment under controlled conditions and field plots. A: Representative disease symptoms of white lupin after *C. lupini* stem inoculation under controlled conditions (12 dpi). Disease score index of 1 = healthy, 3 = minor disease symptoms, 5 = average disease symptoms. 7 = severe disease symptoms and 9 = complete plant decay. Plant shown is the reference accession Feodora. B: Illustration of disease foci (brown) occurring in field plots. Disease score index: 1 = no disease symptoms (0%), 3 = minor disease symptoms (>2 to 5% diseased plants), 5 = moderate disease symptoms (>8 to 14% diseased plants), 7 = severe disease symptoms (>22 to 37% diseased plants), 9 = extremely severe disease symptoms (>61% to 100% diseased plants).

Disease assessments in the field were performed using a 1 to 9 DSI based on a protocol described by Jacob et al. (2017): 1 = no disease symptoms (0%), 2 = very minor disease symptoms (>0 to 2% diseased plants), 3 = minor disease symptoms (>2 to 5% diseased plants), 4 = low to moderate disease symptoms (>5 to 8% diseased plants), 5 = moderate disease symptoms (>8 to 14% diseased plants), 6= moderate to severe disease symptoms (>14 to 22% diseased plants), 7 = severe disease symptoms (>22 to 37% diseased plants), 8= severe to extremely severe disease symptoms (>37 to 61% diseased plants), 9 = extremely severe disease symptoms (>61% to 100% diseased plants). In field plots anthracnose occurs in disease foci as illustrated in **Fig. 1B**. Disease

assessments were performed at least 4 times per growing season, (60 to 70 days 80 to 90 days, 100 to 110 days and 120 to 130 days after sowing) and sAUDPC for field plots ($sAUDPC_{Field}$) and for 1-rows ($sAUDPC_{Row}$) was calculated to assess and compare disease progression. Yield (dt/ha) was assessed for field plots at grain harvest in August. The two high-throughput phenotyping systems, controlled condition and 1-row field, were compared with field plot disease assessments.

Statistical analysis of experimental data

Statistical analyses were performed with R 4.0.3 (R Core Team 2020) using the packages *lme4* (Bates et al. 2015)*, lmerTest* (Kuznetsova et al. 2017) and *emmeans* (Lenth et al. 2019)*,* following a mixed model with factors of interest (i.e. accession, strain) as fixed and environment, environment x accession and replicated block nested in environment as random factor, after confirming the assumptions of normality of residuals and homogeneity of variance. To achieve a normal distribution, data was transformed with a square root or logit transformation. Data are presented as estimated least-squares means using the aforementioned mixed model. Mean separation between lupin accessions and the reference cultivar Feodora was analyzed using Dunnett's test ($p \leq 0.05$). Estimated means of controlled and field conditions were correlated using the Pearson correlation coefficient. Broad sense heritability (H^2) was estimated as: genotypic variance/phenotypic variance, according to (Toker 2004). The number of replicates (n) needed to obtain a least significant difference (LSD) of 1 for $\alpha = 0.05$ was calculated according to the method of Williams and Abdi (2010), with the formula: $n = (t\alpha, df)^2 * 2MSE$. Where n = replicates, *MSE*=mean square error, df = degrees of freedom of residuals, t = t-test distribution and α = the critical *P* value.

Results

Evaluation of phenotyping under controlled conditions

BLASTn analysis of the ITS region confirmed that Swiss strain JA01 (GenBank accession MT741840) belongs to the species *Colletotrichum lupini*, showing 100% sequence identity with *C. lupini* strains corresponding to genetic group 2 (var. *setosum;* GenBank accessions JQ948169, JQ948161, MK463733) and 99.80% (494/495 bp) sequence identity with *C. lupini* strains corresponding to genetic group 1 (var. *lupini;* GenBank accessions JQ948155, JQ948159, JQ948158) at 100% query coverage. Comparing the three disease parameters, the highest broad-sense heritability (H^2) was found for standardized area under the disease progress curve under controlled conditions ($sAUDPC_{CC}$) with a H^2 of 0.82 followed by relative shoot dry weight (SDW_{rel}) with a H^2 of 0.78 and relative lesion size ($Lesion_{rel}$) with a H^2 of 0.75. Stem inoculation proved to be feasible for high-throughput phenotyping as up to 108 plants per m^2 could be phenotyped by one person within 26 days. To detect a least significant $sAUDPC_{CC}$ difference of 1 between two genotypes, a minimum of 4 replications was required, in contrast to single-row analysis with a H^2 of 0.48 which required 15 replicates.

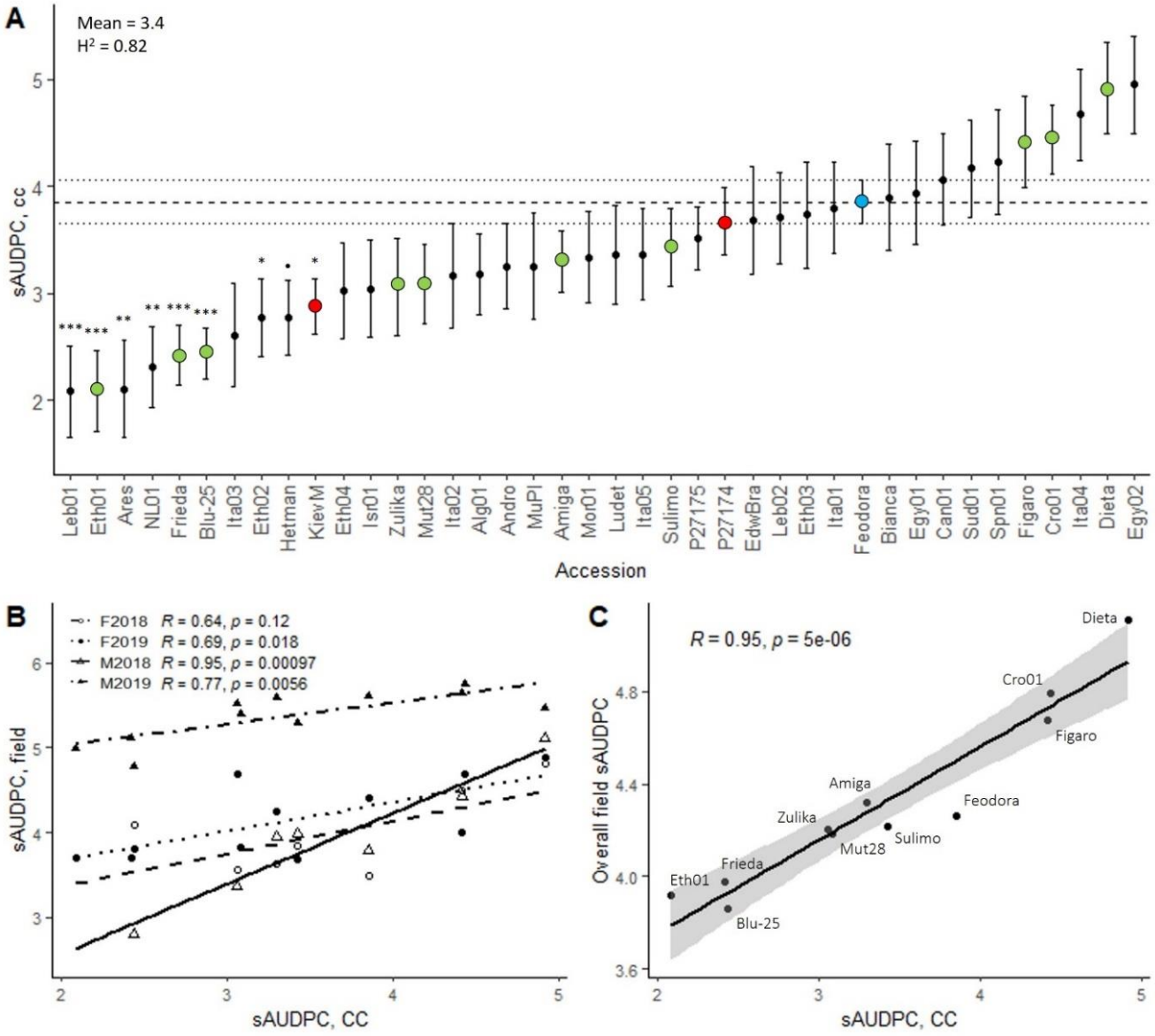

Figure 2. Anthracnose severity under controlled conditions expressed in sAUDPC and its correlation with field performance. A: Estimated means of standardized area under the disease progress curve (sAUDPC) of white lupin accessions phenotyped under controlled conditions (CC). The green dots indicate accessions tested in the field, blue indicates the susceptible reference accession Feodora and red dots indicate the RIL population parental lines, Kiev Mutant and P21714. Dashed line and dotted lines indicate the mean and estimated standard error, respectively, of Feodora. Error bars indicate standard error of the estimated mean. • $P \leq 0.1$, * $P \leq 0.05$, ** $P \leq 0.01$ and *** $P \leq 0.001$ difference with Feodora (Dunnett's test). B & C: Correlation plots of CC sAUDPC and field sAUDPC of each of the four different environments Feldbach (F) 2018 and 2019 and Mellikon (M) 2018 and 2019 individually (B) and combined (C). R indicates Pearson correlation coefficient

Increased levels of anthracnose resistance identified in white lupin

Disease phenotyping under controlled conditions revealed quantitative, rather than qualitative disease resistance as none of the 40 phenotyped white lupin accessions showed complete resistance to anthracnose. Eight accessions (Leb01, Eth01, Ares, NL01, Blu-25, Frieda, Eth02 and Kiev Mutant) showed significantly lower $sAUDPC_{CC}$ means compared with the susceptible reference cultivar Feodora, with differences ranging between -1.8 (Leb01) and -1 (Kiev Mutant; **Fig. 2A**). The accessions Frieda, Eth01 and Blu-25 also were more resistant under field conditions (Figure 2C). The difference between the $sAUDPC_{cc}$ means of accession Hetman and Feodora tended towards significance (P = 0.08). Five of the accessions mentioned above, Frieda, Kiev Mutant, NL01, Blu-25, and Eth01 also showed significantly higher SDW_{rel} means compared with Feodora, with differences ranging between 0.36 (Frieda) and 0.15 (Eth01; **Fig S1A**). The assessment of $Lesion_{rel}$ did not reveal significant differences compared to Feodora (**Fig. S2A**).

Strong correlation between controlled condition phenotyping and field performance

A subset of 11 white lupin accessions was screened in field plots to verify the phenotypes expressed under controlled and 1- row field conditions. Significant genotype (P = 0.01), field site (P = 0.001) and genotype x field interaction (P < 0.001) effects were found for the field plot experiments. Spatial within-trial variation was accounted for by a block effect (P > 0.001). No significant effects were detected for the 1-row experiments. Disease pressure gradually increased over the growing season and was on average lower in 2018 (3.95) compared to 2019 (4.77). Growing conditions in 2018 were warmer (18.35°C) and drier (267 mm) on average, compared to 2019 (16.35°C and 572 mm; **Fig. S4**). Significant Pearson correlation coefficients were found between $sAUDPC_{Field}$ averaged across all four environments and $sAUDPC_{cc}$(r = 0.95, P < 0.0001), SDW_{rel} (r = -0.80, P = 0.003) and $Lesion_{rel}$ (r = 0.89, P < 0.0001; **Table 2, Fig. 2, S1** and **S2**). For each environment individually, correlations with $sAUDPC_{Field}$ ranged between 0.95 and 0.64 for $sAUDPC_{CC}$, -0.86 and -0.52 for SDW_{rel} and 0.95 and 0.44 for $Lesion_{rel}$. When comparing controlled condition parameters with harvested yield (dt/ha) of the field plots averaged across all four environments, a significant correlation of -0.64 (P = 0.03) was found for $sAUDPC_{CC}$. Correlations between yield and SDW_{rel} (r =0.62, P = 0.04) and $Lesion_{rel}$ (r =-0.58, P = 0.064) were or tended towards significance. For each environment individually, correlations with yield ranged between -0.79 and -0.22 for $sAUDPC_{CC}$, 0.71 and 0.19 for SDW_{rel} and -0.86 and -0.25 for $Lesion_{rel}$. High-throughput disease phenotyping within a 1-row field setup ($sAUDPC_{Row}$) showed Pearson correlation coefficients of 0.33 (P = 0.43) and -0.23 (P = 0.56) with $sAUDPC_{Field}$ and yield averaged across all four environments, respectively, when replicated once at one site (**Table 2**). When replicated twice at two sites, Pearson correlations of 0.31 (P = 0.34) and -0.45 (P = 0.17) between $sAUDPC_{Field}$ and yield, respectively, were found. Comparing 1-row experiments to each environment individually showed no significant correlations.

Poor genomic predictions.

The best-predicting model was rrBLUP, with a value of 0.50 for intra-populations predictive ability. The predicted disease scores ranged from 2.12 to 4.70. The useful QTL antr04/05_2 on linkage group ALB04 was particularly rare and was only found in eight genotypes. This included two genotypes from Ethiopia, one of which was included in this study (Eth04) and which combined a very low predicted susceptibility score (2.165) with the simultaneous presence of all three QTLs (**Table 1**). Genomic prediction (GP) values generated by rrBLUB (**Table 1**) revealed no significant correlation with $sAUDPC_{CC}$ (-0.31, $P = 0.26$) or $sAUDPC_{Row}$ values (-0.46, $P = 0.085$; **Table 2**). When comparing GP with SDW_{rel} and $Lesion_{rel}$, significant Pearson correlation coefficients of 0.60 ($P = 0.02$) and -0.55 ($P = 0.03$), respectively, were revealed. Importantly, the parental lines of the RIL population that was used for the QTL study and the genomic prediction model, i.e., P27174 and Kiev Mutant, exhibited an opposite anthracnose resistance pattern to that displayed in their evaluation in Australia (Rychel-Bielska et al. 2020). The resistant parent P27174 ($n = 15$) showed a $sAUDPC_{CC}$ of 3.7 and an SDW_{rel} of 0.5, while the susceptible parent Kiev Mutant ($n = 23$) showed a $sAUDPC_{CC}$ of 2.9 and an SDW_{rel} of 1 (**Fig. 2**, and **S1**). This observation was confirmed by a follow-up experiment where Kiev Mutant, P27174, Feodora and four additional lupin accessions, were inoculated with either the Australian *C. lupini* strain IMI 375715 or the Swiss *C. lupini* strain JA01 (**Fig. S3**). This experiment also indicated that both *C. lupini* strains exhibit a similar virulence pattern on white lupin as no strain ($P = 0.64$) or strain x accession interaction ($P = 0.96$) effects were observed.

Discussion

Considering the lack of resistance to anthracnose disease in white lupin, a reliable high-throughput phenotyping tool to screen global white lupin germplasm is required. The described anthracnose disease phenotyping protocol, inoculating the stems of 14 day old white lupin seedlings grown under controlled conditions with a spore suspension of the pathogen, was useful to identify resistant genotypes, feasible for high-throughput phenotyping and the data corresponded with field performance. Similar stem inoculation approaches have proven to be a reliable tool to speed up selection in resistance breeding for various crops such as cotton (Bolek et al. 2005), sunflower (Schwanck et al. 2016), various *Brassicaceae* (Atri et al. 2019), and different legumes such as pea (Chang et al. 2018), soy (Twizeyimana et al. 2012) and Andean lupin (Falconí 2012). This study showed strong significant correlations ($r > 0.8$) between controlled condition phenotyping and replicated multi-environment field evaluations. This is in line with an anthracnose resistance study performed in Canada, showing that disease evaluations of stem inoculated seedlings of six white lupin cultivars under controlled conditions corresponded to disease evaluations of a 3 year field trial (Bhaskara Reddy et al. 1996). High correlations between stem inoculation under controlled conditions and field evaluations were also reported for other crops such as soybean (Li 2018; Twizeyimana et al. 2012), canola (Devey and Rosielle 1986) and grapevine (Poolsawat et al. 2012). Thus, stem inoculation of white lupin

seedlings under controlled conditions can be considered a reliable tool to speed up white lupin resistance breeding.

Table 2. Pearson correlation coefficients of two high-throughput phenotyping systems, (1) under controlled conditions and (2) with 1-rows under field conditions, with field plot phenotyping and genomic prediction values[a].

Field plot phenotyping, natural infection			**Controlled condition phenotyping, stem inoculation**			**1-row field phenotyping, disease spreader rows[b]**	
Parameter	**Environment**	**Mean**	***s*AUDPC$_{CC}$**	**SDW$_{rel}$**	**Lesion$_{rel}$**	***s*AUDPC$_{Row}$, 1 rep, 1 site[c]**	***s*AUDPC$_{Row}$, 2 reps, 2 sites**
***s*AUDPC$_{Field}$**	F 2018	3.99	0.64	-0.52	0.75•	0.003	-0.19
	F 2019	4.15	0.69*	-0.64*	0.44	0.23	0.48
	M 2018	3.92	0.95***	-0.86*	0.95***	0.19	0.30
	M 2019	5.38	0.77**	-0.68*	0.56•	0.22	0.44
	Average		**0.95*** **	**-0.80** **	**0.89*** **	**0.33**	**0.31**
Yield (dt/ha)	F 2018	42	-0.22	0.19	-0.25	-0.03	-0.26
	F 2019	20	-0.43	0.41	-0.35	-0.18	-0.37
	M 2018	26	-0.79*	0.71•	-0.86*	-0.05	-0.31
	M 2019	14	-0.72*	0.66*	-0.58•	-0.29	-0.54
	Average		**-0.64***	**0.65***	**-0.58•**	**-0.23**	**-0.45**
Genomic prediction (n = 15)			-0.31	0.60*	-0.55*	-0.38	-0.46•

[a] 2018 n = 7; 2019 n = 11, M = Mellikon; F = Feldbach; sAUDPC = standardized area under disease progress curve; SDW$_{rel}$ = shoot dry weight relative to control; Lesion$_{rel}$ = Lesion size relative to total stem length; and n = number of genotypes. • $P \leq 0.1$, * $P \leq 0.05$, ** $P \leq 0.01$ and *** $P \leq 0.001$.

[b] Correlations of the genomic predictions are based on 2 replicates of environment M 2019.

[c] Correlations are comprised of the average of obtained correlation coefficients of all available replications

From the three disease parameters, sAUDPC$_{CC}$ showed the strongest correlation with field performance (r = 0.95, P < 0.0001) and the highest heritability (H^2 = 0.82). In other plant pathosystems, using sAUDPC as a disease parameter was also highly correlated to field performance (Bradley et al. 2006; Li 2018; Trapero et al. 2013). The disease parameters SDW$_{rel}$ and Lesion$_{rel}$ also showed high heritability and correlation to field performance, although these were lower than for sAUDPC$_{CC}$. With lesion$_{rel}$, no accessions could be identified that significantly showed less infection than Feodora. Besides resistance, SDW$_{rel}$ has the advantage of also encompassing tolerance (Maya and Matsubara 2013). It should be taken into account, however, that a distinct symptom of lupin anthracnose is shoot elongation, potentially leading to a slight increase in biomass in minor to moderately infected plants at early stages, and therefore potentially leading to a misinterpretation of SDW$_{rel}$ results. Based on sAUDPC$_{CC}$, eight out of the forty accessions were significantly more resistant to anthracnose than the susceptible reference cultivar Feodora (**Fig. 2A**). Among those are three released cultivars, Frieda, Ares and Kiev Mutant, the breeding line Blu-25 and four genetic resources, Eth01, Eth02, NL01 and Leb01,

which are thus valuable resources for white lupin breeding and cultivation. The developed phenotyping tool should now be applied on a larger set of accessions to find more sources of resistance in white lupin to counter yield loss caused by anthracnose.

Disease phenotyping within a 1-row field setup has been widely applied for resistance breeding (Niks et al. 2011), including lupins (Adhikari et al. 2009; Ruge-Wehling et al. 2009). In this study, 1-row disease evaluations based on $sAUDPC_{Row}$, replicated once at one site, showed no significant correlations with field plot evaluations ($sAUDPC_{Field}$). With four replicates over two sites, correlation with $sAUDPC_{Field}$ improved. Compared to stem inoculation under controlled conditions, 1-row disease phenotyping showed a weaker correlation to field performance and results should be interpreted with caution. However, with sufficient replicates, 1-row disease phenotyping could be an alternative approach to identify anthracnose resistance in white lupin, especially when environmental variation has to be considered.

Predicted resistance values based on the genomic prediction model reported by Rychel-Bielska et al. (2020), taking into account the three major QTLs and possible minor ones, showed no positive correlations with $sAUDPC_{CC}$ and $sAUDPC_{Row,}$ suggesting no association of the reported QTLs with anthracnose resistance in the current study. As discussed by Rychel-Bielska et al. (2020), different inoculation methods, environmental variation and *C. lupini* strain-specific virulence can influence plant resistance and therefore should be taken into account. In this study, stem inoculation through wounding was performed under controlled conditions and might have contributed to the observed lack of correlation and contrasting resistance levels observed for Kiev Mutant and P27174. This, however, does not explain the absence of positive correlations between 1-row field evaluations under natural infection and the predicted values. In blue lupin, a similar unexpected observation occurred when material with strong anthracnose resistance identified under Australian conditions (Yang et al. 2008) turned out to be susceptible in Germany (Fischer et al. 2015). Warmer temperatures up to 25°C and higher rainfall (mm) favour anthracnose disease pressure in lupin (Dubrulle et al. 2020a; Thomas and Sweetingham 2004), highlighting the relevance of varying enviromental conditions. The average growing conditons during the Swiss (16.1 to 18.7°C, 195 to 694 mm, **Fig. S4**) and Australian field experiments (12.9 to 13.1°C, 441 to 566 mm; BOM 2005) indicate the lower temperature of 3 to 6 °C on average at the Australian site may have contributed to the observed inconsistencies. Differences in *C. lupini* strain virulence are unlikely as no strain or accession x strain interaction effects were found and *C. lupini* is suggested to be clonal (Talhinhas et al. 2016). Although QTL mapping and genomic prediction studies for quantitative traits are considered valuable (de Ronne et al. 2020; Haile et al. 2020; Lyra et al. 2020) and are being applied for key traits in white lupin (Annicchiarico et al. 2020; Annicchiarico et al. 2019), the method encounters various limitations (Wang et al. 2018; Xu et al. 2017). Genomic prediction models could effectively be applied to less related populations (Hao et al. 2019), but optimal predictions are achieved with related training populations (Akdemir and Isidro-Sánchez 2019; Albrecht et al. 2011; Berro et al. 2019). We suggest the main reason for poor genomic predictions of anthracnose tolerance in this study was the seemingly completely inconsistent response within the current testing conditions of the two

parent genotypes that represented resistance and susceptibility in the RIL population evaluated in the Australian site, whose data were used for constructing the genomic model.

This study provides a high-throughput phenotyping tool to identify field-relevant resistance against lupin anthracnose under controlled conditions to support white lupin resistance breeding. Phenotyping white lupin germplasm within a 1-row setup under field conditions was deemed to be less reliable as lower correlations with field plot disease assessments were found compared with controlled condition phenotyping. The resistant accessions identified in this study, Frieda, Blu-25, Eth01, Eth02, NL01, Leb01 and Ares, should be incorporated into current breeding programs after field validation. As predicted resistance values could not be validated, further insight on the effect of environmental conditions, infection pathways and the genetic basis underlying anthracnose resistance in white lupin is required. Current research focuses on phenotyping and genotyping a diverse set of 200 white lupin genotypes, including Kiev Mutant, P27174 and the resistant accessions identified in this study using the described phenotyping tool.

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Author contribution

JAA, PH and MMM conceived the original idea for this study. JAA conducted the experiments and took the lead in manuscript writing. CA and AL planned and performed the field trials with help. CA and PA provided seeds of white lupin genotypes. NN and MK established and provided genomic prediction values. JAA analyzed the data with contributions from PH, PA and MMM. JAA designed the figures and tables with input from PH, MMM, PA, RTV and MRF. All authors significantly contributed to data interpretation and provided critical feedback that shaped the final version. PH and MMM acquired the funding for this project.

Data availability

The datasets generated during the current study are available at: https://doi.org/10.5281/zenodo.4917198

Supplementary

Table S1. Pearson correlation coefficients of sAUDPC and disease score at single time point with field plot phenotyping under natural conditions across four environments. •= $P \leq 0.1$, * = $P \leq 0.05$, ** = $P \leq 0.01$ and *** = $P \leq 0.001$.

Controlled condition	H^2	sAUDPC, Field	Yield (dt/ha), Field
4dpi	0.76	0.48	-0.10
8dpi	0.79	0.93***	-0.66*
12dpi	0.82	0.96***	-0.67*
sAUDPC	0.82	0.95***	-0.64*

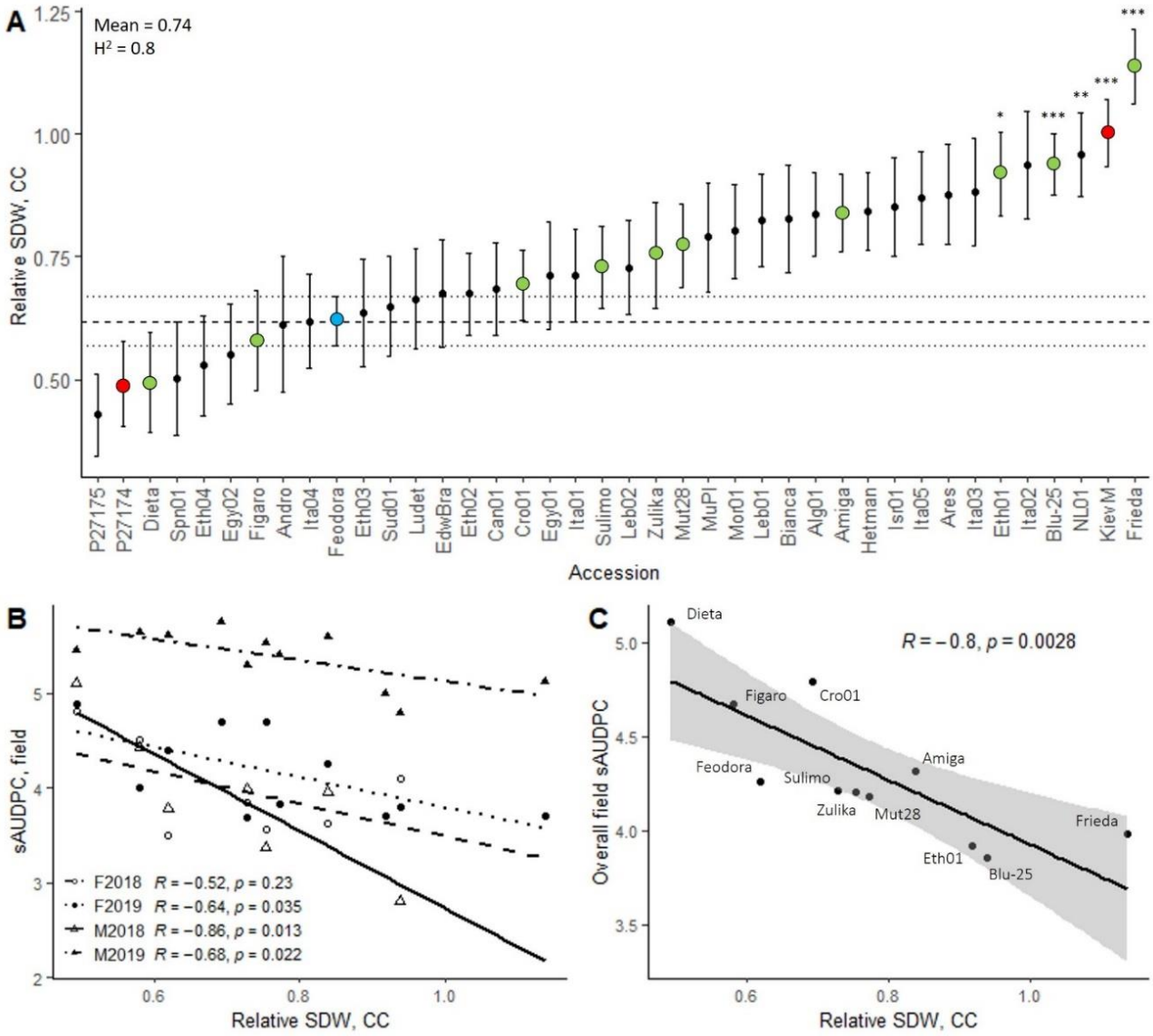

Figure S1. Anthracnose severity under controlled conditions expressed in Relative SDW and its correlation with field performance. A: Estimated means of relative shoot dry weight (SDW) of white lupin accessions phenotyped under controlled conditions (CC). The green dots indicate accessions tested in the field, blue indicates the susceptible reference accession Feodora and red dots indicate the RIL population parental lines, Kiev Mutant and P21714. Dashed line and dotted lines indicate the mean and estimated standard error, respectively, of Feodora. Error bars indicate standard error of the estimated mean. • $P \leq 0.1$, * $P \leq 0.05$, ** $P \leq 0.01$ and *** $P \leq 0.001$ difference with Feodora (Dunnett's test). B & C: Correlation plots of CC sAUDPC and field sAUDPC of each of the four different environments Feldbach (F) 2018 and 2019 and Mellikon (M) 2018 and 2019 individually (B) and combined (C). R indicates Pearson correlation coefficient.

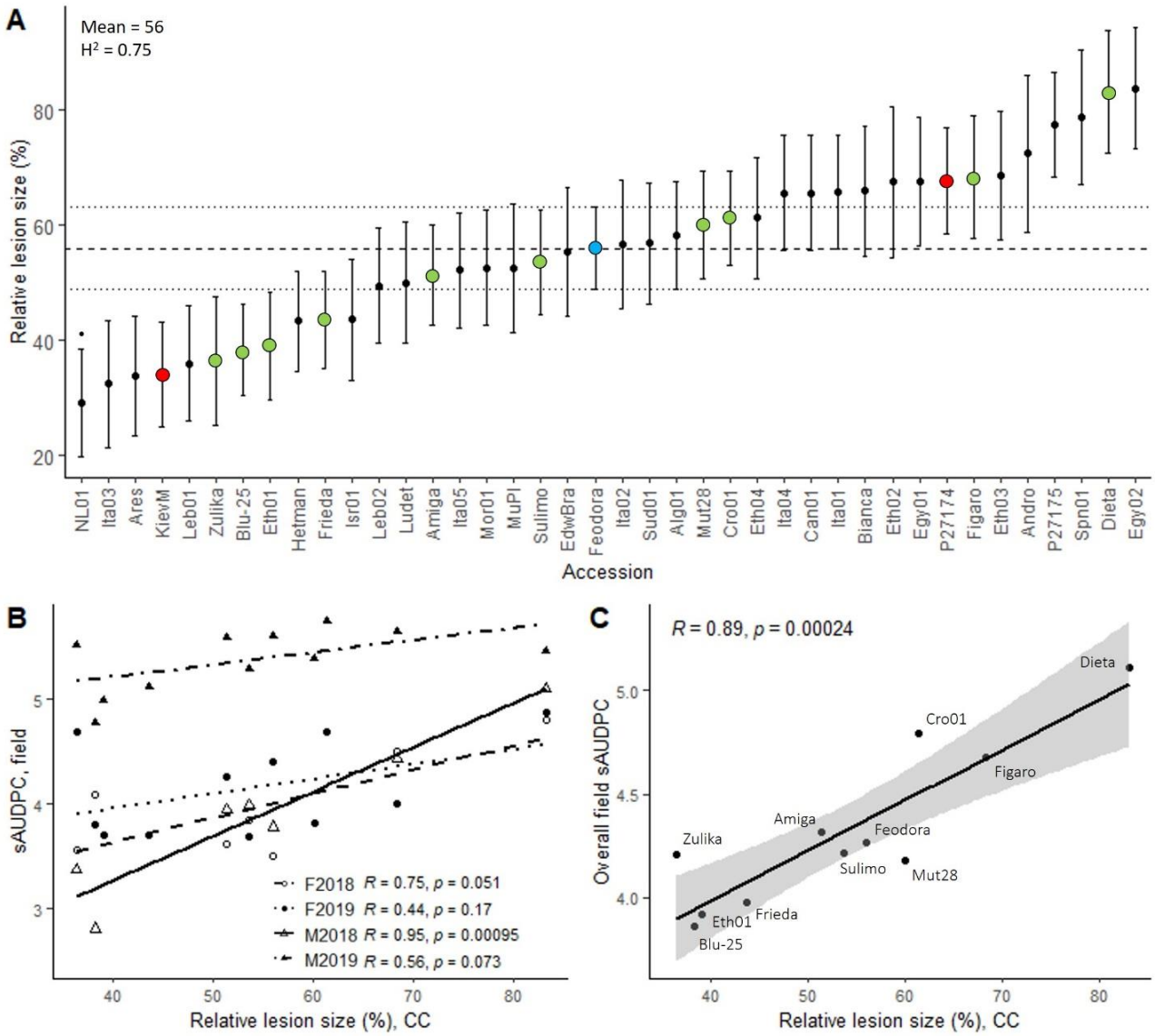

Figure S2. Anthracnose severity under controlled conditions expressed in relative lesion size (%) and its correlation with field performance. A: Estimated means of relative lesion size of white lupin accessions phenotyped under controlled conditions (CC). The green dots indicate accessions tested in the field, blue indicates the susceptible reference accession Feodora and red dots indicate the RIL population parental lines, Kiev Mutant and P21714. Dashed line and dotted lines indicate the mean and estimated standard error, respectively, of Feodora. Error bars indicate standard error of the estimated mean. • $P \leq 0.1$, * $P \leq 0.05$, ** $P \leq 0.01$ and *** $P \leq 0.001$ difference with Feodora (Dunnett's test). B & C: Correlation plots of CC sAUDPC and field sAUDPC of each of the four different environments Feldbach (F) 2018 and 2019 and Mellikon (M) 2018 and 2019 individually (B) and combined (C). R indicates Pearson correlation coefficient.

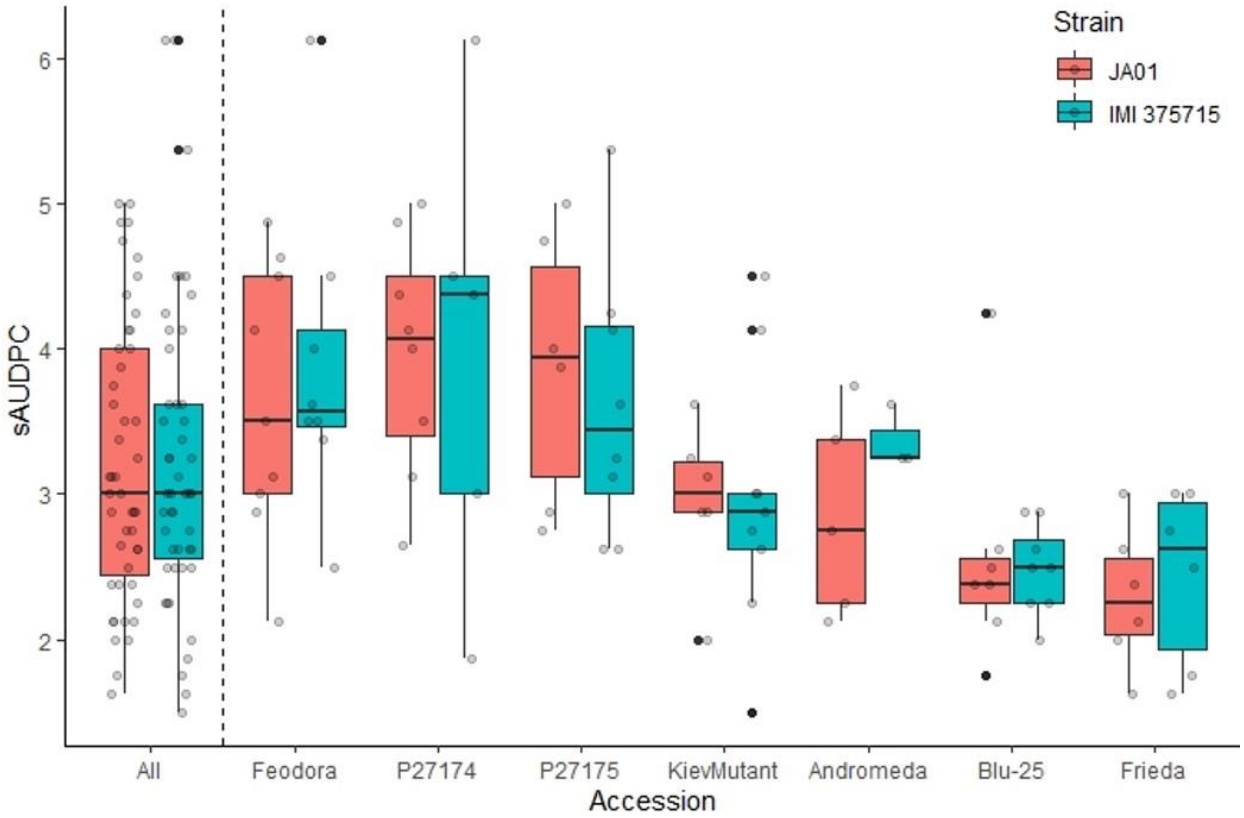

Figure S3. Virulence of *C. lupini* strain JA01 and IMI 37515 on different white lupin (*L. albus*) accessions. Box plot showing the standardized area under the disease progress curve (sAUDPC) of different white lupin accessions inoculated under controlled conditions with the Swiss *C. lupini* strain JA01 (red) or Australian strain IMI 375715 (blue). No strain effect (F-value$_{1\text{-}6}$ = 0.22, *P* = 0.64) and no strain x accession interaction effect (F-value$_{1\text{-}6}$ = 0.23, *P* = 0.96) was observed.

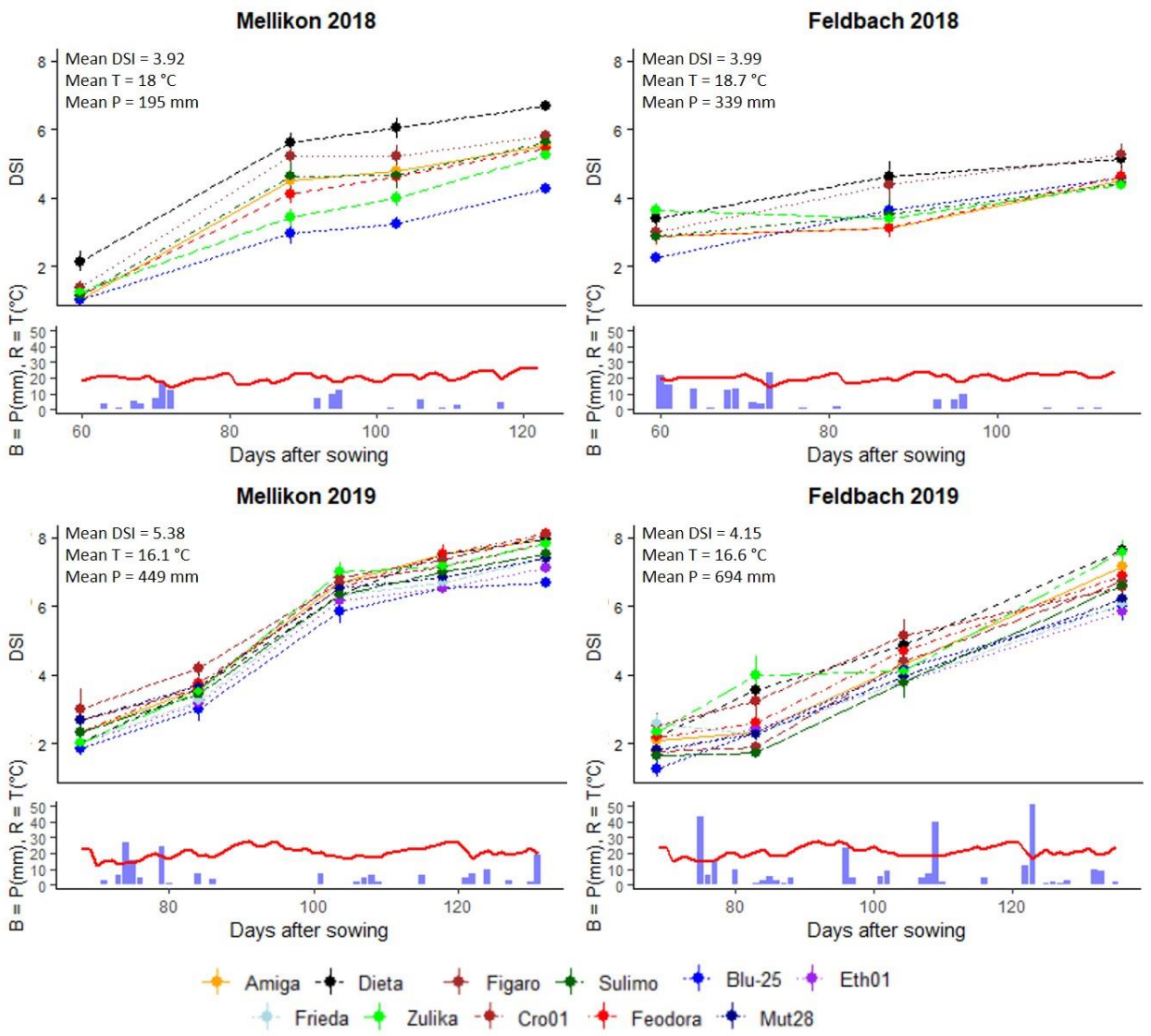

Figure S4. Anthracnose development and weather data throughout growing season in Mellikon and Feldbach in 2018 and 2019. Mean DSI indicates overall mean of disease score index across all accessions, mean T and mean P indicate mean temperature (°C) and mean precipitation (mm), respectively, from sowing to harvest. Error bars indicate standard error of the mean. B indicates blue precipitation bar plots and R indicate the red temperature line. Source weather data is Agrometeo 2019.

Chapter 4

Genome-wide association study reveals white lupin candidate gene involved in anthracnose resistance

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Abstract

White lupin (*Lupinus albus* L.) is a re-emerging protein crop and promising alternative to soybean. Its cultivation, however, is severely threatened by anthracnose disease caused by the fungal pathogen *Colletotrichum lupini*. To dissect the genetic architecture for anthracnose resistance, genotyping-by-sequencing (GBS) was performed on white lupin genotypes collected from the center of domestication and traditional cultivation regions. GBS resulted in 4,611 high-quality single-nucleotide polymorphisms (SNPs) for 181 genotypes, which were combined with resistance data observed under controlled conditions to perform a genome-wide association study (GWAS). Obtained disease phenotypes were shown to highly correlate to overall three-year disease assessments under Swiss field conditions ($r > 0.8$). GWAS results identified two significant SNPs associated with anthracnose resistance on gene Lalb_Chr05_g0216161 encoding a RING zinc-finger E3 ubiquitin ligase potentially involved in plant immunity. Peak SNPs tending towards significance also revealed other loci potentially involved in resistance pathways. Population analysis showed a remarkably fast linkage disequilibrium (LD) decay, weak population structure and grouping of commercial varieties with landraces, corresponding to the slow domestication history and scarce breeding efforts in white lupin. Together with 15 highly resistant genotypes identified in the resistance assay, our findings show promise for further crop improvement. This study provides the basis for marker-assisted selection, genomic prediction and understanding anthracnose resistance mechanisms in white lupin to improve breeding programs worldwide.

Keywords

Colletotrichum lupini, Lupinus albus, GWAS, quantitative trait locus (QTL), resistance breeding, single-nucleotide polymorphism (SNP)

Introduction

White lupin (*Lupinus albus* L., 2n = 50) is a grain legume with a high protein content and various health benefits that shows great potential to complement soybean and fulfill the growing demand for plant-based protein (Annicchiarico 2008; Arnoldi et al. 2015; Boschin et al. 2008; Monteiro et al. 2014; Sujak et al. 2006). White lupin is believed to originate from the North-Eastern Mediterranean, where wild *graecus* types still persist, and has been cultivated for more than 4,000 years across the Mediterranean and Eastern Africa (Wolko et al. 2011). Domestication of white lupin has been slow and systematic breeding efforts scarce. In general, lupin species have long been considered valuable assets in crop rotations due to their unique symbiosis with *Bradyrhizobium lupini,* making them highly efficient nitrogen fixators (Fernández-Pascual et al. 2007; Peix et al. 2015). Specifically, white lupin is one of the few crops that form specialized cluster roots, which increase phosphorus availability by carboxylate secretion, significantly increasing soil fertility (Gallardo et al. 2020; Lambers et al. 2013). Since the development of sweet low alkaloid varieties (Kroc et al. 2017), white lupin has become increasingly interesting for the food and feed industry (Lucas et al. 2015).

Cultivation of lupins, however, is severly compromised by the seed- and air-borne fungal pathogen *Colletotrichum lupini*, causing lupin anthracnose (Damm et al. 2012; Nirenberg et al. 2002; Talhinhas et al. 2016). Infected seeds (primary infection) and rain-splash dispersal (secondary infection) can cause total yield loss under favorable conditions (Thomas and Sweetingham 2004; White et al. 2008). Typical symptoms are stem twisting and bending and nectrotic lesions on stems and pods (Alkemade et al. 2021b). *Colletotrichum lupini* is a member of the *C. acutatum* species complex (clade 1), which contains numerous important plant pathogens (Damm et al. 2012). Contrary to the broad host range seen for most members of this complex, *C. lupini* is host-specific to members of the genus *Lupinus* (Baroncelli et al. 2017; Talhinhas et al. 2016). The current lupin anthracnose outbreak started in the 1970s and coincided with a decrease in lupin production worldwide, especially in Europe (FAOSTAT 2021). The pandemic is caused by a globally dispersed and genetically uniform group (II) of highly aggressive strains originating from South America (Alkemade et al. 2021b; Dubrulle et al. 2020a). Little is known about the interaction between *C. lupini* and its host, but a hemibiotrophic lifestyle is considered likely (De Silva et al. 2017; Dubrulle et al. 2020b)

Disease management of lupin anthracnose is currently focused on planting pathogen-free seed and chemical control, although the latter strategy is not available for the organic sector and is considered problematic due to adverse environmental effects (Thomas et al. 2008a; White et al. 2008). The dispersal of infected symptomless seeds is believed to be the most likely cause of the rapid spread of *C. lupini* strains across the globe. Advanced molecular diagnostics to determine infection levels are being developed, but are not yet routinely available (Kamber et al. 2021; Pecchia et al. 2019). Breeding for resistance is therefore likely to be the most sustainable solution. However, no complete resistance has yet been found in white lupin and the trait is considered polygenic (Adhikari et al. 2009; Alkemade et al. 2021a; Jacob et al. 2017). Quantitative trait locus

(QTL) mapping of anthracnose resistance using a recombinant inbred line population formed with the highly resistant Ethiopian landrace (P27174) and the susceptible cultivar Kiev Mutant, revealed three major resistance QTLs in an Australian experiment (Książkiewicz et al. 2017; Phan et al. 2007; Yang et al. 2010). Unfortunately, genotypes selected based on these QTLs did not show increased resistance either under controlled or Swiss field conditions (Alkemade et al. 2021a). The development of a high-throughput phenotyping system for field-relevant anthracnose resistance, together with the availability of a high-quality white lupin reference genome (Hufnagel et al. 2020), would allow for more in-depth genomic studies. In particular, genome-wide association studies (GWAS) have proven to be a valuable tool to determining the underlying genetics of quantitative traits in diverse populations. Moreover, GWAS have led to the discovery of single-nucleotide polymorphism (SNP) markers and candidate genes associated with traits of interest for numerous crops (Liu and Yan 2019). As an example, GWAS was recently used with the closely related blue lupin (*L. angustifolius* L.) to identify SNP markers for pod shattering (Mousavi-Derazmahalleh et al. 2018b) and climatic adaptation (Mousavi-Derazmahalleh et al. 2018a).

The aim of this study was to identify SNP markers and candidate genes associated with anthracnose resistance in white lupin. A collection of 200 white lupin cultivars, breeding lines and landraces, originating from across the Mediterranean and important cultivation regions, was genotyped-by-sequencing (GBS) and phenotyped for anthracnose resistance under controlled conditions. These accessions were shown to be variable for key agronomic traits (Annicchiarico et al. 2010), such as drought tolerance (Annicchiarico et al. 2018),and grain yield (Annicchiarico et al. 2019). Understanding the genetic architecture of anthracnose resistance in white lupin will provide crucial information to support further crop improvement.

Material and Methods

Germplasm collection

White lupin (*Lupinus albus* L.) accessions were collected across the Mediterranean region, Atlantic islands, Eastern Africa, Europe, Chile and Australia from seed genbanks and local partners. The accessions are described in **Table S1**. In total, the 200 genotypes include commercial cultivars, breeding lines, and traditional land races. The collection includes a large number of landraces from CREA's white lupin world collection (Annicchiarico et al. 2010), widely studied genotypes such as Amiga, Feodora, Kiev Mutant and P27174 (Adhikari et al. 2009; Hufnagel et al. 2021), and recently discovered resistant lines (Alkemade et al. 2021a).

Disease phenotyping

The white lupin collection was phenotyped for anthracnose resistance under controlled conditions (25 ± 2°C, 16 h light and 70% relative humidity) using the high-throughput protocol described by Alkemade et al. (2021a). Stem wound inoculations were performed with the highly virulent *Colletotrichum lupini* strain JA01 (genetic group II; Alkemade et al. (2021b)). Disease was

assessed at 3, 7 and 10 days post inoculation (dpi) with a 1 to 9 disease score index (DSI), with 1 being healthy and 9 completely diseased (Alkemade et al. 2021a). At 10 dpi, lesion size, stem length, and shoot fresh weight were determined. The overall disease score is expressed as the standardized area under the disease progress curve (sAUDPC), the lesion size as relative to overall stem length (LS_{rel}), and the shoot fresh weight is expressed relative to a control (SFW_{rel}). All of the experiments were performed in a randomized complete block design with a minimum of 8 replicates per genotype.

Field trials

Phenotypic data obtained under controlled conditions of twelve genotypes was compared to phenotypic data acquired over three-year field trials in Switzerland (**Table S1**). Field trials were performed within six row plots according to Alkemade et al. (2021a) at four distinct sites: Mellikon in 2018, Rümikon in 2019, Full-Reuenthal in 2020 and Feldbach in 2018, 2019 and 2020. Trials performed in 2018 and 2019 are described in Alkemade et al. (2021a). In 2020, in Full-Reuenthal (47°36'02.8"N 8°11'35.2"E) plot sizes were 1.32 X 3.5 m and in Feldbach (47°14'20.0"N, 8°47'18.8"E) plot sizes were 1.5 X 2.7 m with a seed density of 65 seed/m^2. Total field size was 304 m^2 in Full-Reuenthal and 259 m^2 in Feldbach. Trials were performed in a randomized complete block design consisting of 4 replicates. The field trials relied on natural infection and were scored 80, 100, and 135 days after sowing. The DSI ranged from 1 to 9, as described in Alkemade et al. (2021a). The sAUDPC and yield (dt/ha) were determined.

Phenotypic data analysis

Statistical analyses of the phenotypic data were performed within R 4.0.3 (R Core Team 2020) using the packages *lme4* (Bates et al. 2015)*, lmerTest* (Kuznetsova et al. 2017) and *emmeans* (Lenth et al. 2019)*,* following a mixed model. The factors of interest (i.e. genotype) were included as fixed effects, while environment, environment x genotype and replicated block nested in environment were fitted as random factors, after confirming the assumptions of normality of residuals and homogeneity of variance. To achieve a normal distribution, data were transformed with a square root (yield), log10 (SFW_{rel}), square ($sAUDPC_{CC}$), or logit (LS_{rel}) transformation. The mean separation between lupin genotypes and the overall mean were analyzed using Dunnett's test ($P \leq 0.05$). The data are presented as non-transformed estimated least-squares means obtained using the aforementioned mixed model. Estimated means of controlled and field conditions were correlated using the Pearson correlation coefficient. Broad sense heritability (H^2) was estimated as: genotypic variance/phenotypic variance (Toker 2004).

Genotyping and SNP calling

Genomic DNA was isolated from leaf tissue of three week old plants using DNeasy Plant Mini Kit (Qiagen, Hilden, D) and quantified with a Quant-iT™ PicoGreen™ dsDNA Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA). Samples were genotyped in four different batches referred to as TX2016-1 (88 samples), TX2016-2 (32 samples), EL2018 (40 samples) and EL2020 (40 samples), using slightly different procedures as follows:

Genotyping-by-sequencing (GBS) libraries for TX2016-1 and TX2016-2 were prepared with a modified Elshire et al. (2011) protocol. DNA samples (100 ng) were digested with restriction enzyme *ApeKI* (New England Biolabs, Ipswich, MA, USA) and ligated to unique barcodes and common adapters. Equal volumes of ligated products were pooled and purified with NucleoSpin Gel and PCR Clean-up (Macherey-Nagel, Düren, D). Template DNA (50 ng) was mixed with two primers (**Table S2**) and KAPA Library Amplification Readymix (Roche, Basel, CH). Amplification steps were as follows: 5 min at 72 °C, 30 s at 98 °C, and 10 cycles with 10 s at 98 °C, 30 s at 65 °C and 30 s at 72 °C. Sequencing was performed at the University of Texas (USA) on four Illumina HiSeq 2000 (Illumina Inc., San Diego, CA, USA) lanes, at 100 bp single-end. DNA samples for libraries EL2018 and EL2020 were sent to The Elshire Group Ltd. (Palmerston North, New Zealand) for library preparation and sequencing. Library preparation was performed according to Elshire et al. (2011) as described above with the following changes: libraries were amplified with 14 PCR cycles and prepared using a KAPA HyperPrep Kit (Roche, Basel, CH) following the manufacturer's instructions. Sequencing was performed on a single Illumina HiSeq X lane, at 2X150 bp paired-end.

GBS raw reads were demultiplexed using axe demultiplexer (Murray and Borevitz 2018). Trimming for restriction enzyme remnants, alignment on reference genome and SNP calling were performed using the dDocent pipeline (Puritz et al. 2014). For alignment we used the *L. albus* genome version 1.0 (Hufnagel et al. 2020) which was downloaded from https://www.whitelupin.fr/. The final genotype matrix, in the form of a vcf file, was further filtered for quality using the vcftools software (Danecek et al. 2011) with parameters –minQ 30 –max-non-ref-af 1 –non-ref-af 0.001. The resulting data set of 246,279 SNPS was filtered for monomorphic markers, minor allele frequency (MAF) < 5%, missing SNP marker rate > 10%, and a missing rate per individual > 20% (Pavan et al. 2020). Genotypes that deviated with 3 SD from the mean heterozygosity rate were removed (Marees et al. 2018). Missing data were imputed through Beagle (Browning and Browning 2016) within *statgenGWAS* (van Rossum et al. 2020) in R, resulting in 4,611 high-quality SNPs for 181 genotypes.

Linkage disequilibrium and population structure

Linkage disequilibrium (LD) of SNP markers was calculated as the pairwise squared correlation coefficient (r^2) between markers using *LD.decay* in R (Laido et al. 2014). Significant ($P \leq 0.05$) pair-wise LD estimates were used to calculate average LD decay within a sliding window of 5 kb. LD decay was visualized by plotting r^2 estimates against genetic distance (kb). A pairwise distance matrix derived from Euclidean distance of the full SNP dataset was calculated in R to construct a Ward Hierarchical clustering tree (Murtagh and Legendre 2014) with 1,000 bootstraps using *pvclust* in R (Suzuki and Shimodaira 2006). The tree was generated with *ape* (Paradis and Schliep 2019) in R and modified in iTOL v 6.1 (Letunic and Bork 2007). Principal component analysis (PCA) was performed using the *prcomp* function in R based on 1,292 SNPs filtered for a MAF > 20 and physical distance > 2.5 kb. An Astle kinship matrix (Astle and Balding 2009) was generated using *statgenGWAS* in R using the pruned SNP dataset.

Genome-wide association mapping

A genome-wide association study (GWAS) was performed on the full 4,611 SNP dataset using estimated least-square means for the traits disease score (sAUDPC), LS_{rel} and SFW_{rel}. The association between SNPs and phenotypes was analyzed by performing single-trait GWAS following a mixed linear model within *statgenGWAS* in R following the method described in Kang et al. (2010). The first ten principal components were included as covariates to control for population structure and the Astle kinship matrix was included to account for cryptic relatedness (Astle and Balding 2009; Rincent et al. 2014). An efficient mixed model association (EMMA) algorithm was used to estimate the variance components (Kang et al. 2008). General least squares (GLS) were used to estimate effect size and P value for each SNP. A Bonferroni corrected LOD threshold (–log10(0.05 / number of SNPs)) was used to identify significant SNPs. SNPs within 2.5 kb and ≥ 0.5 r^2 were considered linked and included Manhattan and quantile-quantile (Q-Q) plots were generated within *statgenGWAS*.

Candidate gene selection

Candidate genes were identified when containing a significant SNP, a SNP in LD ($r^2 > 0.5$) with significant SNP or within 10 kb of peak SNPs. Candidate genes were located using the white lupin reference genome (v 1.0) browser (Hufnagel et al. 2020). Protein sequences were acquired and blasted (BLASTp) to find homologs in closely related species i.e. blue lupin (*L. angustifolius*), peanut (*Arachis hypogea*), common bean (*Phaseolis vulgaris*), soybean (*Glycine max*) and model species *Medicago truncatula* (LPWG 2017). The potential function of each candidate gene was derived from annotations, literature, and *in silico* analysis.

Results

Strong differentiation and heritability of anthracnose-related traits

Disease phenotyping of 200 white lupin genotypes under controlled conditions revealed a range of resistant and susceptible genotypes (**Fig. 1**). Strong differences between genotypes ($P < 0.001$) for all three anthracnose related traits, sAUDPC, LS_{rel} and SFW_{rel}, were observed with heritabilities of 0.77, 0.78 and 0.74, respectively. Significant ($P \leq 0.05$) Pearson correlations were found between the three traits (r > (-)0.86) and between these traits and the overall disease assessment means of three-year field trials in Switzerland (r > (-)0.8; **Table S3** and **Fig. S1**). We did not observe complete resistance against anthracnose, which we interpret as evidence that resistance in white lupin is quantitative. For sAUDPC, LS_{rel} and relative SFW_{rel}, 8, 13 and 2 accessions respectively, were more resistant than the respective overall mean ($P \leq 0.05$, **Table S1**). Six of these accessions originated from Ethiopia, two are from Chile and one is the newly available commercial variety Frieda. In contrast, seven accessions were more susceptible than the overall mean. Remarkably, the Ethiopian landrace P27175 was resistant in one seed batch

(FiBL 39) but susceptible in another (FiBL 19). Resistant FiBL025 (R-6020) and susceptible LAP0119b (Aster) seeds showed a black-speckled morphology typical for wild *graecus* types.

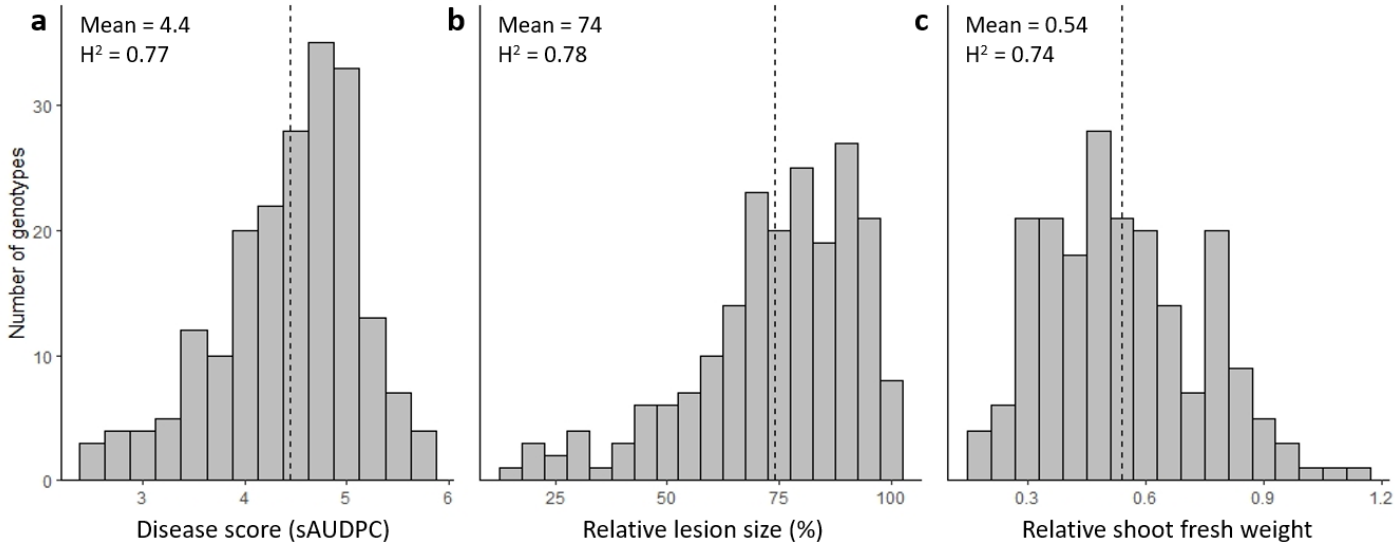

Figure 1: Phenotypic variation of three anthracnose resistance traits among 200 white lupin genotypes. a: Disease score (sAUDPC), **b:** Relative lesion size (%), **c:** Relative shoot fresh weight. Dashed line indicates overall mean.

Weak population structure and fast LD decay

Genotyping-by-sequencing yielded 4,611 high-quality SNPs for 181 genotypes after filtering for monomorphic markers, minor allele frequency (MAF) < 5%, missing SNP marker rate > 10%, missing rate per genotype > 20% and heterozygosity > 0.4 (mean Ho = 0.15). LD decayed to half its maximum value at 2.9 kb ($r^2 = 0.45$) and SNPs were in linkage ($r^2 > 0.5$) over an average distance of 2.5 kb (**Fig. S2**). Cluster analysis on the full SNP dataset distinguished 4 subgroups (I – IV) based on bootstrap support values (BS) > 90 and a branch length threshold of 10 (**Fig. 2a**). These subgroups could also be observed through PCA and Astle kinship analysis after pruning the SNP dataset (MAF > 20% and physical distance > 2.5 kb; **Fig. 2b** and **c**), which revealed overlap between group II, III and IV, while group I was more clearly separated. Group I exclusively contains landraces originating from the South-Eastern Mediterranean. Group II, includes accessions from across the entire study area, encompasses most of the commercial cultivars and breeding lines used in this study (88%). A large proportion (27%) of group II includes landraces from North Africa, half of which originate from Ethiopia. Group III consists mostly of Egyptian (64%) and Ethiopian (23%) landraces, with the Ethiopian landraces strongly clustering together (BS = 100). Group IV contains accessions from across the entire study area, including lines from the Iberian Peninsula (30%) and the Atlantic Isles (26%; **Table S1**). Kinship between genotypes showed relatively close relatedness among sampled genotypes (**Fig. 2c**).

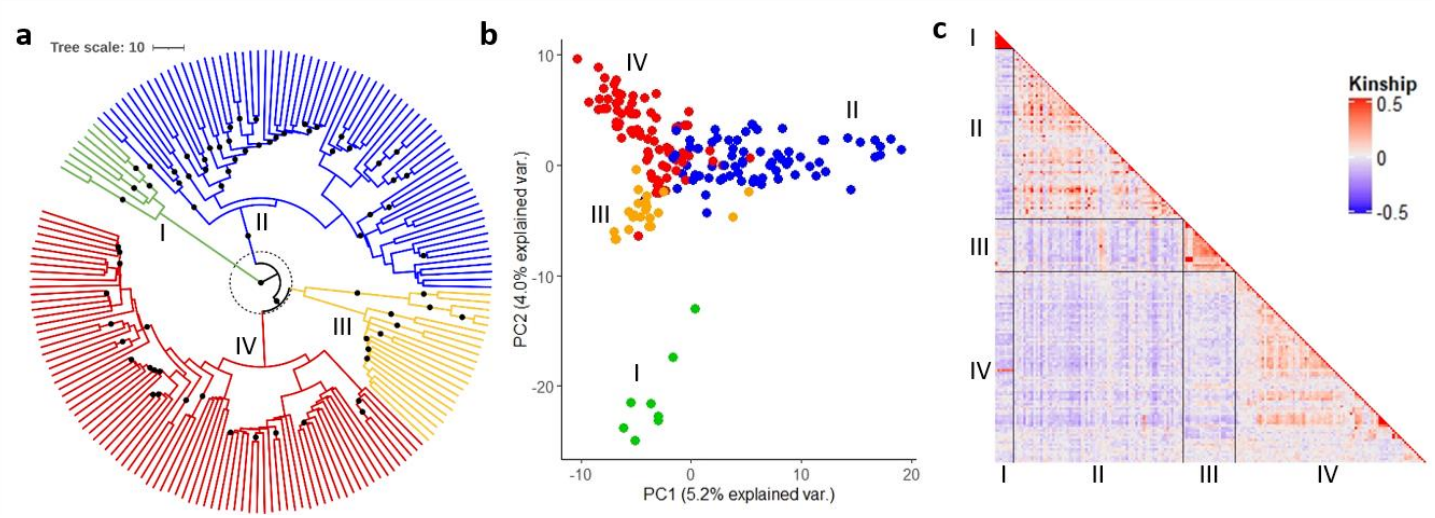

Figure 2: Genetic diversity and population structure of 181 white lupin genotypes. a: Ward cluster analysis (1,000 bootstraps). Colors represent subgroups (I-IV). Black dots represent bootstrap support values (> 90) and dotted circle indicates branch length of 10. **b:** Principal component analysis (PCA). Each dot represents a genotype and colors represent subgroups (I-IV). **c:** Heatmap of the Astle kinship value among genotypes.

Two highly significant SNPs associated with anthracnose resistance

To map genetic variants associated with anthracnose resistance, we performed a genome-wide association study (GWAS) using a linear mixed model. We included the first 10 principal components (PCs) and the Astle kinship matrix to correct for population structure. The first 10 PCs explained 24% of the genetic variance. The resulting Q-Q plots revealed that the model was well calibrated, as we observed a good approximation between the expected and observed P-values (**Fig. 3**). Using a Bonferroni LOD-threshold of 4.96 (*P* = 1.08E-5) and considering SNPs with an $r^2 > 0.5$ to be linked, we identified two highly significant SNPs, Lalb_Chr05_2957601 and Lalb_Chr05_2957940, for both sAUDPC (*P* = 1.07E-08 and 1.64E-07, respectively) and LS_{rel} (*P* = 3.41E-06 and 2.04E-06, respectively). The two SNPs explained 12 to 15% of the observed variation (R^2_{LR}; **Table 1**), were not strongly linked with other SNPs, and were found in exons of the same gene (Lalb_Chr05g0216161; **Fig. 4**). The minor allele frequencies (MAF) of these SNPs were low (10 and 7 %, respectively); the non-reference alleles were significantly associated with increased anthracnose resistance when either heterozygous or homozygous (**Fig. 4c**). The non-reference alleles were only found homozygous for both SNPs in the Chilean genotypes Fibl016 (Blu-25) and LAP0155a & b (Rumbo Baer), which were found to be highly resistant (sAUDPC = 2.55, 2.78 and 3.11, respectively). Other promising SNPs were found on chromosome 5 (Lalb_Chr05_3706534, *P* = 2.46E-05) for LS_{rel}, on chromosome 13 (Lalb_Chr13_12108967, *P* = 2.26E-05), 1 (Lalb_Chr01_15792483, *P* = 6.73E-05) and 8 (Lalb_Chr08_12561556, *P* = 1.20E-04) for SFW_{rel}, and on chromosome 1 (Lalb_Chr01_12025845, *P* = 7.97E-05) for sAUDPC (**Table S4**). The non-reference alleles of these SNPs, with the exception of Lalb_Chr01_12025845, were implicated in decreased anthracnose resistance (**Fig. S3**). SNPs Lalb_Chr01_12025863, Lalb_Chr05_3688076,

Lalb_Chr05_3784474 and Lalb_Chr13_12143224 were linked to, respectively, Lalb_Chr01_12025845, Lalb_Chr05_3706534 and Lalb_Chr13_12108967 (**Table S4**).

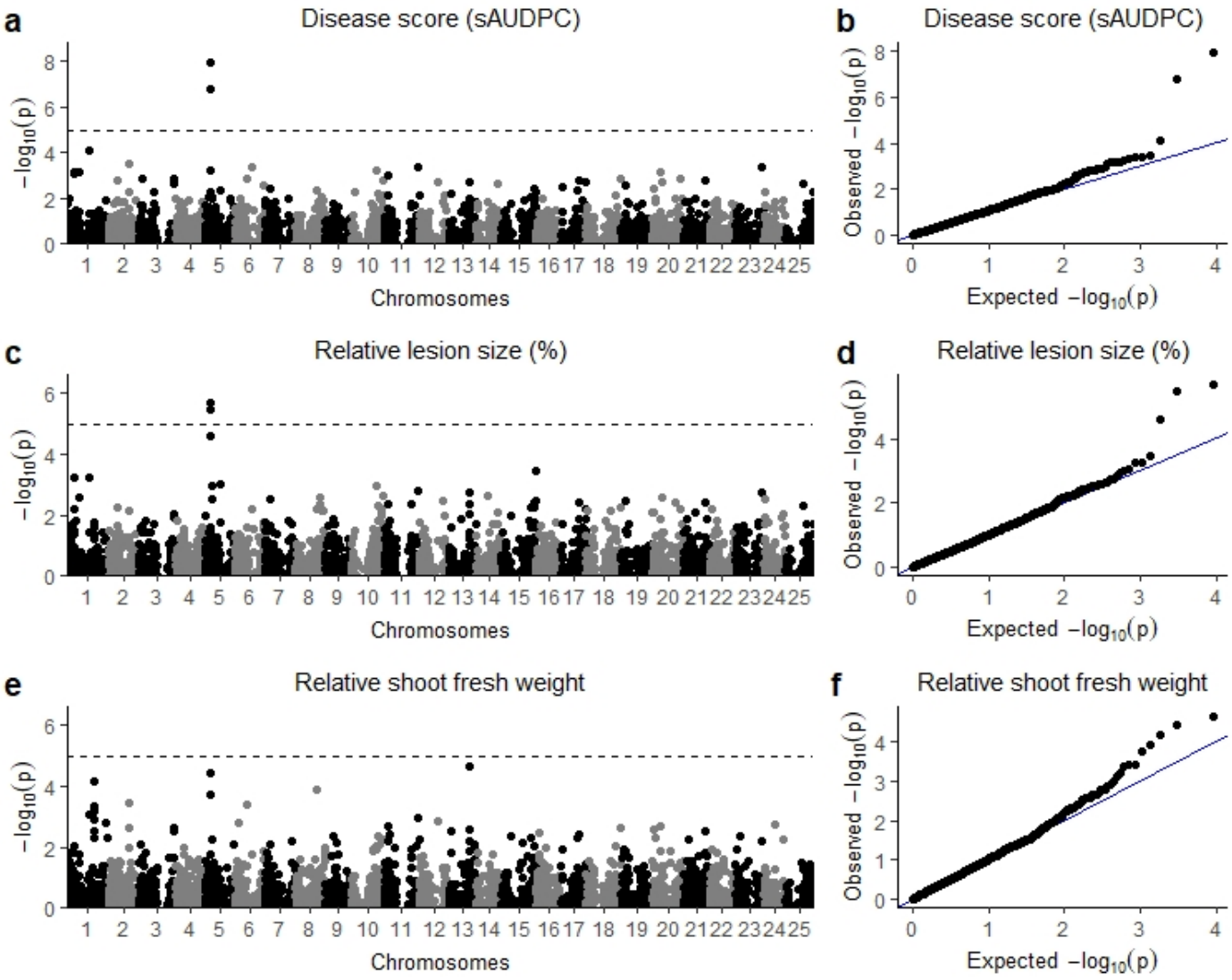

Figure 3: Manhattan and corresponding Q-Q plots showing SNP association with anthracnose resistance. a-b: Disease score (sAUDPC)**, c-d:** Relative lesion size (%), **e-f:** Relative shoot fresh weight. sAUDPC = standardized area under the disease progress curve. Dashed line indicates Bonferroni corrected LOD threshold of 4.96 (*P* = 1.08E-5).

Candidate genes involved in resistance pathways

Candidate genes containing a significant SNP, a SNP linked ($r^2 > 0.5$) to a significant SNP, or located within 10 kb of a significant SNP were considered further. The significant SNPs Lalb_Chr05_2957601 and Lalb_Chr05_2957940 are both located within an exon of the same gene: Lalb_Chr05g0216161 (**Table 1**, **Fig. 4d**). This gene is annotated as a putative chromatin regulator and encodes a protein containing a Von Willebrand factor type A (VWFA) as well as a RING zinc-finger domain (ZF; **Fig. 4d**). Homologs in closely related legume species encode for RING zinc-finger E3 ubiquitin ligases, which are widely associated with plant immunity (Marino et al. 2012). Lalb_Chr13_12108967 is located within an exon of the gene Lalb_Chr13g0297911, which encodes a protease Do-like 9 protein (**Table S4**). One kb downstream from the gene Lalb_Chr13g0297911 is the gene Lalb_Chr13g0297901, which encodes a putative C2H2 transcription factor. Homologs in other closely related species encode for RING-H2 finger proteins. Lalb_Chr05_3706534 is located within the exon of gene Lalb_Chr05g0217341 which encodes a putative transcription regulator and homologs in related species encode the paired amphipathic helix protein Sin3. Linked to this SNP is Lalb_Chr05_3784474 which is located within an exon of the gene Lalb_Chr05g0217471 which encodes for a non-specific serine/threonine protein kinase. Homologs in closely related species encode LRR receptor-like serine/threonine-protein kinases, which are often implicated in plant defense against fungi (Afzal et al. 2008; Tang et al. 2017). Three kb downstream of this gene is Lalb_Chr05g0217461 which putatively encodes an enhanced disease resistance protein. Lalb_Chr01_15792483 is located within the exon of the gene Lalb_Chr01g0015651, which is annotated as a putative protein kinase AGC-RSK-2 and contains a protein kinase and AGC kinase domain. Homologs in closely related species encode serine/threonine-protein kinases. Lalb_Chr01_12025845 and linked SNP Lalb_Chr01_12025863 are both located in close, upstream vicinity (< 35 b) of gene Lalb_Chr01g0012271 which is believed to encode a glycerol kinase. Homologs in closely related species are also annotated as glycerol kinases. Further downstream (8 kb) is the gene Lalb_Chr01g0012261, which putatively encodes a leucine-rich repeat domain and L domain-containing protein. Homologs in closely related species encode for F-box/LRR domain proteins, which are frequently involved in plant defense responses (van den Burg et al. 2008).

Table 1: Significant SNPs and candidate gene associated with anthracnose resistance[a].

Trait	SNP[b]	Alleles	P value	R^2_{LR}	MAF	Location	Candidate gene	Protein	Annotation
Disease Score (sAUDPC)	Lalb_Chr05_2957601	G/A	1.07E-08	0.15	0.10	Exon	Lalb_Chr05_g0216161	KAE9613313.1	Putative chromatin regulator PHD family
	Lalb_Chr05_2957940	C/T	1.64E-07	0.13	0.07	Exon			
Relative lesion size (%)	Lalb_Chr05_2957601	G/A	3.41E-06	0.12	0.10	Exon			
	Lalb_Chr05_2957940	C/T	2.04E-06	0.12	0.07	Exon			
Relative shoot fresh weight	Lalb_Chr05_2957601	G/A	3.65E-05[c]	0.09	0.10	Exon			
	Lalb_Chr05_2957940	C/T	1.81E-04[c]	0.07	0.07	Exon			

[a] SNP = single-nucleotide polymorphism, MAF = minor allele frequency, R^2_{LR} = likelihood-ratio-based R^2. [b] Lalb = *Lupinus albus*, Chr = chromosome, number = position on chromosome, [c] not significant.

Discussion

Disease phenotypes obtained under controlled conditions strongly correlated (r > 0.8) to overall three-year field plot disease assessments in Switzerland, confirming field-relevance of high-throughput phenotyping under controlled conditions (Alkemade et al. 2021a). No complete resistance was observed, but based on sAUDPC, LS_{rel} and SFW_{rel}, a total of 15 different accessions showed to be significantly more resistant to anthracnose compared to the overall mean. Five of these genotypes originated from Ethiopia, which was previously shown to be a good source for white lupin anthracnose resistance (Adhikari et al. 2009; Cowling et al. 1999). However, Ethiopian landrace P27174 (Fibl020 & 38), used as a resistant parent for an anthracnose resistance QTL study (Książkiewicz et al. 2017; Yang et al. 2010), was not shown to be resistant. Resistance in Ethiopian landrace P27175 differed among seed batches, suggesting that seed health or quality may also play a role. High resistance was also found for Chilean cultivar Rumbo Baer and the breeding line Blu-25, both of which appear to derive from a resistant landrace from the Azores (von Baer et al. 2009).

White lupin has long been cultivated across the Mediterranean and North-Eastern Africa, with its primary center of origin believed to be in the Balkans up to Western Turkey where wild *graecus* types are still found (Wolko et al. 2011). This study, which contains accessions collected from across the traditional cultivation regions of white lupin, revealed an exceptionally fast LD decay (2.9 kb). This fast LD decay is consistent with an earlier study by Hufnagel et al. (2021) and the fact that white lupin has a modest rate of outcrossing (Brebaum and Boland 1995). Studies of other grain legume species, including pea (>50 kb; Gali et al. (2019)), soybean (> 240 kb; Wen et al. (2018)), and common bean (> 1 mb; Diniz et al. (2019)), as well as the closely related blue lupin (>77 kb; Mousavi-Derazmahalleh et al. (2018b)), have reported slower rates of LD decay. In addition, we detected a weak population structure, finding four subgroups (I – IV). Principal

component analysis showed overlap between Group II, III and IV, while only Group I, exclusively containing landraces from the South-Eastern Mediterranean, formed a clearly distinct group. Group III consisted primarily of Egyptian and Ethiopian landraces, with Ethiopian genotypes strongly grouping together. Landraces from Ethiopia were previously reported to form a distinct group within white lupin (Raman et al. 2014) and were shown to be most closely related to wild *graecus* types (Hufnagel et al. 2020; Hufnagel et al. 2021), which suggests these landraces derived in isolation and are still little domesticated. In contrast with these results, 10 of the 16 Ethiopian landraces collected in this study, were classified in Group II, containing commercial cultivars and landraces from all across the collection area. Taken together, we interpret the fast LD decay, weak population structure, and the grouping of commercial varieties with landraces to indicate that there have been few recent breeding events in white lupin, which implies that there is great potential for further crop improvement in this re-emerging protein crop.

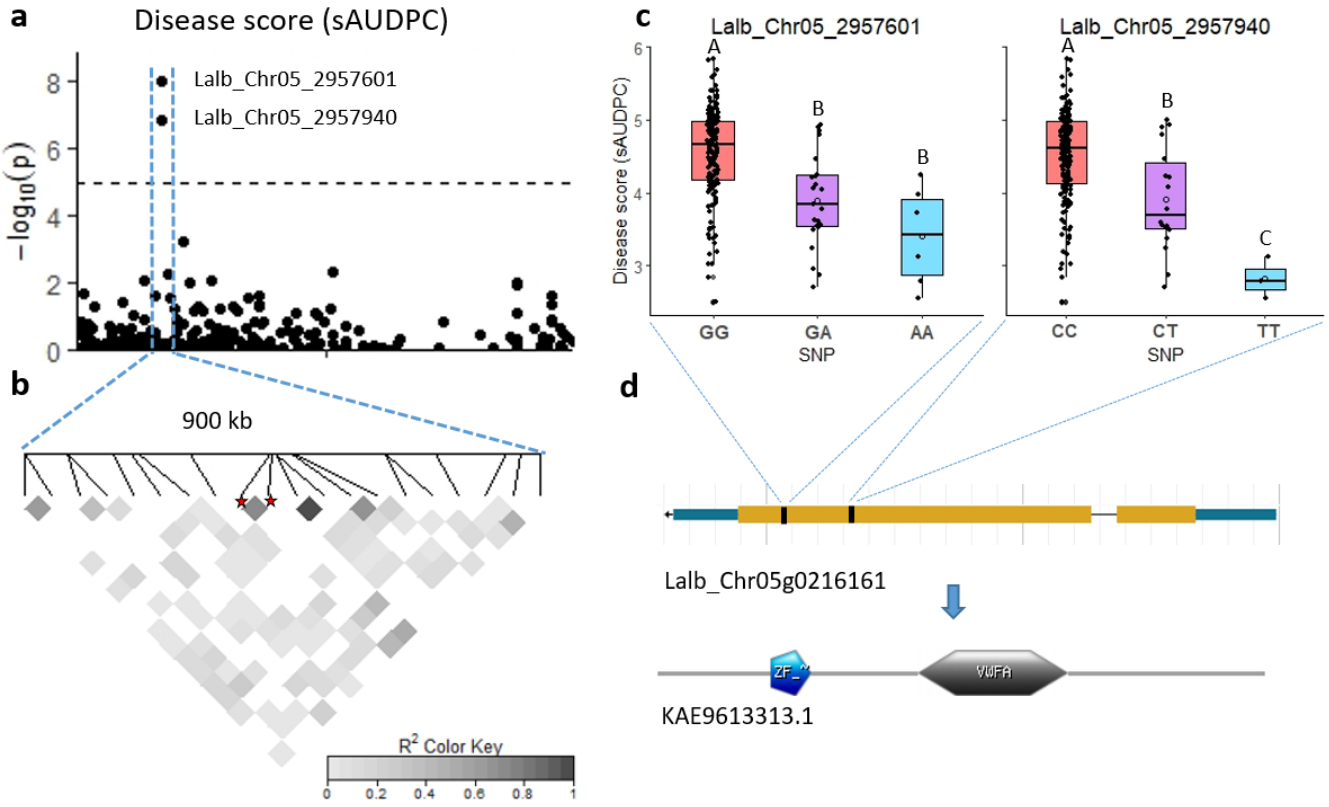

Figure 4: Characterization of SNP Lalb_Chr05_2957601 & -940. a: Manhattan plot of chromosome 5, dashed line indicates Bonferroni corrected LOD threshold of 4.96 (*P* = 1.08E-5). **b:** Linkage disequilibrium (LD) heatmap of 20 SNPs within 500 kb of significant SNPs (stars). **c:** Boxplots showing allele effect on disease score (standardized area under the disease progress curve). Capital letters within plot indicate significant difference (Tuckey-HSD, $P \leq 0.05$), **d:** Candidate gene Lalb_Chr05g0216161, showing protein coding region (orange) and spliced non-coding RNA (blue; www.whitelupin.fr), and corresponding protein KAE9613313.1 with Von Willebrand factor type A (VWFA) and RING zinc-finger domain (ZF; De Castro et al. (2006)).

GWAS analysis identified two significant SNPs, Lalb_Chr05_2957601 and 2957940, on chromosome 5 and SNPs tending towards significance on chromosome 1, 5, 8 and 13, associated with anthracnose resistance. These SNPs do not correspond to previously reported QTLs associated with anthracnose or phomopsis (*Diaporthe toxica*) resistance in white lupin (Cowley et al. 2014; Książkiewicz et al. 2017). Corresponding candidate genes also do not reflect anthracnose resistance genes identified in blue lupin, including *Lanr1, Anman* and *Lanrbo* (Fischer et al. 2015; Yang et al. 2004; Yang et al. 2008). The two significant SNPs together explained 16 to 28% of disease phenotypic variance, confirming anthracnose resistance to be a polygenic trait. The MAF for these SNPs was low and non-references alleles were only homozygous in Chilean, Ethiopian and Moroccan genotypes, but were also present in wild *graecus* types (LD37, GR38, and Batsi; Hufnagel et al. (2021)). Both SNPs are located in the same coding region of Lalb_Chr05g0216161 which encodes for a protein with a RING zinc-finger and VWFA domain. Homologs in closely related legume species encode RING zinc-finger E3 ubiquitin-protein ligases. Lalb_Chr13g0297901, which is located 1 kb downstream of peak SNP Lalb_Chr13_12108967, and its homologs in closely related species, also encode RING (H2) type E3 ubiquitin ligases but lack a VWF domain. E3 ubiquitin-ligases have frequently been shown to be involved in different steps of plant immunity (Duplan and Rivas 2014; Marino et al. 2012; Zhou and Zeng 2017). In pepper (*Capsicum annuum*) the RING finger protein gene, *CaRFP1*, containing a VWFA domain, was shown to act as E3 ubiquitin ligase and was highly upregulated during *C. coccodes* infection (Hong et al. 2007). Other RING type E3 ubiquitin ligases were shown to influence resistance against *Magnaporthe oryzae* in rice (Park et al. 2016), *Xanthonomas* infection in *C. annuum* (Lee et al. 2011), and *Ralstonia solanacearum* in tobacco (Ghannam et al. 2016). Besides biotic stress, RING E3 ubiquitin ligases have shown to improve resistance against abiotic stresses (Cho et al. 2017; Lee and Kim 2011), such as drought (Cheng et al. 2012) and salt stress (Kim and Kim 2013), and were shown to be involved in various plant developmental processes (Shu and Yang 2017), such as root development (Sakai et al. 2012). In conclusion, the identified gene, Lalb_Chr05g0216161, might play an important role in anthracnose resistance in white lupin and should be further investigated.

The genes Lalb_Chr05g0217471 (SNP Lalb_Chr05_3784474) and Lalb_Chr01g0012261 (8 kb downstream of SNP Lalb_Chr01_12025845) both encode for leucine-rich repeat (LRR) containing receptor like proteins with a serine/threonine kinase or F-box domain, respectively, while locus Lalb_Chr01g0015651 (SNP Lalb_Chr01_15792483) only includes a serine/threonine kinase. LRR receptor kinases are well known as resistance genes and for their role in plant immunity (Afzal et al. 2008; Ellis et al. 2000; Tang et al. 2017). In *Arabidopsis,* the LRR receptor kinase BAK1 was involved in resistance against *C. higginsianum* (Yamada et al. 2016). Serine/threonine kinases were shown to be involved in signaling during pathogen recognition and subsequent activation of plant defense mechanisms (Afzal et al. 2008; Goff and Ramonell 2007). LRR receptor-like serine/threonine-protein kinases were shown to confer resistance against apple scab (*Venturia inaequalis*) in apple (Padmarasu et al. 2018) and against rice blast (*Magnaporthe grisea*) in rice (Song et al. 2008). F-box proteins are part of SCF protein complexes, which are the best

characterized type of E3 ubiquitin ligases, and confer substrate specificity (Cardozo and Pagano 2004; Zheng et al. 2002). In plants, F-box genes form a large multigene superfamily and control many important biological functions, such as embryogenesis, seedling development, senescence, and pathogen resistance (Lechner et al. 2006; van den Burg et al. 2008; Xu et al. 2009). Lalb_Chr01g0012271 (SNP Lalb_Chr01_12025845) encodes for a glycerol kinase, which have been shown to mediate in conversion of glycerol to glycerol-3-phosphate (G3P) that contributes to the resistance to *C. higginsianum* in *Arabidopsis* (Mandal et al. 2011; Venugopal et al. 2009). Furthermore, Lalb_Chr05g0217341 (SNP Lalb_Chr05_3706534) encodes for a paired amphipathic helix protein Sin3-like 4 protein, which is known to be involved in powdery mildew (*Podosphaera fusca*) resistance in cucumber (Liu et al. 2021). These identified loci, with a special emphasis on the LRR proteins, have a potential involvement in defense responses against anthracnose in white lupin that requires further investigation.

This study showed that GWAS, thanks to weak population structure, fast LD decay and the availability of a high-quality reference genome, is a powerful tool to identify resistance loci in white lupin and provides the basis for further gene mapping. Further characterization of identified candidate genes, specifically the E3 ubiquitin ligase encoding Lalb_Chr05g0216161, sheds first light on white lupin resistance mechanisms against anthracnose disease. The obtained dataset also provides a basis for marker-assisted selection and the development of genomic prediction models for anthracnose resistance. Overall, this study contributes to understanding the genetic make-up of anthracnose resistance in white lupin and supports future crop improvements.

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Author contribution

JAA, PH and MMM conceived the original idea for this study. PH and MMM acquired the funding for this project. JAA conducted the experiments and took the lead in manuscript writing. JAA analyzed the data with help from NN. CA planned and performed the field trials and provided seeds of genotypes Fibl001 - FiBL040. PA provided seeds of genotypes LAP002a - LAP0156a. BF contributed with providing lab assistance. JAA designed the figures and tables with input from NN, PH, PA, MMM, RTV and MRF, who

also significantly contributed to data interpretation and provided critical feedback that shaped the final version.

Data availability

The datasets generated during the current study are available at: https://doi.org/10.5281/zenodo.5142130

Supplementary

Table S1: White lupin germplasm collection and resistance to anthracnose[a].

Genotype	Accession	Origin	Subgroup	sAUDPC	P[b]	LS_{rel} (%)	P[c]	SFW_{rel}	P[d]	Supplier	Type
Fibl001	**Mutant 28**	**Soviet Union**	**II**	**3.85**	**1**	**61.7**	**1**	**0.68**	**1**	**IPK**	**C**
Fibl002	Hetman	Poland	II	3.53	0.34	45.5	0.02	0.64	1	IPK	C
Fibl003	La572	Algeria	II	4.11	1	71.1	1	0.55	1	IPK	LR
Fibl004	**Zagrebska**	**Yugoslavia**	**II**	**4.60**	**1**	**69.4**	**1**	**0.48**	**1**	**IPK**	**BL**
Fibl005	**Lup 2078**	**Ethiopia**	**II**	**2.49**	**1.3E-03**	**30.3**	**2.6E-03**	**0.80**	**0.75**	**IPK**	**LR**
Fibl006	**Lup 258**	**Ethiopia**	**II**	**3.49**	**0.50**	**43.7**	**0.08**	**0.72**	**0.97**	**IPK**	**LR**
Fibl007	La688	Algeria	NA	4.39	1	77.2	1	0.62	1	IPK	LR
Fibl008	Markt Valencia	Spain	NA	4.41	1	78.5	1	0.54	1	IPK	C
Fibl009	Vir 503	Egypt	II	4.09	1	64.8	1	0.56	1	VIR	LR
Fibl010	Kiev Mutant	Ukraine	II	4.57	1	68.8	1	0.47	1	POL	C
Fibl011	Lup 2076	Ethiopia	II	3.72	0.96	59.3	0.99	0.58	1	IPK	LR
Fibl012	Lup 2079	Ethiopia	II	2.95	1.4E-03	27.0	8.9E-07	0.81	0.39	IPK	LR
Fibl013	Bianca	Italy	II	3.78	1	66.8	1	0.47	1	IPK	C
Fibl014	**Frieda**	**Germany**	**II**	**2.69**	**1.6E-03**	**20.7**	**2.7E-07**	**0.81**	**0.23**	**DSV**	**C**
Fibl015	**Sulimo**	**France**	**II**	**4.71**	**1**	**84.4**	**1**	**0.45**	**1**	**JD**	**C**
Fibl016	**Blu-25**	**Chile**	**II**	**2.56**	**0.01**	**31.4**	**0.02**	**0.82**	**0.70**	**SB**	**BL**
Fibl017	**Feodora**	**France**	**II**	**4.55**	**1**	**73.7**	**1**	**0.47**	**1**	**JD**	**C**
Fibl018	Ten 4776	Tenerife	IV	5.02	1	93.5	0.89	0.50	1	ESP	LR
Fibl019	P27175	Ethiopia	III	4.94	1	72.5	1	0.20	0.01	DPIRD	LR
Fibl020	P27174	Ethiopia	NA	4.72	1	90.9	0.95	0.54	1	DPIRD	LR
Fibl021	**Figaro**	**France**	**II**	**4.84**	**1**	**79.8**	**1**	**0.52**	**1**	**JD**	**C**
Fibl022	**Zulika**	**Czech Republic**	**II**	**4.07**	**1**	**65.5**	**1**	**0.84**	**0.59**	**OSEVA a.s.**	**C**
Fibl023	**Dieta**	**UK**	**II**	**5.84**	**8.6E-04**	**92.2**	**0.48**	**0.19**	**2.5E-03**	**Soya UK**	**C**
Fibl024	Vir 104	Czechoslovakia	II	2.87	0.03	33.3	2.6E-04	0.79	0.89	VIR	LR
Fibl025	R-6020	Poland	II	3.54	0.48	42.0	6.2E-03	0.79	0.92	POL	BL
Fibl026	Lup 559	Ethiopia	II	2.83	0.01	15.2	1.1E-06	0.88	0.33	IPK	LR
Fibl027	Vir 296	Palestine	II	3.24	0.16	31.2	1.8E-03	0.88	0.27	VIR	LR
Fibl028	Por 4154	Portugal	IV	3.02	0.09	21.9	2.0E-04	1.14	0.03	ESP	LR
Fibl029	Ten 4774	Tenerife	IV	4.14	1	58.8	1	0.84	0.86	ESP	LR
Fibl030	Tol 4767	Spain	IV	4.63	1	77.9	1	0.46	1	ESP	LR
Fibl031	Kievskij Skorospelij	Ukraine	II	4.10	1	59.9	0.97	0.80	0.99	ESP	C
Fibl032	Kiev Mutant	Ukraine	II	4.86	1	87.8	0.99	0.47	1	ESP	C
Fibl033	La433	Egypt	II	4.80	1	68.2	1	0.28	0.20	ESP	LR
Fibl034	La441	Egypt	II	4.38	1	64.7	1	0.55	1	UWA	LR

Fibl035	La420	Sudan	II	4.67	1	82.5	1	0.51	1	UWA	LR
Fibl036	Murringo	Australia	II	3.38	0.43	46.6	0.25	0.72	0.96	UWA	LR
Fibl037	SUN6289B	Israel	II	5.25	0.60	88.4	0.99	0.46	1	UWA	BL
Fibl038	P27174	Ethiopia	III	3.49	0.50	68.0	1	0.65	1	UWA	LR
Fibl039	P27175	Ethiopia	III	2.50	7.2E-06	31.2	4.8E-07	0.79	0.36	UWA	LR
Fibl040	Lupini bean	UK	II	4.21	1	63.0	0.98	0.32	0.98	IPK	LR
LAP0002a	Ac045	Azores	IV	5.05	0.97	88.4	0.94	0.43	1	CREA	LR
LAP0002c	Ac045	Azores	IV	4.55	1	89.4	0.97	0.29	0.83	CREA	LR
LAP0003a	Ac050	Azores	IV	3.52	0.47	61.7	0.99	0.76	0.84	CREA	LR
LAP0003d	Ac050	Azores	IV	5.00	0.99	82.8	1	0.30	1	CREA	LR
LAP0005b	Ac079	Azores	IV	4.22	1	72.6	1	0.61	1	CREA	LR
LAP0005c	Ac079	Azores	NA	5.30	0.75	93.9	0.53	0.37	1	CREA	LR
LAP0006b	Ac085	Azores	IV	4.79	1	77.8	1	0.72	1	CREA	LR
LAP0008d	Ac140	Azores	IV	4.30	1	76.0	1	0.64	1	CREA	LR
LAP0011c	Gr003	Greece	NA	4.41	1	85.1	1	0.47	1	CREA	LR
LAP0012d	Gr005	Greece	NA	4.24	1	78.3	1	0.46	1	CREA	LR
LAP0013b	Gr017	Greece	IV	4.63	1	88.3	0.92	0.42	1	CREA	LR
LAP0014b	Gr021	Greece	NA	4.79	1	86.8	0.99	0.57	1	CREA	LR
LAP0015c	Gr025	Greece	NA	5.57	0.29	97.0	0.56	0.31	0.99	CREA	LR
LAP0018c	Gr049	Greece	NA	4.95	1	92.6	0.97	0.32	1	CREA	LR
LAP0019c	Gr056	Greece	IV	5.07	0.99	91.9	0.99	0.62	1	CREA	LR
LAP0019d	Gr056	Greece	IV	3.88	1	69.6	1	0.58	1	CREA	LR
LAP0020c	Gr057	Greece	IV	5.59	0.16	101.3	0.11	0.26	0.91	CREA	LR
LAP0021a	Tr001	Turkey	IV	5.30	0.80	88.3	0.98	0.38	1	CREA	LR
LAP0023a	Tr012	Turkey	IV	4.85	1	80.3	1	0.44	1	CREA	LR
LAP0024b	Tr016	Turkey	IV	3.83	0.99	62.7	1	0.79	0.98	CREA	LR
LAP0026c	Tr021	Turkey	IV	5.34	0.45	93.8	0.69	0.28	0.56	CREA	LR
LAP0027b	La110	Turkey	IV	4.43	1	78.7	1	0.45	1	CREA	LR
LAP0028b	La120	Turkey	II	3.55	0.70	49.0	0.55	0.78	0.81	CREA	LR
LAP0029b	La259	Turkey	IV	5.41	0.49	95.5	0.69	0.34	1	CREA	LR
LAP0030b	La431	Turkey	IV	5.16	0.93	90.2	0.73	0.29	0.94	CREA	LR
LAP0031b	E002	Spain	IV	4.78	1	80.5	1	0.56	1	CREA	LR
LAP0031d	E002	Spain	IV	3.81	0.97	50.8	0.74	0.87	0.51	CREA	LR
LAP0032d	E003	Spain	IV	4.22	1	69.9	1	0.52	1	CREA	LR
LAP0032e	E003	Spain	NA	3.85	0.97	74.8	1	0.60	1	CREA	LR
LAP0033c	E016	Spain	IV	4.53	1	75.2	1	0.61	1	CREA	LR
LAP0035d	E059	Spain	IV	3.86	1	54.6	0.92	0.75	1	CREA	LR
LAP0037c	E068	Spain	IV	3.36	0.25	47.1	0.46	0.72	0.82	CREA	LR
LAP0037d	E068	Spain	IV	3.42	0.81	44.7	0.50	0.45	1	CREA	LR
LAP0038b	E072	Spain	IV	4.35	1	81.1	1	0.56	1	CREA	LR
LAP0039d	E075	Spain	IV	4.82	1	88.2	0.95	0.64	1	CREA	LR
LAP0040a	E191	Portugal	II	4.06	1	66.3	1	0.95	0.29	CREA	LR
LAP0040c	E191	Portugal	IV	4.98	1	83.8	1	0.50	1	CREA	LR

LAP0041b	E080	Portugal	IV	4.43	1	75.7	1	0.45	1	CREA	LR
LAP0041c	E080	Portugal	IV	4.55	1	83.6	1	0.64	1	CREA	LR
LAP0042a	E091	Portugal	IV	4.77	1	78.8	1	0.35	1	CREA	LR
LAP0042b	E091	Portugal	III	5.02	1	78.2	1	0.47	1	CREA	LR
LAP0043b	E099	Portugal	IV	3.41	0.55	37.7	0.15	0.76	0.95	CREA	LR
LAP0043c	E099	Portugal	IV	4.46	1	75.3	1	0.46	1	CREA	LR
LAP0044c	E104	Portugal	IV	4.23	1	71.2	1	0.69	1	CREA	LR
LAP0045c	E107	Portugal	IV	5.00	1	74.7	1	0.38	1	CREA	LR
LAP0045d	E107	Portugal	IV	4.00	1	65.9	1	0.52	1	CREA	LR
LAP0046a	E112	Portugal	IV	4.71	1	83.0	1	0.61	1	CREA	LR
LAP0046b	E112	Portugal	IV	5.05	1	77.9	1	0.57	1	CREA	LR
LAP0047b	E126	Portugal	IV	5.03	1	88.2	1	0.43	1	CREA	LR
LAP0048c	E132	Portugal	IV	4.18	1	69.3	1	0.49	1	CREA	LR
LAP0049c	La127	Algeria	IV	4.66	1	74.3	1	0.67	1	CREA	LR
LAP0050c	La568	Algeria	IV	4.85	1	79.3	1	0.32	0.94	CREA	LR
LAP0051b	La572	Algeria	IV	3.85	0.97	54.1	0.90	0.79	0.99	CREA	LR
LAP0051c	La572	Algeria	IV	4.46	1	76.0	1	0.49	1	CREA	LR
LAP0052b	La686	Algeria	IV	5.03	1	82.7	1	0.53	1	CREA	LR
LAP0052c	La686	Algeria	IV	4.31	1	56.0	0.84	0.44	1	CREA	LR
LAP0053b	La688	Algeria	IV	4.82	1	93.6	0.93	0.27	0.75	CREA	LR
LAP0053d	La688	Algeria	IV	4.86	1	77.6	1	0.24	0.18	CREA	LR
LAP0054a	La060	Morocco	IV	3.63	0.75	66.3	1	0.86	0.46	CREA	LR
LAP0054c	La060	Morocco	IV	4.86	1	84.7	1	0.30	0.98	CREA	LR
LAP0055a	La150	Morocco	II	4.03	1	52.4	0.84	0.59	1	CREA	LR
LAP0055b	La150	Morocco	II	3.97	1	79.3	1	0.87	0.95	CREA	LR
LAP0055c	La150	Morocco	II	4.24	1	74.2	1	0.80	0.93	CREA	LR
LAP0055d	La150	Morocco	II	4.25	1	72.4	1	0.33	1	CREA	LR
LAP0057a	La197	Madeira	IV	4.86	1	94.8	0.48	0.34	0.98	CREA	LR
LAP0057d	La197	Madeira	IV	4.38	1	78.6	1	0.50	1	CREA	LR
LAP0058c	La198	Madeira	IV	4.61	1	71.2	1	0.44	1	CREA	LR
LAP0059a	La641	Canaries	IV	5.16	0.91	87.3	1	0.48	1	CREA	LR
LAP0060c	La642	Canaries	IV	4.50	1	80.2	1	0.53	1	CREA	LR
LAP0060d	La642	Canaries	IV	3.02	0.09	57.1	0.94	0.76	0.75	CREA	LR
LAP0061c	La646	Canaries	IV	4.02	1	59.5	1	0.56	1	CREA	LR
LAP0061d	La646	Canaries	IV	4.83	1	91.7	0.64	0.29	0.89	CREA	LR
LAP0062d	La648	Canaries	IV	4.86	1	81.0	1	0.37	0.92	CREA	LR
LAP0063a	La652	Canaries	II	5.00	0.99	89.1	0.99	0.36	0.80	CREA	LR
LAP0063b	La652	Canaries	IV	5.22	0.93	99.8	0.18	0.27	0.94	CREA	LR
LAP0064b	La653	Canaries	IV	4.12	1	76.6	1	0.58	1	CREA	LR
LAP0065a	La425	Syria	II	4.89	1	81.6	1	0.42	0.99	CREA	LR
LAP0066b	La427	Syria	IV	3.51	0.70	58.3	0.95	0.66	1	CREA	LR
LAP0066c	La427	Syria	I	4.93	1	98.7	0.27	0.27	0.88	CREA	LR
LAP0067a	La547	Syria	IV	5.23	0.96	99.7	0.44	0.31	1	CREA	LR

LAP0068c	La416	Lebanon	I	4.94	1	86.8	1	0.48	1	CREA	LR
LAP0069c	La418	Lebanon	I	4.98	0.99	94.4	0.71	0.30	0.84	CREA	LR
LAP0070a	La673	Lebanon	I	4.89	1	93.6	0.88	0.44	1	CREA	LR
LAP0070c	La673	Lebanon	I	5.10	0.98	92.9	0.76	0.35	0.98	CREA	LR
LAP0071a	La406	Israel	I	4.94	1	86.3	0.98	0.33	0.99	CREA	LR
LAP0071d	La406	Israel	I	4.46	1	92.3	0.95	0.37	1	CREA	LR
LAP0072b	La409	Israel	II	5.70	0.37	76.6	1	0.25	0.84	CREA	LR
LAP0073d	La415	Jordan	I	4.99	1	95.7	0.78	0.43	1	CREA	LR
LAP0074c	La432	Jordan	III	5.09	1	81.6	1	0.49	1	CREA	LR
LAP0075d	La654	Kenya	IV	4.41	1	88.9	0.98	0.57	1	CREA	LR
LAP0076a	La655	Kenya	II	5.14	0.99	98.5	0.28	0.27	0.90	CREA	LR
LAP0077a	La656	Kenya	II	4.05	1	64.3	1	0.75	0.99	CREA	LR
LAP0077c	La656	Kenya	II	5.00	1	94.8	0.94	0.41	1	CREA	LR
LAP0078a	La020	Ethiopia	II	4.36	1	83.1	1	0.57	1	CREA	LR
LAP0079a	La559	Ethiopia	II	4.83	1	92.3	0.92	0.35	1	CREA	LR
LAP0079d	La559	Ethiopia	II	4.86	1	75.6	1	0.28	0.94	CREA	LR
LAP0080a	La399	Ethiopia	II	3.19	0.12	48.8	0.22	1.02	0.04	CREA	LR
LAP0080b	La399	Ethiopia	II	4.36	1	68.0	1	0.42	1	CREA	LR
LAP0081b	La420	Sudan	II	4.47	1	67.5	1	0.63	1	CREA	LR
LAP0081d	La420	Sudan	II	4.12	1	67.8	1	0.78	0.98	CREA	LR
LAP0082b	La422	Sudan	II	4.85	1	87.1	0.99	0.66	1	CREA	LR
LAP0082d	La422	Sudan	II	4.95	1	93.0	0.75	0.48	1	CREA	LR
LAP0083a	La629	Sudan	II	4.99	1	94.0	0.95	0.34	1	CREA	LR
LAP0083d	La629	Sudan	III	4.68	1	58.6	0.96	0.30	0.56	CREA	LR
LAP0084c	Ethiopie98	Ethiopia	III	4.39	1	83.7	1	0.37	0.99	CREA	LR
LAP0084d	Ethiopie98	Ethiopia	III	4.27	1	91.2	0.98	0.53	1	CREA	LR
LAP0086a	Egypte011	Egypt	III	5.41	0.49	88.4	1	0.21	0.23	CREA	LR
LAP0086d	Egypte011	Egypt	II	4.77	1	69.0	1	0.53	1	CREA	LR
LAP0087c	Egypte026	Egypt	III	4.99	1	88.2	0.99	0.27	0.58	CREA	LR
LAP0087d	Egypte026	Egypt	III	5.11	0.99	78.9	1	0.36	1	CREA	LR
LAP0088a	Egypte016	Egypt	IV	4.46	1	61.4	0.96	0.48	1	CREA	LR
LAP0089b	Egypte022	Egypt	III	4.55	1	70.9	1	0.70	1	CREA	LR
LAP0089d	Egypte022	Egypt	III	4.20	1	73.6	1	0.55	1	CREA	LR
LAP0090c	Egypte038	Egypt	III	5.05	1	79.1	1	0.59	1	CREA	LR
LAP0090d	Egypte038	Egypt	III	4.44	1	73.7	1	0.69	1	CREA	LR
LAP0091a	Egypte055	Egypt	II	3.89	0.99	56.4	0.99	0.95	0.33	CREA	LR
LAP0091b	Egypte055	Egypt	III	4.85	1	87.9	1	0.57	1	CREA	LR
LAP0092b	Egypte064	Egypt	IV	4.14	1	67.2	1	0.44	1	CREA	LR
LAP0092d	Egypte064	Egypt	III	3.30	0.26	43.3	0.23	0.63	0.99	CREA	LR
LAP0093c	Egypte076	Egypt	III	3.58	0.62	26.6	4.2E-04	0.80	0.98	CREA	LR
LAP0093d	Egypte076	Egypt	III	3.16	0.09	19.6	2.7E-05	0.76	0.90	CREA	LR
LAP0094b	Egypte093	Egypt	II	5.13	0.99	96.7	0.58	0.55	1	CREA	LR
LAP0094d	Egypte093	Egypt	III	4.89	1	71.5	1	0.47	1	CREA	LR

LAP0095a	La356	Egypt	III	5.30	0.70	97.7	0.52	0.36	0.97	CREA	LR
LAP0095b	La356	Egypt	III	4.82	1	75.0	1	0.47	1	CREA	LR
LAP0096a	La364	Egypt	II	4.43	1	79.2	1	0.59	1	CREA	LR
LAP0096d	La364	Egypt	II	5.19	0.93	72.0	1	0.43	1	CREA	LR
LAP0097a	Ita001	Italy	II	4.85	1	72.3	1	0.76	1	CREA	LR
LAP0097b	Ita001	Italy	NA	5.00	1	87.8	0.94	0.58	1	CREA	LR
LAP0098c	Ita002	Italy	NA	4.74	1	86.7	1	0.47	1	CREA	LR
LAP0098d	Ita002	Italy	NA	3.95	1	85.1	1	1.11	0.07	CREA	LR
LAP0099a	Ita037	Italy	II	4.10	1	62.3	1	0.84	0.99	CREA	LR
LAP0100b	Ita042	Italy	IV	5.82	6.6E-03	100.5	0.09	0.17	2.3E-03	CREA	LR
LAP0101b	Ita051	Italy	NA	5.68	0.03	98.2	0.30	0.20	0.01	CREA	LR
LAP0101c	Ita051	Italy	NA	4.24	1	70.4	1	0.58	1	CREA	LR
LAP0102d	Ita019	Italy	NA	4.25	1	66.1	1	0.89	0.45	CREA	LR
LAP0104d	Ita049	Italy	NA	4.32	1	91.2	0.97	0.62	1	CREA	LR
LAP0105a	Ita057	Italy	NA	4.91	1	88.2	1	0.30	0.98	CREA	LR
LAP0105d	Ita057	Italy	IV	3.60	0.73	40.2	0.06	0.77	0.74	CREA	LR
LAP0106a	Ita058	Italy	IV	4.04	1	68.7	1	0.67	1	CREA	LR
LAP0107b	La246	Madeira	IV	3.74	0.91	55.4	0.78	0.83	0.63	CREA	LR
LAP0108a	Calabria	Italy	IV	4.17	1	74.9	1	0.82	0.97	CREA	LR
LAP0110a	Maroc78	Morocco	NA	4.00	1	55.3	0.83	0.49	1	CREA	LR
LAP0110d	Maroc78	Morocco	IV	4.83	1	92.7	0.78	0.45	1	CREA	LR
LAP0111a	Ares	France	II	4.49	1	74.1	1	0.57	1	CREA	C
LAP0112b	Lublanc	France	II	5.39	0.61	91.4	0.79	0.36	0.46	CREA	C
LAP0113b	**Amiga**	**France**	**II**	**4.39**	**1**	**69.7**	**1**	**0.67**	**1**	**FD**	**C**
LAP0115b	Energy	France	II	5.18	0.73	83.5	1	0.25	0.02	CREA	C
LAP0117b	Adam	France	II	3.97	1	80.1	1	0.45	1	CREA	C
LAP0118b	Luxe	France	IV	5.62	0.01	92.4	0.63	0.44	1	CREA	C
LAP0119b	Aster	France	II	5.47	0.05	95.2	0.22	0.38	0.84	CREA	C
LAP0123d	Molise	France	IV	4.27	1	66.2	1	0.69	1	CREA	C
LAP0124b	Lecce	France	IV	4.57	1	91.1	1	0.38	1	CREA	C
LAP0126a	Ludet	France	II	5.10	0.87	95.0	0.47	0.36	1	CREA	C
LAP0150/4	Lucky	France	II	4.73	1	92.8	0.90	0.38	1	CREA	C
LAP0151d	MB-38	Italy	II	5.04	1	93.5	0.76	0.45	1	CREA	C
LAP0153/1	Imane	France	II	3.93	1	70.0	1	0.59	1	CREA	C
LAP0155a	Rumbo Baer(a)	Chile	II	2.78	0.04	50.3	0.37	0.92	0.19	SB	C
LAP0155b	Rumbo Baer(b)	Chile	II	3.11	0.12	48.7	0.29	0.94	0.15	SB	C
LAP0156a	Multitalia	Italy	IV	4.72	1	87.4	1	0.59	1	CREA	C

[a] Bold are accessions tested in the field, sAUDPC = standardized area under the disease curve. LS_{rel} = relative lesion size (%), SFW_{rel} = relative shoot fresh weight. [b] P-value sAUDPC compared to overall mean (Dunnett's test), [c] P-value LS_{rel} compared to overall mean (Dunnett's test), [d] P-value SFW_{rel} compared to overall mean (Dunnett's test). UWA: University of Western Australia, Centre for Legumes in Mediterranean, Western Australia; ESP: Instituto Nacional de Investigacion y Tecnologia Agraria y Alimentaria, Centro Nacional de Recursos Fitogeneticos, Spain; DPIRD: Department of Primary Industries and

Regional Development, Agriculture and Food division, Western Australia; POL: Poznan Plant Breeders Ltd., Poland; VIR: Vavilov Research Institute of Plant Industry, Russia; DSV: Deutsche Saatveredelung AG, Germany; IPK: Leibniz Institute of Plant Genetics and Crop Plant Research, Germany; JD: Jouffray Drillaud, France; SB: Semillas Baer, Chile; FD: Florimond Desprez, France; CREA: Research Centre for Animal Production and Aquaculture, Italy. C: Cultivar; BL: Breeding line; LR: Landrace.

Table S2: Primers used for library preparation.

Primers	**Sequence**
PCRprimer1	AAT GAT ACG GCG ACC ACC GAG ATC TAC ACT CTT TCC CTA CAC GAC GCT CTT CCG ATC T
PCRprimer2	CAA GCA GAA GAC GGC ATA CGA GAT CGG TCT CGG CAT TCC TGC TGA ACC GCT CTT CCG ATC T

Table S3: Correlation between disease assessments under controlled and three-year Swiss field conditions [a].

Trait	sAUDPC Field	Yield (dt/ha) Field	sAUDPC CC	LS_{rel} CC	SFW_{rel} CC
sAUDPC Field	1.00				
Yield (dt/ha) Field	-0.79*	1.00			
sAUDPC CC	0.86*	-0.56•	1.00		
LS_{rel} CC	0.87*	-0.61*	0.96*	1.00	
SFW_{rel} CC	-0.80*	0.53•	-0.92*	-0.86*	1.00

[a] sAUDPC = standardized area under the disease progress curve, LS_{rel} = relative lesion size (%), SFW_{rel} = relative shoot fresh weight, CC = controlled conditions, correlations are expressed as Pearson correlation coefficients. * $P \leq 0.05$, • $P \leq 0.1$.

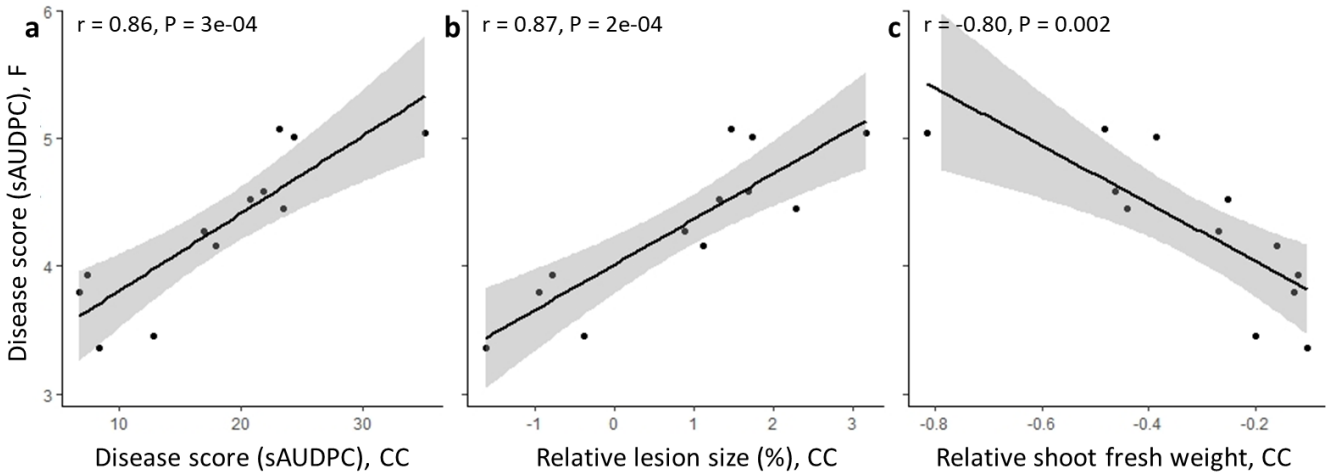

Figure S1: Correlations between anthracnose-related traits assessed under controlled conditions and three-year averaged disease scores assessed in the field. CC = controlled conditions, F = field, sAUDPC = standardized area under the disease progress curve, r = Pearson correlation coefficient.

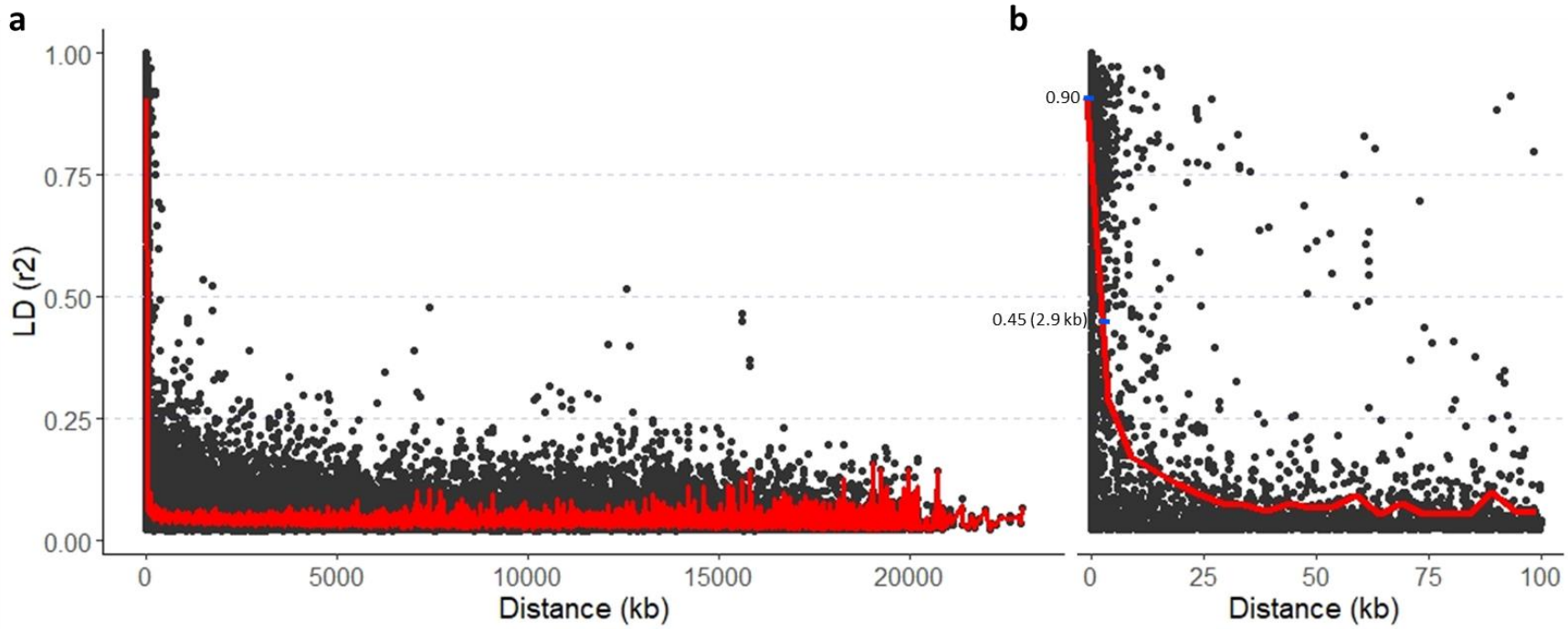

Figure S2: Genome-wide linkage disequilibrium (LD) decay based on 181 white lupin genotypes. a: Whole genome ~23000 kb. **b:** 100 kb, the decay of LD with physical distance between SNPs to half of the maximum values occurred at 2.9 kb (r^2 =0.45).

Table S4: SNPs tending towards significance associated with anthracnose resistance [a].

Trait	SNP[b]	Alleles	P value	R^2_{LR}	MAF	Candidate gene(s)	Location[c]	Annotation
Disease Score (sAUDPC)	Lalb_Chr01_12025845	A/G	7.97E-05	0.08	0.08	Lalb_Chr01g0012271	36b >	Putative glycerol kinase
	Lalb_Chr01_12025863	A/G	9.60E-01	0.00	0.06	Lalb_Chr01g0012261	8 kb <	Putative leucine-rich repeat domain, L domain-containing protein
Relative Lesion size (%)	Lalb_Chr05_3706534	A/G	2.46E-05	0.10	0.16	Lalb_Chr05g0217341	Exon	Putative transcription regulator Others family
	Lalb_Chr05_3688076	A/G	5.83E-01	0.00	0.16	Lalb_Chr05g0217331	Exon	Putative RNA helicase
	Lalb_Chr05_3784474	A/G	6.64E-01	0.00	0.15	Lalb_Chr05g0217471	Exon	Putative non-specific serine/threonine protein kinase
						Lalb_Chr05g0217461	3 kb <	Putative protein enhanced disease resistance 2
Relative shoot fresh weight	Lalb_Chr01_15792483	T/C	6.73E-05	0.08	0.11	Lalb_Chr01g0015651	Exon	Putative protein kinase AGC-RSK-2 family
	Lalb_Chr08_12561556	A/G	1.20E-04	0.08	0.12	Lalb_Chr08g0239611	Intron	Putative ribosomal protein S30Ae/sigma 54 modulation protein
						Lalb_Chr08g0239621	2.5 kb >	Putative transcription regulator mTERF family
	Lalb_Chr13_12108967	G/A	2.26E-05	0.09	0.43	Lalb_Chr13g0297911	Exon	protease Do-like 9
						Lalb_Chr13g0297901	3 kb <	Putative transcription factor C2H2 family
	Lalb_Chr13_12143224	C/A	1.27E-02	0.03	0.09	Lalb_Chr13g0297971	Intron	Putative oxidoreductase

[a] SNP = Single-nucleotide polymorphism, MAF = minor allele frequency, R^2_{LR} = likelihood-ratio-based R^2. [b] Lalb = *Lupinus albus*, Chr = chromosome, number = position on chromosome. SNPs in italics are linked. [c] < = downstream, > = upstream.

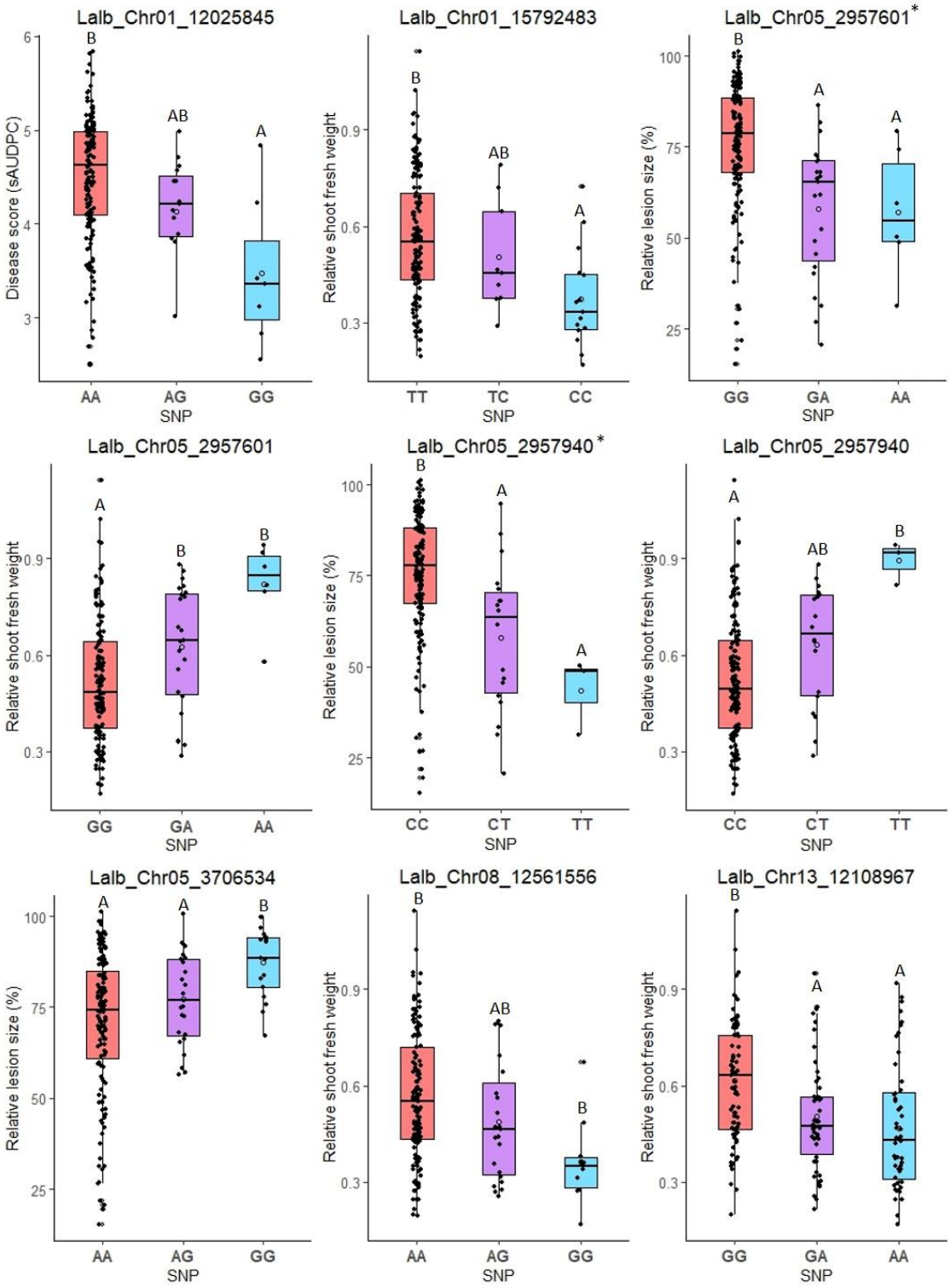

Figure S3: Boxplots showing allele effect on disease score, relative lesion size (%) and relative shoot fresh weight for SNPs associated with anthracnose resistance. Capital letters within plot indicate significant difference (Tuckey-HSD, $P \leq 0.05$). Asterisks (*) indicate significant SNPs. sAUDPC = standardized area under the disease progress curve.

Chapter 5

The potential of non-synthetic seed treatments to control anthracnose disease in white lupin

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Abstract

White lupin (*Lupinus albus* L.) is a promising crop to fulfill the rising global demand for plant-based protein. The seed-borne pathogen *Colletotrichum lupini*, however, threatens lupin cultivation worldwide. Seed dressings of synthetic fungicides were shown effective to reduce infection levels, but their negative environmental impact and exclusion from organic production calls for more sustainable solutions. In this study, a total of eleven different non-synthetic seed treatments was tested in field trials in Switzerland between 2018 and 2021. Treatment types consisted of hot water, steam, electron, long-term storage, vinegar, plant extracts and biological control agents (BCAs). The BCAs were tested for potential antagonistic activity against *C. lupini* during white lupin infection under controlled conditions prior to field trials. Long-term storage and vinegar treatments successfully reduced disease incidence and increased yield to levels similar to those observed for certified seeds, without significantly affecting germination rate. Three BCAs showed significant disease reductions under controlled conditions but not in the field, highlighting the importance of field validations. Besides lowering disease severity, two BCAs also reduced *C. lupini* DNA in stem tissue. Although this study was able to identify promising alternative seed treatments, more research is necessary to explore the optimal application conditions to further improve white lupin seed quality and successfully eradicate *C. lupini*.

Keywords

Biological control agents, seed dressing, *Colletotrichum lupini, Lupinus albus*, storage, vinegar.

Introduction

White lupin (*Lupinus albus* L.) is a grain legume known for its high protein content (36 to 38 %), nutritional value and its rare capability of forming specialized cluster roots that can mobilize poorly available phosphorus sources and drastically improve nutrient acquisition (Annicchiarico 2008; Arnoldi et al. 2015; Gallardo et al. 2019; Lambers et al. 2013). Since the development of sweet, low alkaloid varieties (Kroc et al. 2017), white lupin has received increasing attention from the food and feed industry (Lucas et al. 2015). As the demand for animal protein is projected to double by 2050 (Westhoek et al. 2011), the demand for plant-based protein is expected to rise as well. To full-fill this increasing demand, white lupin could be a sustainable addition to imported soybean in temperate regions. One of the main threats limiting cultivation is anthracnose disease, caused by the seed- and air-borne ascomycete *Colletotrichum lupini* (Damm et al. 2012; Talhinhas et al. 2016). The current global outbreak is caused by highly aggressive strains of genetic group II (Alkemade et al. 2021b; Dubrulle et al. 2020a). *C. lupini* is presumed to be a hemi-biotrophic pathogen (De Silva et al. 2017; Dubrulle et al. 2020b), colonizing the host endophytically and causing the typical disease symptoms of stem and pod twisting and bending upon flowering. This is followed by the formation of necrotic lesions containing orange masses of conidia which are rain-splash dispersed within the crop, leading to secondary infections (Thomas and Sweetingham 2004; White et al. 2008). Infected seeds are the primary source of inoculum, and small amounts of infected seeds (0.1%) can already cause severe yield losses (Thomas and Sweetingham 2004). As infected seeds are often symptomless they are the most important vehicles for spreading aggressive *C. lupini* strains across the world (Elmer 2001).

Current disease control mainly relies on planting pathogen-free seeds and foliar fungicide application (White et al. 2008). Pathogen-free seeds are produced under strict phytosanitary control in environments unfavorable for the disease and molecular detection methods have been developed to determine seed infection levels (Kamber et al. 2021; Pecchia et al. 2019). Fungicide seed treatments, such as thiram, can reduce inoculum viability and transmission (Talhinhas et al. 2016; Thomas et al. 2008a) but are considered problematic due to their environmental impact (Zubrod et al. 2019) and are not suitable for organic cultivation systems. Alternative non-synthetic seed treatments showed to be successful against fungal seed-borne pathogens in various vegetable crops (Mancini and Romanazzi 2014). Seed treatments through dry-heat (Falconí and Yánez–Mendizábal 2016; Thomas and Adcock 2004), UV (Falconí and Yánez-Mendizábal 2018) or long-term storage (Thomas and Sweetingham 1999) showed promising results in reducing *C. lupini* infection in lupin. Hot water seed treatments have been effective against many seed-borne pathogens (Sharma et al. 2015), and showed to reduce *Colletotrichum* inoculum viability in various crops (Mangwende et al. 2020; Yamagishi et al. 2015). The mustard powder based product Tillecur and thyme oil showed promising results against *C.*

lindemuthianum in bean and *Ascochyta* spp. in pea (Tinivella et al. 2009). Although these treatments are promising, none have been tested adequately in the field.

Biological control agents (BCAs) also offer great potential to control seed-borne disease (Mancini and Romanazzi 2014; Rocha et al. 2019; Tinivella et al. 2009). In blue and Andean lupin, seed treatments with the widely used BCA *Bacillus subtilis* effectively reduce anthracnose incidence (Mandrik et al. 2007; Yánez-Mendizábal and Falconí 2018). The bacterial species *Paraburkholderia phytofirmans* PsJN induces resistance mechanisms in plants through seed application (Esmaeel et al. 2018), and is able to colonize and induce growth promotion in lupin (Kost et al. 2014). *Streptomyces griseoviridis* seed treatment was effective in reducing *C. lupini* incidence in blue lupin (Mandrik et al. 2007), while the BCA *Pseudomonas fluorescens* reduced anthracnose disease in common bean (Amin et al. 2014), but not in lupin (Mandrik et al. 2007). The fungus *Clonotastachys rosea* is widely used as BCA is effective against *C. lindemuthianum* in bean (Tinivella et al. 2009) and *C. acutatum* in blue berry (Verma et al. 2006). *Trichoderma* spp. are known as potent fungal BCAs (Sharma and Gothalwal 2017) and, among other act antagonistic on *C. truncatum* infecting soybean (Begum et al. 2010), and induced resistance in pre-treated chili and bean seeds against *C. truncatum* (Yadav et al. 2021) and *C. lindemuthianum* (Amin et al. 2014), respectively. Taken together, alternative seed treatments show potential to reduce *C. lupini* incidence in white lupin, but systematic research is required to identify a treatment against this notorious pathogen which is effective under field conditions.

This study aimed to identify sustainable non-synthetic seed treatments to reduce anthracnose disease in white lupin. Prior to field trials, six different bacterial and fungal BCAs were screened under controlled conditions. Four BCAs and seven other non-synthetic seed treatments (including hot water, steam, electron, long-term storage, vinegar and plant extracts) were tested on infected seeds under field conditions in Switzerland in an attempt to identify sustainable seed treatments that can considerably improve seed health and disease management of *C. lupini* in white lupin.

Materials and Methods

Biological control agent treatments under controlled conditions

Six biological control agents (BCAs) were selected, four bacterial species: *Pseudomonas fluorescens* G308 (Pflu-G308), *Bacillus subtilis* HG77 (Bsub-HG77), *Paraburkholderia phytofirmans* PsJN (Pphy-PSjN) and *Streptomyces griseoviridis* (Mycostop), and two fungal species: *Clonostachys rosea* (PreStop), *Trichoderma asperellum* (T-Gro; **Table 1**). BCA efficacy was tested on stem wound inoculated plants as described in Alkemade et al. (2021a), applying 5 µl of *C. lupini* (strain JA01) spore suspension (10^5/ml) on 14 day old seedlings. Certified white lupin seeds of cv. Feodora were used, obtained from Jouffray-Drillaud (Cissé, FR). Two different application methods, seed dressing and stem wounding, were tested (**Table 1**). For stem

wounding, BCA suspensions were mixed with the *C. lupini* spore suspension prior to inoculation. For seed dressing, seeds were soaked for 30 s in 5 ml BCA suspension prior to sowing. Experiments were performed in a randomized complete block design with a minimum of five replicates per experiment, which was repeated thrice. Disease was assessed at 3, 7 and 10 days post inoculation (dpi) using a 1 to 9 disease index score (DSI) to calculate the standardized area under disease progress curve (sAUDPC) as described in Alkemade et al. (2021a). At 10 dpi, lesion size and stem length were determined to calculate lesion size relative to overall stem length.

DNA extraction and qPCR

After stem wound application of the four BCAs: Bsub-HG77, Pphy-PSjN, Mycostop and PreStop, quantitative real time PCR (qPCR) was performed to quantify *C. lupini* DNA in stem tissue 1 cm above the inoculation site at 10 dpi. Genomic DNA was extracted following a cetyltrimethylammonium bromide (CTAB) extraction protocol described by Kamber et al. (2021). *C. lupini* DNA was quantified by performing qPCR on the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene in a S1000 Thermal Cycler (Bio-Rad, CA, USA) using the primers, GAPDH_F "5'-CCCACGGCAAAAGAGTCAGAA-3'" and GAPDH_R "5'-CAATCATGCCGAAACAGCCG-3'", and a fluorogenic hydrolysis probe GAPDH_P "5'-CGTCGTGTCATTACAACAAGCCC-3'" as described in Kamber et al. (2021). The reaction was performed using a Rotor Gene Q cycler (Qiagen, CH). Threshold was automatically determined by Rotor-Gene Q Series Software 2.3.1. *C. lupini* DNA quantity was expressed as 45 minus obtained cycle numbers until threshold (CT) values.

Seed treatments in the field

Eleven seed treatments with a total of twenty-one different conditions were tested on infected seeds of white lupin cv. Feodora (**Table 2**). Six of those treatments were also tested on certified seeds (**Table S2**). Untreated infected and certified seeds were used as controls. Hot water and steam treatments were performed at Sativa (Rheinau, CH), see **Table 2**. The thyme oil emulsion (0.1%) and table vinegar (pH = 3) were applied by soaking seeds for 30 minutes and re-dried overnight at room temperature. Tillecur (Biofa, Münsingen, DE), a mustard-based product, was applied as powder on the seeds according to manufacturer's instructions. Electron treatments were performed at Evonta (Radeberg, DE) with different penetration depths and intensities. BCA Bsub-HG77 and Pphy-PSjN were applied by soaking seed for one hour in a bacterial solution of an optical density $(OD)_{600}$ of 0.25, seeds were re-dried overnight at room temperature. BCA product Mycostop was applied as powder on the seeds according to manufacturer's instructions. BCA product Prestop was foliar applied three times, one time at plant emergence, 10 days after plant emergence and once at flowering according to manufacturer's instructions. Field trials were conducted between 2018 and 2021 in plots following a randomized complete block design in Switzerland in Feldbach, Mellikon, Rümikon and Full-Reuenthal as described in Alkemade et al.

(2021a). Seed germination was assessed by counting germinated plants 2 x 1 meter per plot, 30 days after sowing. Disease assessments were performed using a 1 to 9 DSI as described in Alkemade et al. (2021a), with "1" being completely healthy and "9" completely diseased. Disease assessments were performed three to four times per growing season (60, 80, 100 and 120 days after sowing). The standardized area under the disease progress curve (sAUDPC) was calculated to assess and compare disease progression (Jeger and Viljanen-Rollinson 2001). Yield (dt/ha) was assessed at harvest in mid or end August. SAUDPC, yield and germination rate are visualized relative to the untreated (infected) control.

Data analysis

Statistical analyses were performed with R 4.0.3 (R Core Team 2020) using the packages lme4 (Bates et al. 2015), lmerTest (Kuznetsova et al. 2017) and emmeans (Lenth et al. 2019), following a mixed linear model with factors of interest (i.e. treatment) as fixed and environment, environment x treatment, and replicated block nested in environment as random factors, after confirming the assumptions of normality of residuals and homogeneity of variance. For yield and germination rate field data, data was normalized to the "infected seed" control for each environment (**Table S1**). Field data are presented as estimated least-squares means using the aforementioned mixed model. To achieve a normal distribution, data were square root transformed. Mean separations between treatments and the non-treated control were analyzed using Dunnett's test ($p \leq 0.05$).

Table 1. Overview of biological control agent treatments tested under controlled conditions.

BCA	Species	Strain	Conditions SW	Conditions SD	Supplier
Pflu-G308	*Pseudomonas fluorescens*	G308	0.25 / 5 µl[a]	0.25 / 30 s[a]	Hohenheim University, DE
Bsub-HG77	*Bacillus subtilis*	HG77	0.25 / 5 µl[a]	0.25 / 30 s[a]	Hohenheim University, DE
Pphy-PSjN	*Paraburkholderia phytofirmans*	PSjN	0.25 / 5 µl[a]	0.25 / 30 s[a]	Austrian Institute of Technology, AT
Mycostop*	*Streptomyces griseoviridis*	-	1 x 10^6 / ml[b]	2.68 x 10^8 / ml[b]	Verdera, Espoo, FI
PreStop*	*Clonostachys rosea*	J1446	1 x 10^6 / ml[b]	2.68 x 10^8 / ml[b]	Verdera, Espoo, FI
T-Gro*	*Trichoderma asperellum*	kd	1 x 10^6 / ml[b]	2.68 x 10^8 / ml[b]	Andermatt Biocontrol, Grossdietwil, CH

SW: Stem wounding, SD: Seed dressing, [a] Concentrations in OD_{600}, [b] concentration in spores / ml, * Commercial product.

Table 2. Overview of infected seed treatments tested under Swiss field conditions.

Treatment	Description	Year	n	E	Source / company
Inf_seed	Infected seeds	2018, 19, 20, 21	35	7	Infected field harvest, half a year old
Cert_seed	Certified seeds	2018, 19, 20, 21	32	7	Jouffray-Drillaud, Cissé, FR
Hotwater_1	55℃ / 5 min	2018	4	2	Sativa, Rheinau, CH
Hotwater_2	55℃ / 10 min	2019, 20	14	4	Sativa, Rheinau, CH
Hotwater_3	65℃ / 10 min	2020	8	2	Sativa, Rheinau, CH
Hotwater_4	68℃ / 5 min	2020	8	2	Sativa, Rheinau, CH
Steam_1	63℃ / 270 s	2018	4	2	Sativa, Rheinau, CH
Steam_2	68℃ / 270 s	2018, 19	10	4	Sativa, Rheinau, CH
Steam_3	75℃ / 120 s	2019, 20	14	4	Sativa, Rheinau, CH
Steam_4	80℃ / 270 s	2020	8	2	Sativa, Rheinau, CH
Steam_5	80℃ / 300 s	2020	8	2	Sativa, Rheinau, CH
Storage	3 years / room temperature	2020	8	2	Infected field harvest Mellikon, CH (2017)
Thyme	Thyme oil, 0.1% / 30 minutes	2019	6	2	Thymian Thymol bio, Primavera, Oy-Mittelberg, DE
Tillecur	Mustard based powder, 1 kg / 100 kg seeds	2018	4	2	Biofa, Münsingen, DE
Vinegar	30 minutes	2020, 21	11	3	Coop, CH
E6	Penetration depth 1, intensity 2	2020	8	2	Evonta, Radeberg, DE
E7	Penetration depth 2, intensity 1	2020	8	2	Evonta, Radeberg, DE
E9	Penetration depth 3, intensity 1	2020	8	2	Evonta, Radeberg, DE
E11	Penetration depth 4, intensity 1	2020	8	2	Evonta, Radeberg, DE
Bsub-HG77[a]	0.25 (OD_{600}) / 1 h	2019	6	2	Hohenheim University, DE
Pphy-PSjN[a]	0.25 (OD_{600}) / 1 h	2019	3	1	Austrian Institute of Technology, AT
Mycostop[a]	1 kg / 150 kg seeds	2021	3	1	Verdera, Espoo, FI
PreStop[a]*	5 kg / ha (5 g / L), 3 times	2021	3	1	Verdera, Espoo, FI

n: number of replicates, E: environment, a: see **Table 1**. * Foliar application

Results

Pphy-PSjN and Prestop consistently reduce anthracnose severity under controlled conditions

The stem wound application of Prestop (3.2, $P < 0.001$), Pphy-PSjN (3.9, $P = 0.0015$) and T-Gro (4, $P = 0.02$) significantly reduced sAUDPC compared with the untreated control (4.8; **Fig. 1**). Application of Pflu-G308, Bsub-HG77 and Mycostop did not show a significant effect on sAUDPC, but the mean of Bsub-HG77 (4.3; $P = 0.06$) tended towards a significant reduction. For lesion size, application of Prestop, Pphy-PSjN and Bsub-HG77 significantly reduced mean lesion size to 29 % ($P < 0.001$), 43 % ($P = 0.001$) and 46 % ($P = 0.006$) of total stem length, respectively, compared with the untreated control (65%). Quantification of *C. lupini* DNA, 1 cm above the point of inoculation, showed a significant reduction for Bsub-HG77 (45-CT = 8.8, $P < 0.001$) and Prestop (45-CT = 10.8, $P < 0.001$) compared with the control (45-CT = 15.9; **Fig. 2**). For seed dressing, none of the tested BCAs showed a significant effect on sAUDPC or lesion size, with the mean sAUDPC of Mycostop (3.8, $P = 0.057$) tending towards a significant reduction compared to the untreated control (4.9; **Fig. 1**). Based on these results, BCAs Bsub-HG77, Pphy-PSjN, Mycostop and Prestop were tested under field conditions. Seed dressing with PreStop reduced germination rate by 70%, and was therefore foliar applied according to manufacturer's recommendations in the field trials.

Seed storage and vinegar reduce anthracnose incidence under field conditions

The seed treatments long-term storage (4.22, $P = 0.021$) and vinegar (4.46, $P = 0.049$) significantly reduced sAUDPC in the field compared to untreated infected seeds (5.37; **Fig. 3**) and are similar to the mean sAUDPC observed for certified seeds (4.46, $P > 0.05$). The disease reduction observed for hot water treatment 2 tended towards significance (4.56, $P = 0.063$). Yields were highly variable between environments (**Table S1**) and are therefore expressed as relative to infected (**Fig. 3**) or certified control (**Fig. S1**). Long-term storage tripled yield (3, $P < 0.001$), compared to the infected untreated control and was similar compared to relative yields observed for certified seeds (2.1, $P = 0.37$). None of the treatments showed a significant reduction in disease incidence or increase in yield compared to certified seeds. When applied on certified seeds, the different treatments did not result in any additional reduction in disease severity compared to the certified seed control (**Fig. S1**). Hot water treatment 3 and steam treatment 4, however, caused almost complete yield loss.

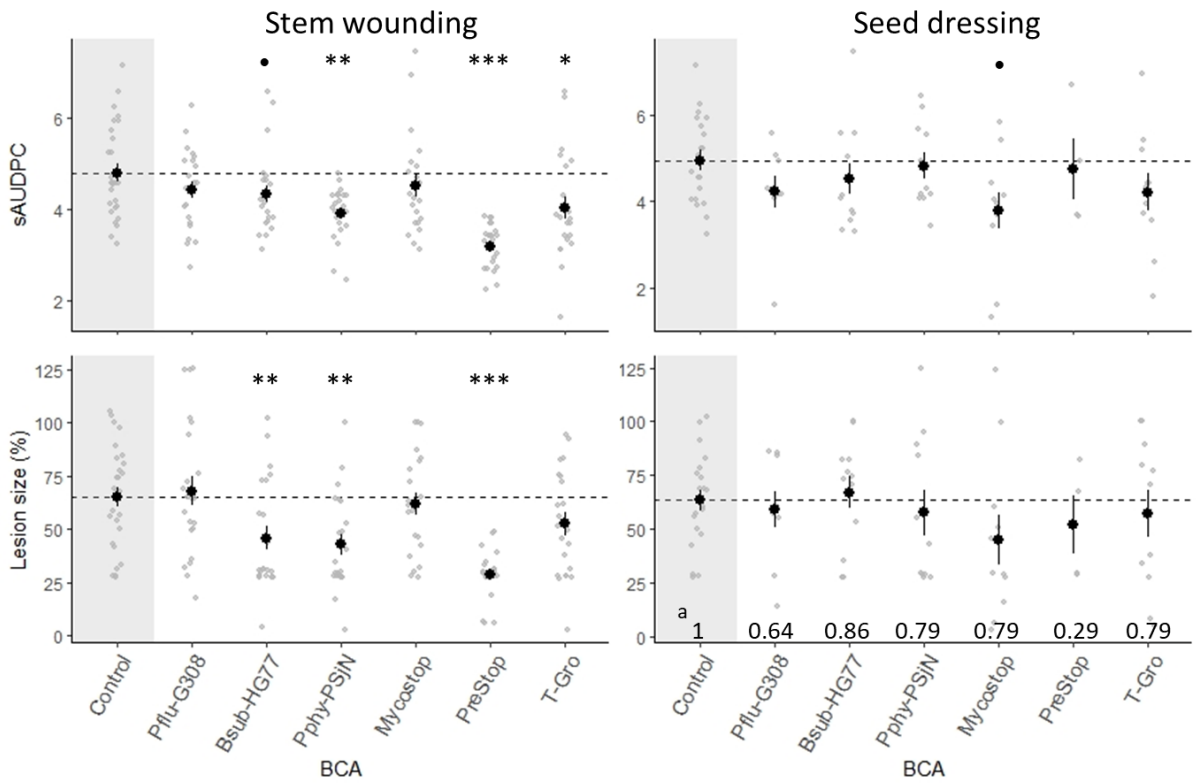

Figure 1. Biological control agent (BCA) performance under controlled conditions. Disease incidence is expressed as standardized area under the disease progress curve (sAUDPC). Lesion size is expressed as relative to total stem length. Thick black dots indicate mean and error bars indicate standard error. Gray dots indicate normalized data points. • $P \leq 0.1$, * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, difference with control (Dunnett's test). a = Germination rates relative to untreated control. See **Table 1** for description of treatments.

Seed treatments can affect germination rates

On infected seeds, the seed treatments hot water 3 (0.26, $P < 0.001$), steam 5 (0.67, $P < 0.001$), Bsub-HG77 (0.72, $P = 0.016$) and steam 4 (0.77, $P = 0.04$) significantly reduced germination rate relative to untreated infected seeds (**Fig. 3**). Especially hot water treatment 3 strongly affected germination, reducing mean germination rate by 74 %. None of the other tested treatments significantly affected seed germination but Pphy-PSjN (0.69, P = 0.09) tended towards significance. Interestingly, none of the treatments on infected seeds that affected germination rate resulted in yield loss. On certified seeds, hot water 3 and steam 4 also strongly affected seed germination by -84 % (0.16, $P < 0.001$) and -88 % (0.12, $P < 0.001$), respectively. This explains the yield reductions and absence of disease severity data points for these treatments in **Fig. S1**. In contrast to infected seeds, applications of the BCAs Bsub-HG77 and Pphy-PSjN on certified seeds did not affect germination. None of the treatments significantly improved germination rate.

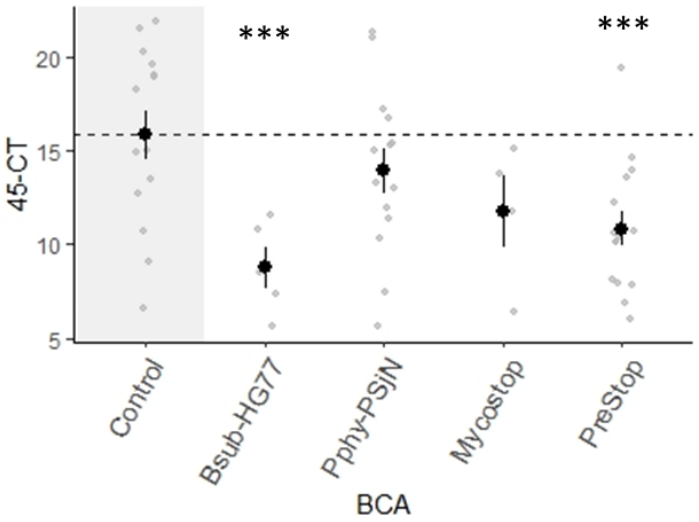

Figure 2. *Colletotrichum lupini* presence in stem tissue. Determined at 1 cm above inoculation point, expressed as 45 (total cycles) minus obtained CT (cycle number upon reaching threshold). Thick black dots indicate mean and error bars indicate standard error. Gray dots indicate normalized data points. • $P \leq 0.1$, * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, difference with control (Dunnett's test). See **Table 1** for description of treatments.

Discussion

Seed health is crucial for successful crop production. In lupin, the seed-borne fungal pathogen C. lupini can cause devastating crop losses. Various biological control agents (BCAs) have been successfully applied to reduce seed-borne disease in numerous crops, including legume species (Mancini and Romanazzi 2014; Rocha et al. 2019; Tinivella et al. 2009). Our results, where we tested six such BCAs, indicated that bacterial agents Bsub-HG77 (B. subtilis HG77), Pphy-PSjN (P. phytofirmans PSJN) and commercial products Prestop (C. rosea J1446) and T-Gro (T. asperellum kd) can successfully reduce anthracnose disease on white lupin when applied simultaneously with the pathogen to the wounded stem. These observations are in line with reported antagonistic activity of B. subtilis to C. lupini in through antifungal lipopeptide secretion (Yánez-Mendizábal and Falconí 2018) and induced systemic resistance in Andean lupin (Yánez-Mendizábal and Falconí 2021). Biological control through the secretion of cell-wall-degrading enzymes, the production of antifungal secondary metabolites, and the induction of systemic resistance have often been observed for P. phytofirmans PSJN (Depoorter et al. 2016; Esmaeel et al. 2018), C. rosea (Sun et al. 2020) and T. asperellum (de los Santos-Villalobos et al. 2013; Mukherjee et al. 2012) in other plant pathosystems. Treatments with Bsub-HG77 and Prestop both reduced lesion size and detected C. lupini DNA in stem tissue, whereas treatment with Pphy-PSjN only reduced lesion size, suggesting different modes of action. When applied under field conditions, through seed dressing (Bsub-HG77, Pphy-PSjN, Mycostop) or foliar application (Prestop), disease reducing effects could not be confirmed. Treatment of infected seeds with Bsub-HG77 and Pphy-PSjN even showed reduced germination rates. Changing application conditions, such as concentration and exposure time, might strongly influence disease reducing capacity and germination rate.

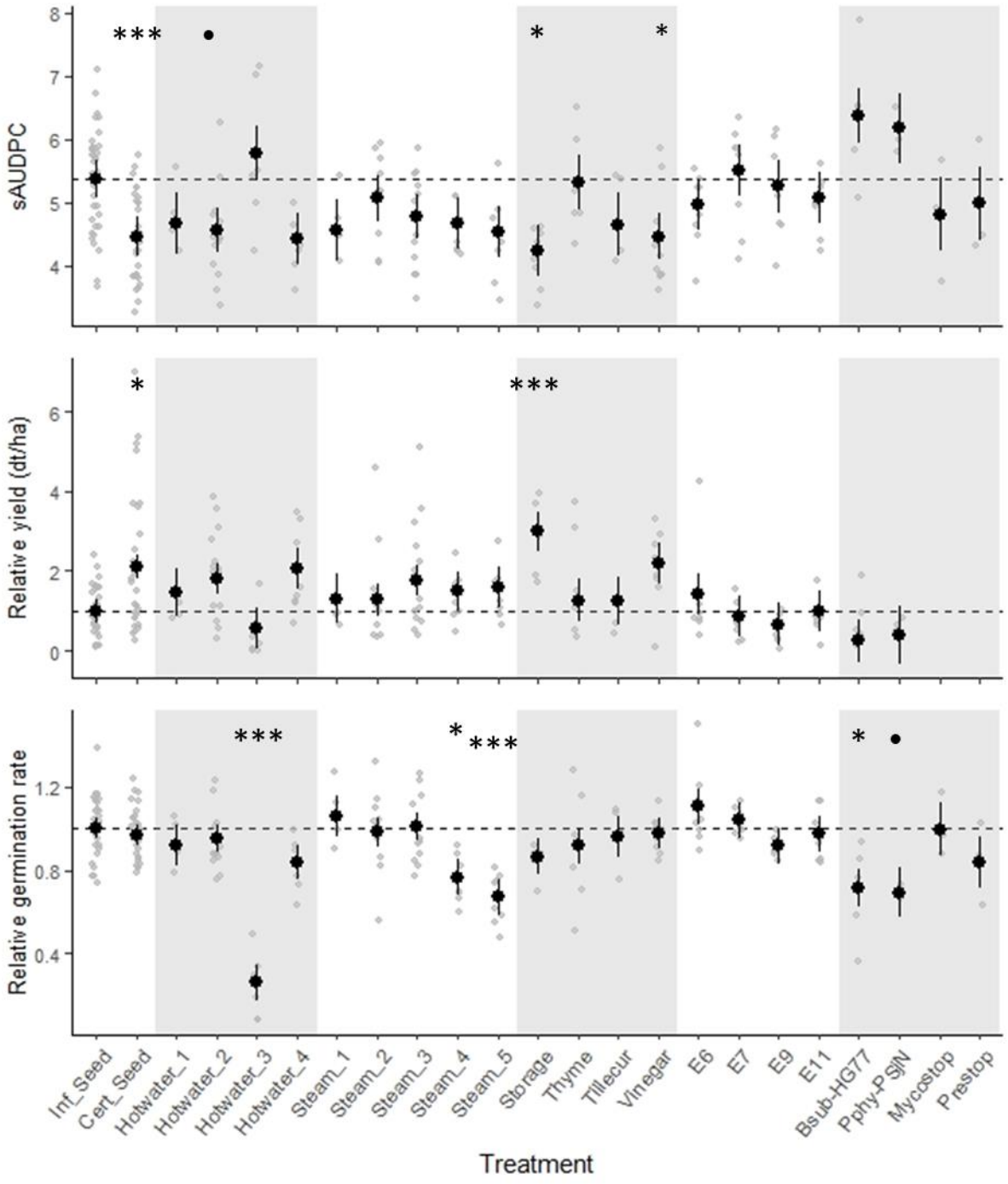

Figure 3. Seed treatments on infected seeds tested under field conditions in Switzerland. Disease incidence is expressed the standardized area under the disease progress curve (sAUDPC). Yield (dt/ha) and germination rate are expressed as relative to untreated infected seeds. Thick black dots indicate estimated means and error bars indicate estimated standard error. Jitter indicates normalized data points. • $P \leq 0.1$, * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, difference with control (Dunnett's test). See **Table 2** for description of treatments.

Long-term storage (three years) of infected seeds at room temperature resulted in the strongest reduction in disease severity and increase in yield of all treatments whilst germination rates were not affected. Similar observations were made by Thomas and Sweetingham (1999) and Cwalina-Ambroziak and Kurowski (2005), showing significant reductions of *C. lupini* infection after more than six months of storage. This suggests that *C. lupini* resting structures cannot survive for long times in white lupin seeds. Major disadvantages of long-term storage, however, are the long duration and required storage facility, making it very costly on a large scale. Artificial seed aging through Elevated Partial Pressure of Oxygen (EPPO) might be a promising alternative to speed up the process (Groot et al. 2012). Soaking seeds in vinegar for 30 minutes also resulted in a clear reduction of disease incidence and did not affect germination rates. Vinegar has been used as seed treatment against common bunt (*Tilletia caries*) and *Fusarium graminearum* in wheat, is considered cost effective and has been approved for usage in the organic sector (Borgen et al. 2021; Gao et al. 2020). Vinegar (acetic acid) and other acids at concentrations of 2.5% or higher reduced seed-associated bacteria (Van der Wolf et al. 2008), indicating that certain acid concentrations can reduce seed-borne pathogen viability without hampering seed germination. The effect on *C. lupini* viability of long-term storage and external seed treatments, such as acid, heat, UV, and fungicides (Falconí and Yánez-Mendizábal 2018; Falconí and Yánez–Mendizábal 2016; Thomas and Sweetingham 2003; Thomas and Adcock 2004), indicate colonization of the seed coat rather than the embryo or endosperm (Shade et al. 2017), as observed for other *Colletotrichum* species (Begum et al. 2008; Harman 1983).

Overall, long-term storage and vinegar treatments showed similar performance compared to certified seeds, making them promising tools to improve seed health of infected seeds. Treating certified seeds did not reduce disease or improve yield but could hamper germination rates, indicating that treatment of certified seeds might not give additional benefits. Further research is required to optimize application conditions. Exploring the optimal storage conditions could shorten required storage time to reduce *C. lupini* viability, whilst the optimal acidity and soaking duration could improve vinegar treatment effectiveness. Especially for BCA treatments, different concentrations, modes of application or combining BCAs could improve effectiveness, but more research is required to transfer observed controlled condition effects to field conditions.

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Author contribution

JAA, PH and MMM conceived the original idea for this study. PH and MMM acquired the funding for this project. JAA conducted experiments and took the lead in manuscript writing. CH conducted experiments at FiBL (Switzerland). JAA and CH analyzed the data. CA planned and performed the field trials and provided seeds. JAA designed the figures and tables with input from PH, MMM, RK, RTV and MRF, who also significantly contributed to data interpretation and provided critical feedback that shaped the final version.

Supplementary

Table S1. Mean sAUDPC and yield under Swiss field conditions.

Location	Year	sAUDPC[a]		Yield (dt/ha)	
		Mean	SE	Mean	SE
Mellikon	2018	4.15	0.18	17	2.5
Feldbach	2018	4.20	0.21	42	4.0
Rümikon	2019	5.52	0.13	4	0.4
Feldbach	2019	4.37	0.16	8	1.6
Full-Reuenthal	2020	5.17	0.16	8	1.2
Feldbach	2020	5.57	0.21	4	0.9
Full-Reuenthal	2021	4.90	0.47	NA	NA

[a] standardized area under the disease progress curve

Table S2. Overview of certified seed treatments tested under Swiss field conditions.

Treatment	Description	Year	n	E	Source / company
Control	Certified seeds	2018, 19, 20, 21	35	7	Infected field harvest, half a year old
Control_F	Infected seeds	2018, 19, 20, 21	32	7	Jouffray-Drillaud, Cissé, FR
Hotwater_1	55℃ / 5 minutes	2018	4	2	Sativa, Rheinau, CH
Hotwater_2	55℃ / 10 minutes	2019, 20	6	2	Sativa, Rheinau, CH
Hotwater_3	65℃ / 10 minutes	2020	8	2	Sativa, Rheinau, CH
Steam_1	63℃ / 270 seconds	2018	4	2	Sativa, Rheinau, CH
Steam_2	68℃ / 270 seconds	2018, 19	10	4	Sativa, Rheinau, CH
Steam_3	75℃ / 120 seconds	2019, 20	6	2	Sativa, Rheinau, CH
Steam_4	80℃ / 270 seconds	2020	8	2	Sativa, Rheinau, CH
Thyme	Thyme oil, 0.1% / 30 minutes	2019	6	2	Thymian Thymol bio, Primavera, Oy-Mittelberg, DE
Tillecur	Mustard based powder, 1 kg / 100 kg seeds	2018	4	2	Biofa, Münsingen, DE
Bsub-HG77[a]	0.25 (OD_{600}) / 1 h	2019	6	2	Hohenheim University, DE
Pphy-PSjN[a]	0.25 (OD_{600}) / 1 h	2019	3	1	Austrian Institute of Technology, AT

n: number of replicates, E: environment, a: see **Table 1**.

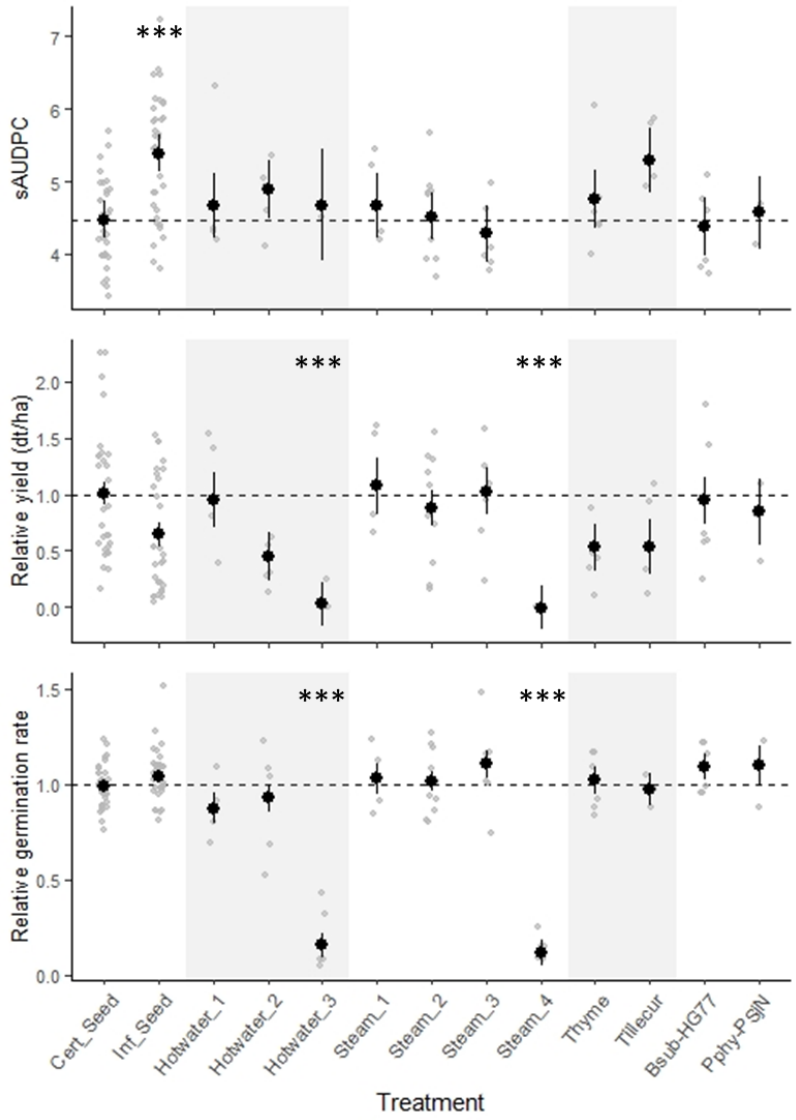
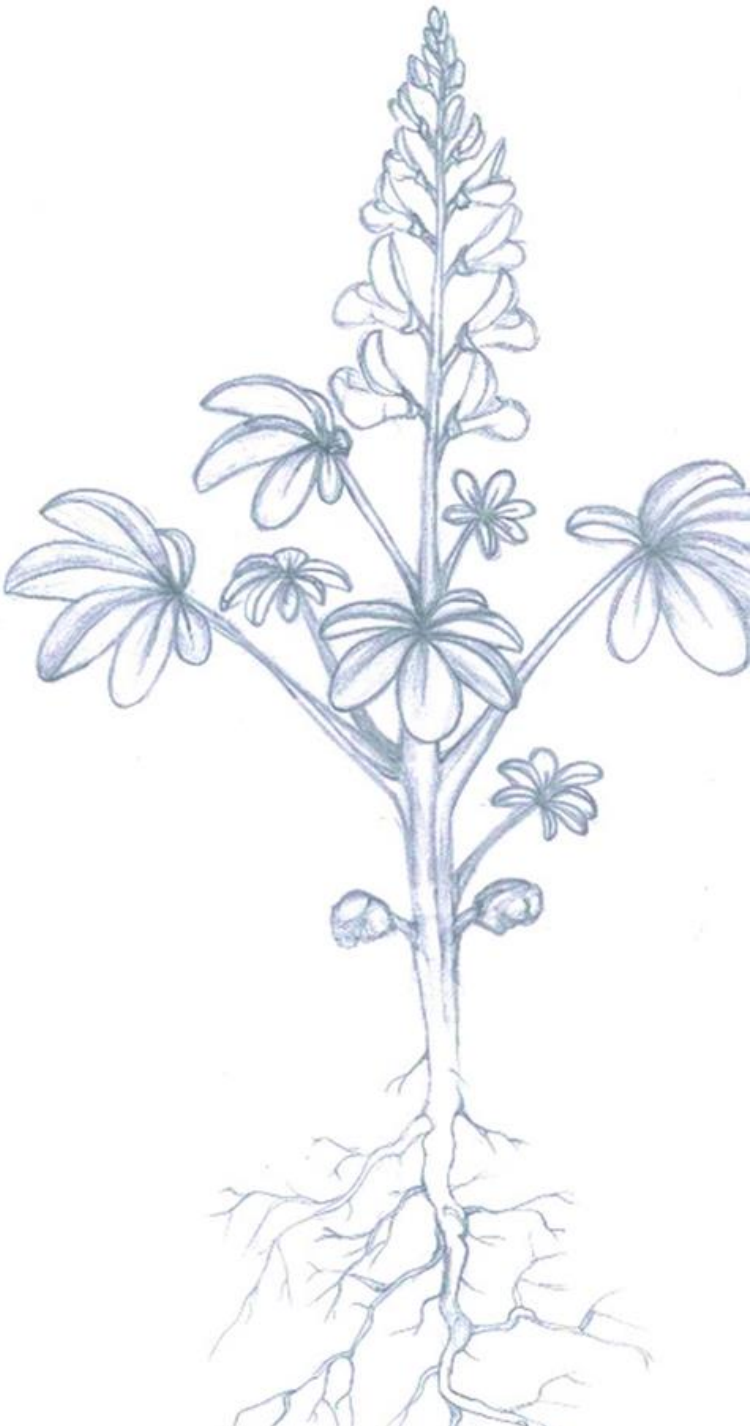


Figure S1. Seed treatments on certified seed tested under field conditions in Switzerland. Disease incidence is expressed the standardized area under the disease progress curve (sAUDPC). Yield (dt/ha) and germination rate are expressed as relative to untreated certified seeds. Thick black dots indicate estimated means and error bars indicate estimated standard error. Jitter indicates normalized data points. • $P \leq 0.1$, * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, difference with control (Dunnett's test). See **Table S2** for description of treatments.

Chapter 6

General Discussion



In order to successfully re-introduce white lupin cultivation in Europe, our understanding on how to sustainably control anthracnose disease need to be improved. In this PhD thesis I explored the phylogeography, genetic diversity and virulence of the notorious lupin pathogen *C. lupini* to better understand the current anthracnose outbreak and to improve disease management strategies. Secondly, we provided adequate tools to reliably study *C. lupini*-host interactions and effectively identify sources of resistance in white lupin. Thirdly, we analyzed a white lupin population structure and identified candidate genes for anthracnose resistance, to provide a basis for further gene mapping and improve breeding programs. At last, we tested potential non-synthetic treatments to sustainably reduce infection levels in white lupin seeds, which are the primary source of disease inoculum. This thesis, therefore, provides crucial knowledge to further study the *C. lupini*-white lupin pathosystem and a solid basis for a successful re-introduction of white lupin in Europe, a crop that perfectly fits its goals to improve agricultural resilience and reduce its dependence on imported soybeans.

Colletotrichum lupini: a diverse lupin pathogen

Characterization of a global collection of *C. lupini* isolates showed intraspecific diversity to be greater than previously reported, distinguishing a total of six genetic groups and ten distinct morphotypes (Chapter 2). The highest diversity was found in the South American Andes, indicating a South American origin. The South American Andes is also home to a highly diverse population of wild lupin species (Drummond 2008; Nevado et al. 2016). This high diversity in lupin species might have contributed to the diversification within *C. lupini* in this region. A South American origin of *C. lupini* is in line with the fact that *C. lupini* is a member of clade 1 of the *C. acutatum* species complex, which solely consists of *Colletotrichum* species with a South or Central American origin (Bragança et al. 2016; Damm et al. 2012). The different genetic groups found across South America showed distinct virulence patterns on Andean (*L. mutabilis*) and white lupin accessions (Chapter 2). With some strains being highly aggressive on Andean accessions, but not on white lupin and vice versa. A study testing Ecuadorian *C. lupini* isolates on different Andean lupin accessions also showed different virulence patterns per strain accession combination (Falconí et al. 2013). This suggests the existence of different physiological races within *C. lupini*, which has been observed as well for *C. lindemathеum* on common bean (Falleiros et al. 2018), *C. sublineola* on sorghum (Xavier et al. 2018), and *C. truncatum* on lentil (Armstrong-Cho et al. 2012), but is in general considered uncommon for *Colletotrichum*. Increased *C. lupini* sampling in the South American Andes from different lupin species, complemented with virulence screenings on a broad spectrum of lupin species is required to get more insight into the intraspecific diversity and pathogenicity of this species.

The lupin anthracnose pandemic

The first serious incursion of lupin anthracnose was recorded around the 1950's in North America. ITS sequencing and vegetative compatibility analysis indicated that this outbreak was caused by strains belonging to genetic group I (*C. lupini* var. *lupini*) (Nirenberg et al. 2002; Shivas et al. 1998). Besides North America, strains belonging to this group were also found in France,

the Ukraine and Costa Rica, suggesting a widespread dissemination (Damm et al. 2012). Around the 1970's a second, more severe outbreak was reported and coincided with the steady decrease in lupin cultivated area worldwide. This second global outbreak persists until this day and is caused by strains belonging to the genetically uniform group II (*C. lupini* var. *setosum*) (Chapter 2). The high uniformity among strains of group II, their identical morphology (Chapter 2), and non-observed sexual morph (Damm et al. 2012), indicates clonality as suggested by Talhinhas et al. (2016).

Isolates belonging to group II were shown in Chapter 2 and 3, and by Guilengue et al. (2020) to be highly aggressive on white and Andean lupin, and have often been reported to cause devastating losses on blue, yellow and ornamental lupins as well (Adhikari et al. 2011; Elmer et al. 2001; Rosskopf et al. 2014; Shea et al. 2008). Also, wild *L. cosentinii* are known to be susceptible and act as inoculum reservoir in Australia (Shea et al. 2008), indicating a broad host-range among lupin species. Virulence assays in Chapter 2 showed that two group II isolates from Chile could overcome resistance of elite white lupin breeding material, suggesting a higher virulence compared to group II isolates from Switzerland and Australia. This observation should be confirmed and whole genome sequencing (WGS) followed by comparative genomics could give insight into this difference between apparent clonal strains.

A study by Shivas et al. (1998), hypothesized that isolates of group II entered Australia in 1994 via symptomless infected seeds imported from Germany and suggested that group II first originated in Chile. The results of this thesis support a South American origin of *C. lupini* and the presence of group II strains in Chile now confirms this theory (Chapter 2). The possibility of even more aggressive strains escaping South America highlights the need to establish international phytosanitary protocols and detection methods to avoid infected seed transports and prevent additional introductions of different *C. lupini* strains across the world.

White lupin population structure

White lupin has a long domestication history, but unlike blue lupin, modern breeding efforts have been relatively scarce (Wolko et al. 2011). Compared to soybean, chickpea and pea, lupin breeding in general, is still in its infancy. Recent efforts in white lupin genomics, including the availability of a high-quality reference genome and transcriptomic resources (Hufnagel et al. 2020; Xu et al. 2020), enable the development of modern breeding strategies and improve our understanding of this species. Analyzing a diverse white lupin population, including landraces and modern cultivars showed a weak population structure and a remarkable low linkage disequilibrium (LD) decay (Chapter 4). This was also observed by Hufnagel et al. (2021), through examination of a white lupin pangenome, a collection of all genomes within a clade, highlighting white lupin's slow and sporadic domestication process. In contrast to blue and yellow lupin (Berger et al. 2012; Iqbal et al. 2020; Mousavi-Derazmahalleh et al. 2018a), no breeding-associated genomic bottleneck occurred in white lupin (Hufnagel et al. 2021). Altogether, this provides unexplored potential for modern crop improvement. Recent studies already identified loci and characterized molecular mechanisms involved in important agronomic traits, such as

alkaloid concentrations, flowering time and cluster root formation (Hufnagel et al. 2021; Rychel et al. 2019; Xu et al. 2020). As host resistance forms the basis for successful disease control, the next step would be to identify loci conferring resistance to anthracnose disease.

The quest for host resistance

A reliable high-throughput phenotyping protocol is vital to systematically screen the widely available white lupin germplasm for anthracnose resistance (Wolko et al. 2011). As the current pandemic is caused by highly virulent group II strains, breeding for resistance against this group of strains is most important. We developed a high-throughput phenotyping protocol, based on stem inoculation of 14 day old white lupin seedlings, to identify field-relevant anthracnose resistance under controlled conditions and study pathogen-host interactions (Chapter 3). It should be investigated if the protocol also works for blue and yellow lupin, where spray inoculation of (>) 3 week old plants and single-row field trials are the norm (Adhikari et al. 2011; Fischer et al. 2015). Single-row field assessments, often used in white lupin breeding (Adhikari et al. 2009; Arncken et al. 2018), did not correlate well to field plot or controlled condition disease assessments. Reasons for this could be, for example, variable environmental conditions and a lack of sufficient replicates to take those into account or the difference in mode of infection and developmental stage. However, with sufficient replicates, single-row disease phenotyping could still be a good approach to identify anthracnose resistance in white lupin, especially when abiotic stresses have to be considered. In Chapter 3 and 4, Ethiopian landrace P27174, which was identified as resistant in single-row field trials in New Zealand and Australian studies (Adhikari et al. 2009; Cowling et al. 1999), and used as a resistant parent in a quantitative trait locus (QTL) analysis (Książkiewicz et al. 2017), did not show to be resistant. Disease predictions from a whole-genome selection model based on the aforementioned QTL study (Rychel-Bielska et al. 2020), did not correspond to disease values found in Chapter 3. This highlights the need to establish internationally standardized phenotyping protocols, for both controlled and field conditions, to evaluate anthracnose resistance in lupin species to facilitate global data comparison and prevent further inconsistencies.

Understanding the genetic background and molecular mechanisms of resistance against anthracnose is essential to achieve durable resistance. In this thesis, a genome wide association study (GWAS) identified two significant single nucleotide polymorphisms (SNPs), explaining up to 28% of disease phenotypical variance, within gene Lalb_Chr05_g0216161 on chromosome 5, encoding a RING zinc-finger E3 ubiquitin ligase (Chapter 4). E3 ubiquitin-ligases have frequently been shown to be involved in plant immunity (Duplan and Rivas 2014; Marino et al. 2012; Zhou and Zeng 2017), and confer resistance against many fungal pathogens including *C. coccodes* and *Xanthonomas* in pepper (Hong et al. 2007; Lee et al. 2011)*, Magnaporthe oryzae* in rice (Park et al. 2016)*,* and *Ralstonia solanacearum* in tobacco (Ghannam et al. 2016). Although numerous E3 ubiquitin ligases have been recognized to regulate plant immunity, their targets and subsequent molecular mechanisms initiating plant defense often remain unknown. Peak SNPs tending towards significance on chromosome 1, 5, 8 and 13, also revealed loci encoding proteins that are

potentially involved in resistance pathways, such as leucine-rich repeat (LRR) receptor kinases which are well known for pathogen recognition (Tang et al. 2017). The identified candidate genes did not correspond to previously reported QTLs for anthracnose resistance in white lupin (Książkiewicz et al. 2017; Phan et al. 2007; Yang et al. 2010) or resistance genes identified in blue lupin (Fischer et al. 2015; Yang et al. 2004; Yang et al. 2008). This is in line with Książkiewicz et al. (2017), showing high synteny between blue and white lupin, but a different genetic control of important agronomic traits such as phomopsis stem blight resistance and vernalization independence. The identification of anthracnose resistance candidate genes in this thesis provides a solid basis for gene mapping in white lupin. Upon validation, resistance conferring gene variants could be crossed into elite breeding material.

Improving seed health

Besides crop resistance, seed health is vital to ensure production levels. Infected seeds are the primary source of anthracnose during lupin cultivation. Under controlled conditions the biological control agents (BCAs) *Bacillus subtilis* HG77, *Paraburkholderia phytofirmans* PSJN and the commercial product Prestop (*Clonostachys rosea* J1446), showed to reduce disease severity and lesion size, see Chapter 5. These effects however, were not observed under field conditions. In Andean lupin, treatment with *B. subtilis* (CtpxS2-1), which produces high amounts of antifungal lipopeptides, induced systemic resistance against anthracnose (Yánez-Mendizábal and Falconí 2021). Antifungal lipopeptides produced by other *B. subtilis* strains have also been shown antagonistic to *C. gloeosporioides* (Kim et al. 2010) and *C. capsici* (Kumar et al. 2021), and to induce systemic resistance and promote growth in numerous crops (Kloepper et al. 2004), highlighting their importance in pathogen control. *P. phytofirmans* PsJN is a beneficial endophyte that is able to colonize a wide range of plants and is known for its ability to promote plant growth and induce systemic resistance. As it can successfully colonize white lupin (Kost et al. 2014), induced resistance might play a role in the observed decrease in anthracnose incidence in Chapter 5. As a mycoparasite, *C. rosea* exhibits great biological control activity against numerous fungal plant pathogens (Sun et al. 2020), including *C. acutatum* (Verma et al. 2006) and *C. truncatum* (Rodríguez et al. 2011). Secretion of cell-wall-degrading enzymes, the production of (antifungal) secondary metabolites and the induction of plant resistance are the main biocontrol mechanism attributed to *C. rosea* (Chatterton and Punja 2009; Fatema et al. 2018). Further research is required to explore underlying biological control mechanisms of the above mentioned BCAs and to successfully establish biological control in the field.

Long-term storage and vinegar (acetic acid) treatments of infected seeds reduced disease incidence and increased yield under Swiss field conditions to levels comparable to certified seeds (Chapter 5). Similar observation were made by Thomas and Sweetingham (1999) and Cwalina-Ambroziak and Kurowski (2005), showing significant reductions of *C. lupini* infection after more than six months of storage. This suggests that *C. lupini* resting structures cannot survive for long times in white lupin seeds. Long-term storage, however, is costly on a large scale. Artificial seed aging through Elevated Partial Pressure of Oxygen (EPPO) might be a promising alternative to

speed up the process (Groot et al. 2012). Acetic acid has been used as seed treatment against common bunt (*Tilletia caries*; Borgen et al. 2021), *Fusarium graminearum* in wheat (Gao et al. 2020) and seed-associated bacteria (Van der Wolf et al. 2008), and as fumigation to prevent post-harvest decay (Sholberg and Gaunce 1995). The treatment is considered cost effective and has been approved for usage in the organic sector (Borgen et al. 2021; Gao et al. 2020). The effect on *C. lupini* viability of long-term storage and external seed treatments, such as acid, heat, UV, and fungicides (Falconí and Yánez-Mendizábal 2018; Falconí and Yánez–Mendizábal 2016; Thomas and Sweetingham 2003; Thomas and Adcock 2004), indicate colonization of the seed coat as observed for other *Colletotrichum* species (Begum et al. 2008; Harman 1983), rather than the embryo or endosperm (Shade et al. 2017). These results highlight the potential of sustainable non-synthetic treatments as alternative to seed dressing fungicides. It should be investigated if these treatments are also effective in reducing *C. lupini* infection in blue, Andean and yellow lupin, in which alternative treatments have already been shown successful (Falconí and Yánez-Mendizábal 2018; Falconí and Yánez–Mendizábal 2016; Thomas and Adcock 2004; Yánez-Mendizábal and Falconí 2018). Seed-borne anthracnose disease also causes significant yield losses in other legumes, such as soybean, chickpea, pea and common bean. In these crops some alternative seed treatments have been shown successful (Begum et al. 2010; Faruk and Khatun 2020; Silva et al. 2012; Tinivella et al. 2009), but are not widely applied yet. Exploring the effectivity and mode of action of inexpensive and easy applicable seed treatments, that can be broadly applied to reduce anthracnose infection in legume species, could greatly increase sustainability of legume production.

Understanding white lupin–*C. lupini* interaction

Understanding the life cycle and infection mechanisms of *C. lupini* is crucial to further improve disease management and white lupin resistance breeding. It is well established that infected seed are the primary source of infection (Talhinhas et al. 2016; Thomas and Sweetingham 2004; White et al. 2008). It is still unclear, however, how *C. lupini* colonizes the host after seed germination. Under field conditions anthracnose symptoms become most noticeable upon flowering (Chapter 3; White et al. 2008). This is in line with controlled condition experiments on Andean and blue lupin, showing highest plant susceptibly during flowering (Falconi et al. 2015; Ruge-Wehling et al. 2009). After flowering, secondary infections quickly spread throughout the crop which are favored by high humidity and temperatures (Dubrulle et al. 2020a; Thomas and Sweetingham 2004; Thomas et al. 2008b). Upon artificial inoculation, *C. lupini* was shown to represent a hemibiotrophic lifestyle comparable to other members of the *C. acutatum* species complex (De Silva et al. 2017; Gomes et al. 2009; Peres et al. 2005), with appressoria formation and a switch from biotrophy to necrotrophy observed 24h and 48h post inoculation, respectively (Dubrulle et al. 2020b). The development of a qPCR method to quantify *C. lupini* presence *in planta* (Kamber et al. 2021), could offer additional insights into the pathogen's life cycle by monitoring *C. lupini* at different plant developmental stages.

Members of the *C. acutatum* species complex are relatively closely related but harbor a wide variety of host-spectra, infection strategies and reproduction modes (Damm et al. 2012; Peres et al. 2005). As *C. lupini* is highly host specific, in contrast to most other *C. acutatum* species complex members (Baroncelli et al. 2017), it offers great potential to act as a model species to study the evolution of host-speciation. Applying high-resolution sequencing on the worldwide *C. lupini* collection shown in Chapter 2, could provide valuable information on global population structure and major genetic re-arrangement events. Genomes of representative strains of group I and II have recently been sequenced and published (Baroncelli et al. 2021; Dubrulle et al. 2020b). Performing WGS on isolates representing recently identified genetic groups and morphotypes, with distinct virulence patterns on white and Andean lupin, offers great possibilities for comparative genomics within *C. lupini* and the *C. acutatum* species complex. This would allow to identify species-specific regions and those undergoing strong positive selection, involved in i.e. pathogenicity or virulence, providing insight into the evolution of host-speciation and virulence mechanisms within the genus *Colletotrichum*. Together with available transcriptomic and proteomic data of the first stages of white lupin infection (Dubrulle et al. 2020b), important pathogenicity regions could be identified, which could greatly support white lupin breeding.

Controlling anthracnose disease in white lupin

Controlling anthracnose disease in white lupin cultivation is vital to improve yield stability and economic security. Fungicide application has been shown effective but is not always accessible and not considered a sustainable long-term solution (Thomas et al. 2008a; White et al. 2008; Zubrod et al. 2019). Resistance breeding offers an economical and effective solution to control anthracnose disease. Thanks to the weak population structure, a fast LD decay, and the absence of a genetic bottleneck, white lupin breeding has great potential (Chapter 4; Hufnagel et al. 2021). This thesis provides a solid basis for further gene mapping and gives a first insight into white lupin resistance mechanisms against anthracnose disease. Upon further characterization and validation of the identified candidate genes (Chapter 4), genetic markers and genomic prediction models could be developed to speed up breeding efforts (Rychel-Bielska et al. 2020). Understanding the resistance mechanisms in white lupin using the available genomic resources (Annicchiarico et al. 2020; Hufnagel et al. 2020; Hufnagel et al. 2021) and deploying precision breeding techniques could further accelerate resistance breeding (Veillet et al. 2020; Zhu et al. 2020). Anthracnose resistance in white lupin is considered polygenic (Yang et al. 2010) and none of the screened white lupin accessions showed complete resistance against a *C. lupini* group II strain from Switzerland (JA01). In order to achieve durable resistance in white lupin a diverse set of resistance genes should therefore be integrated and applying genomic prediction strategies, regarding the entire genome instead of a few major genes, might be unavoidable. Breeding can significantly improve resistance, but high disease pressures or the introduction of novel highly aggressive *C. lupini* strains could still severely threaten production.

As primary disease inoculum is seed-borne, and low infection levels can already cause significant yield losses, starting with clean seeds is crucial. Chapter 5 showed that using certified white lupin

seeds decreased disease incidence and could potentially double yield compared to field-saved infected seeds. Certified seeds are currently produced under fungicidal control. Moving seed propagation to environments unfavorable to anthracnose disease, while applying strict phytosanitary measures and, if available, non-synthetic control during flowering and pod development, certified-seed production could become more sustainable. If certified seeds are not accessible, non-synthetic seed treatments, such as dry-heat, hot-water, long term storage and vinegar, could reduce *C. lupini* disease incidence to levels comparable to certified seeds (Chapter 5; Thomas and Adcock 2004). Before sowing, seed infection levels should be determined by performing qPCR (Kamber et al. 2021), to prevent disease introductions. As infected seeds are often symptomless, this technique could also help to prevent the spreading of novel *C. lupini* strains, from especially South America (Chapter 2), across the world. Establishing international guidelines to control lupin seed health for cultivation and trade, through standardized testing procedures according to international seed health organizations, such as the International Seed Testing Association (ISTA; Aveling 2014), could significantly reduce the risk of new outbreaks.

Besides improving seed health and deploying resistant cultivars, cropping systems can also greatly influence disease pressure. It has long been known that monocultures are highly vulnerable to disease outbreaks (Anderson and May 1986; King and Lively 2012). More diverse systems, such as intercropping, have often shown to be more resilient to biotic and abiotic stresses (2009; Boudreau 2013; He et al. 2019; Sapoukhina et al. 2010; Zhang et al. 2019). Compared to monocultures, intercropping systems were shown to reduce air-borne fungal disease by 73% (Boudreau 2013). In Ethiopia, intercropping common bean with sorghum significantly reduced bean anthracnose (*C. lindemuthianum*) incidence (Hailu 2019), and in Cameroon intercropping practices also showed to reduce anthracnose (*C. kahawae*) in coffee cultivation (Bedimo et al. 2007). White lupin's capabilities of nitrogen fixation, phosphorus mobilization and ecosystem services make it an ideal crop for mixed cropping systems (Dissanayaka et al. 2015; Fijen et al. 2021; Fujita et al. 1992). Intercropping white lupin with barley or wheat resulted in higher total yield compared to monoculture (Jannasch and Martin 1999; Mariotti et al. 2009), whereas intercropping with oats decreased total yield (Arncken et al. 2015). Exploring optimal white lupin cropping partners and mixing schemes for disease reducing capabilities could further increase agricultural productivity and resilience. Taken together, an integrative approach including modern breeding efforts, disease prevention strategies and mixed cropping systems, is therefore recommended to successfully and sustainably control anthracnose disease in white lupin.

Re-introducing white lupin cultivation in Europe

White lupin represents a promising alternative to imported soybean and is a great crop to diversify European agriculture. Its high-protein content, ability to improve soil fertility and additional health benefits, have raised the interest of the feed and food industry. The scarcity of modern breeding efforts, resulting in a lack of suitable/locally-adapted cultivars, make white lupin yields still highly variable (Cernay et al. 2015). As already lined out above, one of the most

important problems is anthracnose disease. The recent released commercial cultivars Frieda and Celina, already showed a significant increase in resistance to anthracnose, both under Swiss and German field and controlled conditions (Chapter 3 and 4). Validating and integrating identified candidate resistance genes and employing disease-free seeds of resistant cultivars could drastically decrease the anthracnose problem currently observed for lupin cultivation in Europe (Chapter 4 and 5). However, to successfully introduce white lupin, other key agronomic traits should be improved as well. Evaluating a diverse collection of white lupin accessions showed great potential to improve yields (Annicchiarico et al. 2019). As especially in temperate Europe growing seasons are short, early flowering is essential to achieve sufficient ripening. Adhikari et al. (2013) showed that early flowering is combinable with increased anthracnose resistance. Other important traits for successful cultivation in Europe are tolerance to cold (Annicchiarico and Iannucci 2007), calcareous soils (Annicchiarico and Alami 2012) and drought (Annicchiarico et al. 2018). It is also crucial to maintain a low alkaloid content below 0.02% to enter the market (Boschin et al. 2008).

Besides agronomical traits, socio-economic factors play a major role as well. The initial decline in lupin cultivation was partly caused by low and volatile margins for lupin grain and EU policies favoring soybean imports (de Visser et al. 2014; Zander et al. 2016). Additional benefits provided by white lupin cultivation, such as ecosystem services, improving soil fertility, and reducing emissions, are often underestimated and are currently not translated into economic benefits (Lucas et al. 2015; Reckling et al. 2016; Zander et al. 2016). Restricting soybean imports and implementing payments for provided ecosystem services could help to make white lupin cultivation more attractive. Establishing a more profitable market for high-quality locally produced plant-based protein might also contribute to increasing white lupin cultivation. To successfully re-introduce white lupin into European cropping systems breeding efforts should be expanded to improve yield stability and policies to make cultivation more profitable should be implemented.

Altogether, this thesis explored the origins and phylogeography of the notorious lupin pathogen *C. lupini*, improved our understanding on the *C. lupini*-lupin pathosystem, provided a reliable high-throughput phenotyping protocol, identified candidate genes for anthracnose resistance in white lupin and identified potential treatments to sustainably reduce infection levels in white lupin seeds. Therefore, this thesis provides the basis for a successful re-introduction of white lupin, a crop that perfectly fits the European goals of reducing its dependence on imported soybeans and improving its agricultural diversity and resilience.

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Acknowledgments

Working on this project about white lupin and anthracnose disease has been a great experience. First of all I would like to thank my daily supervisors Pierre Hohmann and Monika Messmer who gave me the opportunity to come to FiBL and start working with lupins. Both have been of great support throughout the full three and a half years that I worked on this thesis, thanks a lot for that! Pierre, I am really grateful for all the time you spend on improving my work during all our weekly meetings and writing processes. Even though you were often occupied with lots of other projects, you had always time for me and responded quick to all my questions. You were often able to point out different angles of the problem which helped me a lot to improve the research. During my last year at FiBL we were unfortunately not able to meet so often anymore due to corona. During that time you found a new job in Spain and I wish you all the best for this new adventure! Monika, even though you were always really busy, somehow you always found space to catch up with me and discuss about the project and daily life. I always had the feeling you were running more than 100 projects at the same time, but still you knew every detail about my subject and somehow you were always right in every discussion we had about it. I think I never met someone who knew so much about so many different topics as you did, and I really admire you for that. I will really miss our unplanned talks in the hallway and the occasional celebrations with FiBL sekt! I wish you all the best at FiBL and with the plant breeding group!

Very important to my research were the lupin field trials. Without Christine Arncken I would never have been able to do them. Christine, thanks a lot for all your efforts and help, every season again. As I spend most of my time at the university in the lab, working in the field was relatively new to me. You taught me a great amount on how to prepare, sow, maintain and harvest a field trial. I really enjoyed the hot summer days at the field, having discussions under plum trees during lunch, swimming in the Rhine and jumping in the beautiful lake of Zürich at Feldbach after work. Also thanks a lot for providing the seeds I needed for my indoor trials. Good luck with all the field trials and the lupin breeding in the coming years.

I would also like to thank my direct colleagues of the plant breeding group. Benedikt and Lukas you were great office buddies. Benedikt, I really enjoyed our lunches together at the Fibl terrace and our talks in the office. I hope you will find a nice job and wish you all the best with during your new phase in your life as a father! Seraina, you really helped me a lot during my first days at FiBL. You made sure I could find everything, had all my necessary documents and made me feel welcome. Also thank you for all your help scoring lupins in the field in that first season, when we still had to figure everything out. Nachelli, you also made me really feel at home right from the start. Thanks for your help in the lab and funny conversations during lunch. Gracias por todos y los merjores deseos para tu nueva carrera! Leo, I was not sure to mention you together with my colleagues, because you have also been my first roommate in Switzerland and above all a good friend throughout my time in the mountain country. Thanks a lot for all our evening discussions and your hospitality at your home.

During my thesis I was lucky to have received help from many interns and students. Thanks you Christa for your efforts during your master thesis, your commitment to try kill *C. lupini* in those lupin seeds was great. Jan and Katharina thanks for your help inoculating, scoring and harvesting more than thousands lupin seedlings. As plants don't care about holidays and weekends you even helped me out on your free days! Carol and Jack, you were great to work together with in the lab and the field. I won't forget our trip to the lake of Geneva and France to get some lupin samples. Also thank you Esther for all the work on lupin seeds you already did when I just started at FiBL. Furthermore, I want to thank Maggi, Hassan, Pauline and Marco for your help in the field and the nice coffee breaks! I would also like to thank Thomas Oberhänsli for all his assistance in the molecular lab. It was always fun to work together. Also thank you Toni and Adolfo for the occasional help in the chemical and soil labs.

Besides working, FiBL was a great place to meet many different kind of people from all around the world. During my time there I made quite some good friends and I will really miss hanging out all together at the FiBL guesthouses. Timo you were very welcoming from the start and invited me to my first guesthouse party, already on my first day of arrival! You often wasted significant parts of my day when we met in the hallways and we kept discussing about all kinds of interesting stuff. Also thank you for introducing me to bouldering! Joken, I still wonder what your first impression was when you arrived from the Indian Himalayan Mountains into our super messy flat after a guesthouse party. Luckily we became really good friends and you invited us to your home in India! I which you all the best with the yaks! Eva, Simon and Hund, thank you for the great times at FiBL, mountains and other places! Of course also thanks to Eva W, Anita, Jana, Giulia, Sarheed, Jini, Dennis and everybody else for your friendships at FiBL! Danke Vielmal!

As my workplace was in Switzerland, I lived for more than three years quite far away from my family and friends. Despite the distance, you have been of great support during my thesis. Thanks for all the great advice! The most important, however, was the welcome distraction from my thesis during my visits back in the Netherlands. Also thanks to my girlfriend for the support during the research and writing of my thesis. Thanks for putting things in perspective when I worried too much, and thanks a lot for the amazing lupin drawing!

At last but not least, I would like to thank my promotor Ralf T. Vögele and co-promotor Maria R. Finckh, Paolo Annicchiarico, Nelson Nazzicari, Cesar E. Falconi, Amelia W. Huaringa, and everybody else that contributed to this thesis.

Appendix

List of scientific contributions

Scientific publications

Alkemade, J. A., Messmer, M. M., Arncken, C., Leska, A., Annicchiarico, P., Nazzicari, N., Książkiewicz, M.,
Vögele, R. T., Finckh, M. R. and Hohmann, P. (2021). A high-throughput phenotyping tool to identify field-relevant anthracnose resistance in white lupin. *Plant Disease;* 105, https://doi.org/10.1094/PDIS-07-20-1531-RE

Alkemade, J. A., Messmer, M. M., Vögele, R. T., Finckh, M. R. and Hohmann, P. (2021). Genetic diversity of
Colletotrichum lupini and its virulence on white and Andean lupin. *Scientific Reports, 11:13547,* https://doi.org/10.1038/s41598-021-92953-y

Alkemade, J. A., Nazzicari N., Messmer M.M., Annicchiarico P., Ferrari B., Voegele R.T., Finckh M.R., Arncken C., Hohmann P. (2022) Genome-wide association study reveals white lupin candidate gene involved in anthracnose resistance. Theoretical and Applied Genetics, 135: 1011-1024. https://doi.org/10.1007/s00122-021-04014-7

Alkemade J.A., Arncken C., Hirschvogel C., Messmer M.M., Leska A., Voegele R.T., Finckh M.R., Kölliker R., Groot S.P.C., Hohmann P. (2022) The potential of alternative seed treatments to control anthracnose disease in white lupin. Crop Protection, 158: 106009. https://doi.org/10.1016/j.cropro.2022.106009

Kamber, T., Malpica-López, N., Messmer, M. M., Oberhänsli, T., Arncken, C., **Alkemade, J. A.**, Hohmann, P. (2021). A qPCR assay for the fast detection and quantification of *Colletotrichum lupini*. *Plants, 10, 1548.* https://doi.org/10.3390/plants1008154

Conference talks

2021 **Zürich Mycology Symposium 2021.** Oral presentation: Genetic diversity and pathogenicity of the lupin pathogen *Colletotrichum lupini*. Online. Originally: Zürich, Switzerland.

2020 **Colletotrichum workshop 2020** (in conjunction with ECFG15)**.** Oral presentation: Genetic diversity of *Colletotrichum lupini* and its virulence on white lupin (*Lupinus albus*). Rome, Italy

Conference poster sessions

2021 **EUCARPIA Liveseed conference 2021 - Breeding and Seed sector innovations for organic food Systems.** Poster and pitch: GWAS to identify loci conferring resistance to anthracnose in white lupin. Online. Originally: Cēsis, Latvia.

2020 **European Conference of Fungal Genetics 15 (ECFG15).** Poster: Genetic diversity of *Colletotrichum lupini* and its virulence on white lupin (*Lupinus albus*). Rome, Italy.

PSC symposium 2020: Connectivity - Plant interactions reloaded. Poster: Genetic diversity within *Colletotrichum lupini* and its virulence on white lupin (*Lupinus albus*). Online. Originally: Zürich, Switzerland.

2018 **PSC symposium 2018: Breakthroughs in Plant Sciences.** Poster: Quantifying resistance and lifestyle assessments of *Colletotrichum* in lupin. Zürich, Switzerland.

Nationale Bioforschungstagung. Poster: Vorstufenselektion auf Anthraknosetoleranz bei Weisser Lupine. Frick, Switzerland.

Curriculum Vitae

Joris A. Alkemade MSc

Key Qualifications

An ambitious, goal-orientated researcher in the field of molecular phytopathology. Specialized in fungal plant pathogens causing disease in less studied crops. Combines experiences from the field and greenhouse with lab work and molecular data analysis. Good team-player, shows initiative, independent, internationally orientated and promotes a friendly and open working atmosphere.

Expertise: Molecular phytopathology, resistance breeding, fungal plant pathogens, population genomics, plant-microbe interactions, microbiology, field and greenhouse trials.

Education

2018-2021 **PhD in molecular phytopathology and resistance breeding.** Research on anthracnose disease, caused by the fungal pathogen *Colletotrichum lupini*, in white lupin.
Promotor: Prof. Dr. Ralf T. Vögele (University of Hohenheim, Germany). **Co-promotor:** Prof. Dr. Maria R. Finckh (University of Kassel, Germany). **Daily supervisors**: Dr. Pierre Hohmann and Dr. Monika M. Messmer (FiBL, Switzerland). **Graduate school:** Plant Science Center (PSC) from the ETH Zürich, University of Zürich and University of Basel, Switzerland. **Funding:** Liveseed (EU horizon 2020)

2015-2018 **MSc Plant Biotechnology.** Wageningen University & Research (WUR), the Netherlands.
Thesis 1. Unravelling the potential role of three *Verticillium dahliae* effector proteins in zinc acquisition, competition and virulence. Laboratory of Phytopathology, WUR.
Thesis 2. The interaction between *Globodera pallida* RBP-1 effectors and the WPP domain of RanGAP2 and their effect on resistance suppression. Laboratory of Nematology, WUR.
Research internship. Mapping the *Fusarium oxysporum* f. sp*. cubense* population in Costa Rica. Cooperación Nacional Bananera (CORBANA). La Rita, Costa Rica.
Specialization: Molecular Plant Breeding and Pathology

2012-2015 **BSc Plant Sciences,** Wageningen University & Research, Wageningen
BSc Thesis: Screening for resistance against *Fusarium oxysporum* f. sp. *cubense* Tropical Race 4 in "Nain" (*Musa acuminata* spp. *malaccensis*) and different accessions of bananas. Department of Tropical Phytopathology, WUR.
Specialization: Plant Genomics and Health

Courses with certificates: Methods and Advances in Plant Protection (2021); Get going with statistics in functional genomics (2020); Writing a Post-Doctoral Grant (2020); Genetic Diversity: Techniques (2019); Good Scientific Practice & Scientific Integrity (2019); Challenges in Plant Science (2018).

Working experience

2018-2021 **PhD candidate** at the Plant Breeding group, Research Institute of Organic Agriculture (FiBL), Frick, Switzerland. Main responsibilities in addition to my PhD: I supervised MSc theses; BSc projects and interns, assisted with educational field days for local farmers and conducted field trials, greenhouse and lab experiments.

Teaching and supervision

MSc thesis supervision

2020-2021 Project 2: Biological control of anthracnose disease (*Colletotrichum lupini*) in white lupin (*Lupinus albus*). ETH, Zürich, Switzerland.

2018 Project 1: Molecular characterization and control of *Colletotrichum lupini*, the causal agent of anthracnose disease, in white lupin (*Lupinus albus*). ETH, Zürich, Switzerland.

Internship supervision (full-time)

2019-2020 Supervised four interns from Germany, Switzerland (Zürich University, ETH Zürich and Basel University), South Korea (Seoul National University) and the Czech Republic full time. Activities took place in the lab, growth chamber and field.

Internship supervision (part-time)

2020 Supervised two interns from Switzerland and Spain (University of Granada). Activities took place in the lab, growth chamber and field.

BNF (National Qualification Program) supervision

2019-2021 Supervised three academic professionals from Switzerland, Iran and Poland during their 6 month BNF projects at FiBL.

Scientific publications

Alkemade, J. A., Messmer, M. M., Arncken, C., Leska, A., Annicchiarico, P., Nazzicari, N., Książkiewicz, M., Vögele, R. T., Finckh, M. R. and Hohmann, P. (2021). A high-throughput phenotyping tool to identify field-relevant anthracnose resistance in white lupin. *Plant Disease;* 105, https://doi.org/10.1094/PDIS-07-20-1531-RE

Alkemade, J. A., Messmer, M. M., Vögele, R. T., Finckh, M. R. and Hohmann, P. (2021). Genetic diversity of *Colletotrichum lupini* and its virulence on white and Andean lupin. *Scientific Reports, 11:13547,* https://doi.org/10.1038/s41598-021-92953-y

Alkemade, J. A., Nazzicari N., Messmer M.M., Annicchiarico P., Ferrari B., Voegele R.T., Finckh M.R., Arncken C., Hohmann P. (2022) Genome-wide association study reveals white lupin candidate gene involved in anthracnose resistance. Theoretical and Applied Genetics, 135: 1011-1024. https://doi.org/10.1007/s00122-021-04014-7

Alkemade J.A., Arncken C., Hirschvogel C., Messmer M.M., Leska A., Voegele R.T., Finckh M.R., Kölliker R., Groot S.P.C., Hohmann P. (2022) The potential of alternative seed treatments to control anthracnose disease in white lupin. Crop Protection, 158: 106009. https://doi.org/10.1016/j.cropro.2022.106009

Kamber, T., Malpica-López, N., Messmer, M. M., Oberhänsli, T., Arncken, C., **Alkemade, J. A.**, Hohmann, P. (2021). A qPCR assay for the fast detection and quantification of *Colletotrichum lupini*. *Plants, 10, 1548.* https://doi.org/10.3390/plants1008154

Acquisition

2021 **Federation of European Microbiology Societies (FEMS), Research and Training Grant.** Project description: Understanding *C. lupini* population dynamics and getting insight in genomic regions potentially involved in pathogenicity and host speciation. (€3500)

2021 **BSPP Plant Pathology Junior fellowship Fund.** Project description: Understanding *C. lupini* population dynamics and getting insight in genomic regions potentially involved in pathogenicity and host speciation. (£ 3000)

Conferences and workshop sessions

2021 **Zürich Mycology Symposium 2021.** Oral presentation: Genetic diversity and pathogenicity of the lupin pathogen *Colletotrichum lupini*. Online. Originally: Zürich, Switzerland.

EUCARPIA Liveseed conference 2021 - Breeding and Seed sector innovations for organic food Systems. Poster and pitch: GWAS to identify loci conferring resistance to anthracnose in white lupin. Online. Originally: Cēsis, Latvia.

2020 **European Conference of Fungal Genetics 15 (ECFG15).** Poster: Genetic diversity of *Colletotrichum lupini* and its virulence on white lupin (*Lupinus albus*). Rome, Italy.

Colletotrichum workshop 2020 (in conjunction with ECFG15)**.** Oral presentation: Genetic diversity of *Colletotrichum lupini* and its virulence on white lupin (*Lupinus albus*). Rome, Italy.

PSC symposium 2020: Connectivity - Plant interactions reloaded. Poster: Genetic diversity within *Colletotrichum lupini* and its virulence on white lupin (*Lupinus albus*). Online. Originally: Zürich, Switzerland.

2019 **FiBL open day.** Poster: Bekämpfung der Anthraknose im Lupinenanbau. Frick, Switzerland.

2018 **PSC symposium 2018: Breakthroughs in Plant Sciences.** Poster: Quantifying resistance and lifestyle assessments of *Colletotrichum* in lupin. Zürich, Switzerland.

Nationale Bioforschungstagung. Poster: Vorstufenselektion auf Anthraknosetoleranz bei Weisser Lupine. Frick, Switzerland.

22- 09-2021

Date **Signature**

PhD program courses

University of Zurich[UZH] | ETH zürich

List of activities

Student Joris Alkemade
Matriculation number
Faculty / Department Faculty of Agricultural Sciences (PhD student)

Title# *Module number*	*Category /* *Type*	*Lecturer /* *Offered by*	*Location /* *End date*	*Credits /* *Hours*
Introduction to Scientific Integrity	Compulsory activity Transferable skills	Anna Deplazes UZH	Y03 G 85 at Irchel Campus 2019-07-11	0.00 2 h
Writing a Post-Doctoral Grant	Core elective activity Transferable skills	Dr. Andrea Degen, Dr. Melanie Paschke ETH	Zurich 2020-09-29	1.00 30 h
Get going with statistics in functional genomics	Core elective activity Transferable skills	Anne Roulin, Jean-Claude Walser ETH	Zurich 2020-10-07	1.00 30 h
Challenges in Plant Science	Compulsory activity Transferable skills	Samuel Zeeman, Luisa Last ETH	Zurich 2018-09-29	2.00 60 h
Genetic Diversity: Techniques	Core elective activity Methods	Dr. Aria Minder ETH	Zurich 2019-11-20	2.00 60 h
15th European Conference on Fungal Genetics	Elective activity Conference	Prof. Dr. Riccardo Baroncelli other	Rome, Italy 2020-02-20	1.00 30 h
Methods and Advance in Plant Protection P15M	Core elective activity Transferable skills	Prof. Dr. Maria Finckh other	Kassel, Germany 2021-05-21	6.00 180 h
Total				13.00 392 h

Signature of Official Supervisor: 2021.06.09 15:16:48 +02'00'

Date:

Affidavit

Annex 3

Declaration in lieu of an oath on independent work

according to Sec. 18(3) sentence 5 of the University of Hohenheim's Doctoral Regulations for the Faculties of Agricultural Sciences, Natural Sciences, and Business, Economics and Social Sciences

1. The dissertation submitted on the topic

Anthracnose in white lupin: Genetic diversity, virulence and host resistance

.....

is work done independently by me.

2. I only used the sources and aids listed and did not make use of any impermissible assistance from third parties. In particular, I marked all content taken word-for-word or paraphrased from other works.

3. I did not use the assistance of a commercial doctoral placement or advising agency.

4. I am aware of the importance of the declaration in lieu of oath and the criminal consequences of false or incomplete declarations in lieu of oath.

I confirm that the declaration above is correct. I declare in lieu of oath that I have declared only the truth to the best of my knowledge and have not omitted anything.

Utrecht, 16-09-2021

Place, Date

Signature