Phylogenetic Diversity and Effect of Temperature on Pathogenicity of *Colletotrichum lupini*

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Abstract

Although lupin anthracnose caused by *Colletotrichum lupini* is a significant threat for spring and winter lupin crops, it has been poorly studied so far. This study aimed at characterizing the (i) phylogenetic, (ii) morphological, and (iii) physiological diversity of collected isolates from anthracnose-affected lupins. The genetic identification of representative isolates ($n = 71$) revealed that they were all *C. lupini* species, further confirming that lupin anthracnose is caused by this species. However, multilocus sequencing on these isolates and 16 additional reference strains of *C. lupini* revealed a separation into two distinct genetic groups, both of them characterized by a very low genetic diversity. The diversity of morphological characteristics of a selected subset of *C. lupini* isolates was further evaluated. To the best of our knowledge, microsclerotia production observed for some isolates has never been reported so far within the *Colletotrichum acutatum* species complex. Finally, the modeling of growth responses of a subset of *C. lupini* strains revealed the capacity

of some strains to grow in vitro at 5°C. This ability was also evidenced in planta, because *C. lupini* DNA was detectable in plants from 14 days postinoculation at 5°C onward, whereas symptoms began to appear a week later, although at a very low level. Since lupin crops are planted during winter or early spring, growth studies in vitro and in planta demonstrated the capability of the species to grow at temperatures ranging from 5 to 30°C, with an optimum close to 25°C. In this study, *C. lupini*-specific primers were also designed for real-time quantitative PCR on fungal DNA and allowed the detection of *C. lupini* in asymptomatic field samples. These results open perspectives to detect earlier and limit the development of this pathogen in lupin crops.

Keywords: aggressiveness, *Colletotrichum acutatum* species complex, DNA quantification, Lupin anthracnose, microsclerotia

The genus *Lupinus* belongs to the Fabaceae family and includes annual and perennial species originating from Europe, Africa, and South America (Cowling et al. 1998). Lupins have a broad range of interesting agronomic characteristics, including self-sufficiency to nitrogen supply owing to its leguminous properties and a deep root system able to mobilize nutrients from low soil horizons. In addition, lupin seeds have a high protein content (up to 35%) (Lee et al. 2016). Several lupin species originating from Europe are cultivated for food and feed production, including white lupin (*Lupinus albus*), yellow lupin (*L. luteus*), and narrow-leaved or blue lupin (*L. angustifolius*). In France, white lupin breeding has led to the adoption of vernalizing types, which may be sown early in the autumn. These cultivars resist freezing damage over winter and are less subjected to abiotic stress owing to an early flowering period (N. Harzic, *personal communication*).

Although the interest in lupin crops has increased over the past few years, one of the major limiting factors for lupin production remains its susceptibility to diseases, the most destructive of which is anthracnose mainly caused by *Colletotrichum lupini* (Damm et al. 2012; Lotter and Berger 2005; Talhinhas et al. 2016). This species belongs to the *Colletotrichum acutatum* species complex (CAsc) (Jayawardena et al. 2016) that mainly includes plant pathogens causing anthracnose.

The first report of white lupin anthracnose established in 1912 described the causal agent as *Gloeosporium lupinus* in the São Paulo region of Brazil (Bondar 1912). In the 20th century, several studies isolated causal agents of lupin anthracnose and described them as *Glomerella lupinicola* (Seymour 1929) or *C. gloeosporioides* and its teleomorph *Glomerella cingulata* (Weimer 1943, 1951, 1952; Wells and Bell 1969; Wells and Forbes 1967). It was further demonstrated that inoculation with the sexual form of *C. gloeosporioides* induced disease symptoms on various lupin species (TeBeest 1988; Weidemann et al. 1988). The agent of lupin anthracnose was subsequently identified as *C. gloeosporioides* or *C. acutatum* before being taxonomically identified as *C. lupini* since 2002 based on the sequencing of six loci (Damm et al. 2012; Nirenberg et al. 2002). Although most species within the CAsc are polyphagous, a growing body of evidence suggests that *C. lupini* is a host-specific pathogen (Baroncelli et al. 2015a, b; Nirenberg et al. 2002; Sreenivasaprasad and Talhinhas 2005). However, it is not clear whether isolates from additional species are also able to cause lupin anthracnose and if so, to which degree.

Beyond the correct identifications of the pathogens causing lupin anthracnose, the magnitude of intraspecific diversity is also essential to the selection of appropriate disease control strategies. According to Guerber et al. (2003), the *C. acutatum sensu lato* species was divided into at least eight distinct molecular groups using RFLP methods based on glutamine synthase and sequences of the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene. The affiliation to these groups was closely associated with phenotypic diversity, host association, and geographical distribution. Among these groups, isolates from lupin were found to belong to haplotype J2 with two isolates from France and Canada and haplotype J6 with four isolates from New Zealand and the United Kingdom (Guerber et al. 2003). Using AFLPs, PCR, and multilocus sequencing based on internal transcribed spacer (ITS), β -tubulin 2 (TUB2), and histone 4 (HIS4), Talhinhas et al. (2002) showed that *Colletotrichum*

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isolates from lupin were close to *C. acutatum* and that they were divided into two subgroups A1 and A2. Eight distinct molecular groups were further characterized in *C. acutatum* s.l. species by Sreenivasaprasad and Talhinhas (2005) based on ITS region (ITS 1, 5.8S, ITS2) and TUB2 gene sequences, with *C. lupini* belonging to the A1 group, which solely contained isolates causing lupin anthracnose. Phylogenetic analyses of *C. lupini* among the CAsc were refined using previous classifications and data on six loci, and they again demonstrated that *C. lupini* isolates were clustered into one clade (namely, clade 1) among five within CAsc (Damm et al. 2012).

Colletotrichum species are distributed worldwide (Dean et al. 2012). Early disease symptoms on lupin begin with twisting of stems, pods, and petioles; these are later accompanied with necrotic spots on which orange conidial masses can appear (i.e., acervuli) (Sweetingham et al. 1998b). In infected pods, seeds become brown and wrinkled. If able to germinate, the plantlet can exhibit necrosis in cotyledons or the hypocotyl (Gondran and Pacault 1997).

Infected lupin seeds are generally considered the primary inoculum for lupin anthracnose (Decker 1947). Previously, studies demonstrated that 0.1% of seeds infected by anthracnose induced over 50% of yield loss (Gondran et al. 1990). Infected seedlings are also able to contaminate healthy neighboring seedlings (Diggle et al. 2002). At flowering, spores can infect flowers and pods, ultimately resulting in seed contamination (White et al. 2008). Inoculum dispersal is also possible by splashing of ascospores from residues and stubble (White et al. 2008). High humidity and temperatures >10°C have been shown to be conducive to conidial germination and disease onset (Sweetingham et al. 1998a), although the influence of lower temperatures has yet to be studied. It is not known whether the fungus is able to grow endophytically at low temperatures during winter, resulting in a latency phase before expressing symptoms when the conditions are favorable. Such knowledge may help devise appropriate control strategies notably through an early diagnosis in crop.

In this context, the objectives of our study were to determine (i) taxonomic diversity among 71 *C. lupini* isolated strains from lupin with anthracnose symptoms using a multilocus sequencing method of eight loci, (ii) the morphological and physiological diversity by studying the effect of temperatures on fungal growth among a subset of six representative strains, and (iii) their aggressiveness under optimal and suboptimal temperature conditions. Furthermore, in order to detect *C. lupini* and determine its growth in plant samples inoculated in the laboratory and collected from the field, species-specific primers were designed for quantitative PCR (qPCR) analyses.

Materials and Methods

Strains. The strains studied came from the CBS collection (23 strains), the IMI collection (14 strains), or reference isolates (8 strains), or they were isolated from diseased lupin plants collected in western France between 2014 and 2017 (71 strains) (Table 1).

Fungal isolation from symptomatic plants. Diseased plants were associated with metadata describing the locality, collector, date, symptomatic tissue, lupin variety, preceding crop, and phytosanitary product usage.

Diseased plant specimens were cut in 5- to 10-mm fragments and surface sterilized for 1 min in a solution of 1% sodium hypochlorite before being rinsed twice for 2 min in sterile distilled water. After drying using sterile blotting papers, plant fragments were placed on potato dextrose agar (PDA; Becton Dickinson) media with antibiotics (streptomycin 0.05 g/liter, penicillin 0.05 g/liter, and chlortetracycline 0.05 g/liter; Sigma Aldrich). After 5 to 7 days at 25°C, suspected *Colletotrichum* spp. were identified and subcultured onto a new antibiotic-amended PDA plate. *C. lupini* isolates were provided by the Culture Collection of the University of Western Brittany (UBOCC).

Plant sampling for early detection of diseases in the field. Lupin samples were collected in 2017 in 10 fields in the Loire-Atlantique department in France (5 in the north and 5 in the south of the Loire

River). Nine entire lupin plants were collected in each field every week for 5 weeks starting from 3 weeks before flowering. For each collection, fields were considered as symptomatic when symptoms were observed and asymptomatic when no symptoms were detected. When symptoms appeared in a field, they continued to be observed during the next collection dates. Leaves and stems of all samples were separated and lyophilized for 48 h.

Fungal culture and morphological study. Morphological observations (colony color, texture, zonation, growing margin, and color of the reverse side) of a subset of nine strains (Table 1) were made on cultures grown on PDA plates incubated at room temperature (~20°C) under natural daylight over 2 weeks (Cannon et al. 2008). The nine strains were selected considering phylogenetic results, geographical origins, and isolation date. Observations and measurements of conidial size and shape were made by microscopic observation of spores (50 randomly chosen, $\times 1,000$, Olympus BX61 microscope; Shinjuku) harvested after 14 days of incubation and mounted in cotton blue (Cannon et al. 2008).

The mycelium of *C. lupini* pure cultures was scraped from PDA plates after 7 to 10 days at 25°C with a sterile scalpel before DNA extraction with the FastDNA Spin kit (MP Biomedicals) following the manufacturer's instructions.

Multilocus phylogenetic analysis of *C. lupini*. A phylogenetic study was performed on 117 isolates using the ITS region (White et al. 1990), partial sequence of the GAPDH gene (Guerber et al. 2003), partial sequence of the TUB2 gene (exons 3 through 6, including introns 2 through 4) (Woudenberg et al. 2009), partial sequences of the chitin synthase 1 (CHS-1) gene (modified from Carbone and Kohn 1999), partial sequence of the actin (ACT) gene (modified from Carbone and Kohn 1999), partial sequence of the HIS3 gene (Crous et al. 2006), partial sequence of the conserved HMG box region (HMG), the intron included in the conserved HMG box region (Baroncelli et al. 2015b), and the Apn2-Mat1-2-1 intergenic (APN/MAT1) spacer and partial mating type gene (this study).

These regions were amplified using PCR reactions performed in a 25- μ l volume using 0.025 U/ μ l of GoTaq Flexi DNA polymerase (Promega), 1 $\times$ GoTaq Flexi buffer (Promega), 25 to 50 ng of template DNA, 0.08 μ M each primer, 2 mM MgCl₂, and 0.2 mM of 10 mM dNTP mix (Promega). For GAPDH and TUB2 genes, primer concentrations were increased to 0.2 μ M, whereas the dNTP mix concentration was decreased to 0.08 mM. A list of primers pairs and PCR conditions used in this study is reported in Table 2.

PCR products were visualized on a 1% (wt/vol) agarose gel in 1 $\times$ TBE (tris, borate, EDTA) buffer (40 mM Tris-acetate and 1 mM EDTA, pH 8.0) and stained with a GelRed solution under ultraviolet (UV) light. PCR products were sent to Eurofins MWG (Ebersberg, Germany) for purification and sequencing in forward and reverse directions. ABI trace files were analyzed, and consensus sequences were generated using Geneious 10.0.6 (Biomatters; <https://www.geneious.com>) (Kearse et al. 2012).

The sequences obtained were aligned using MAFFT v. 7.304 online software (Katoh and Standley 2013), and they were manually checked and trimmed to optimize the alignment.

Multiple sequence alignments were exported to MEGA7 (Kumar et al. 2016), and the best-fit substitution model was calculated for each separate sequence dataset. The multilocus concatenated alignment (ITS, CHS-1, TUB2, ACT, HIS3, GAPDH, HMG, and APN/MAT1) was performed using Geneious 10.0.6. Using MrBayes 3.2.6 (Ronquist and Huelsenbeck 2003), the Markov chain Monte Carlo (MCMC) algorithm was performed to generate phylogenetic trees with Bayesian posterior probabilities for combined sequence datasets using, for each locus, the nucleotide substitution models determined by MEGA7. Four MCMC chains were run simultaneously for random trees for 5,000,000 generations. Samples were taken every 1,000 generations. The first 25% of trees were discarded as the burn-in phase of each analysis, and posterior probabilities were determined from the remaining trees.

Table 1. Isolation details of *Colletotrichum lupini* strains and references used in this study (fl, flower; fr, fruit; l, leaf; pe, petiole; po, pod; se, seed; st, stem; tw, twig)

Strain	Species	Collection code	Isolate	Origin	Isolation date	Host	Organ
RB017 ^a	<i>C. tamarilloi</i>	CBS 129954	Tom-21	Colombia		<i>Cyphomandra betacea</i>	
RB018 ^a	<i>C. tamarilloi</i>	CBS 129955	Tom-12	Colombia	1998	<i>Cyphomandra betacea</i>	fl
RB020 ^{a,b}	<i>C. lupini</i>	UBOCC-A-118135	PT30	Azores, Portugal	1999	<i>L. albus</i>	st
RB021 ^a	<i>C. fioriniae</i>	CBS 129946	PT170	Torres vedras, Portugal	2002	<i>Olea europaea</i>	
RB022 ^a	<i>C. fioriniae</i>	CBS 129947	CR46	Portugal	2000	<i>Vitis vinifera</i>	
RB042 ^a	<i>C. lupini</i>	CBS 129944	CMG12	Lisbon, Portugal	1996	<i>Cinnamomum zeylanicum</i>	
RB096 ^a	<i>C. salicis</i>	CBS 129972	MP1	U.S.A.		<i>Acer Platanoides</i>	l
RB111 ^a	<i>C. fioriniae</i>	IMI 504882	PJ7	New Zealand		<i>Fragaria × ananassa</i>	
RB116 ^a	<i>C. lupini</i>		CSL 1294	United Kigndom		<i>L. polyphyllus</i>	
RB119 ^a	<i>C. lupini</i>		G52	Germany		<i>L. albus</i>	
RB120 ^a	<i>C. lupini</i>		96A649	Australia		<i>L. polyphyllus</i>	
RB121 ^{a,b}	<i>C. lupini</i>	IMI 504884	HY09	Canada		<i>L. albus</i>	
RB122 ^a	<i>C. lupini</i>		C3	Poland		<i>L. lateus</i>	
RB123 ^a	<i>C. lupini</i>	IMI 504885	SHK788	South Africa		<i>L. albus</i>	
RB124 ^a	<i>C. lupini</i>		BBA 70555	Chile		<i>L. albus</i>	
RB125 ^a	<i>C. lupini</i>	CBS 109224	BBA 70399	Austria		<i>L. albus</i>	
RB127 ^a	<i>C. lupini</i>		PT702	Spain		<i>Olea europaea</i>	
RB147 ^a	<i>C. lupini</i>	IMI 350308		Kent, United Kingdom	1991	<i>Lupinus</i> sp.	
RB151 ^a	<i>C. phormii</i>	CBS 118191	AR3787	South Africa	2002	<i>Phormium</i> sp.	l
RB156 ^a	<i>C. salicis</i>	IMI 345585	CSL 1044; CPC 19376	New Zealand		<i>Fragaria × ananassa</i>	pe
RB157 ^a	<i>C. salicis</i>	CBS 607.94		Netherlands	1994	<i>Salix</i> sp.	l
RB163 ^a	<i>C. nymphaeae</i>	IMI 360928	CPC 18926	Switzerland		<i>Fragaria × ananassa</i>	fr
RB170 ^a	<i>Colletotrichum</i> sp.	CBS 101611		Costa Rica		fern	
RB172 ^a	<i>C. lupini</i>	CBS 109221; UBOCC-A-117269	BBA 70352	Germany		<i>L. albus</i>	
RB173 ^{a,b,c}	<i>C. lupini</i>	CBS 109225; UBOCC-A-117263	BBA 70884	Ukraine		<i>L. albus</i>	
RB177 ^a	<i>C. acutatum</i>	CBS 112996	ATCC 56816; STE-U 5292	Australia	1965	<i>Carica papaya</i>	fr
RB178 ^a	<i>C. acutatum</i>	CBS 113008	STE-U 4463	South Africa		<i>Hakea sericea</i>	
RB179 ^a	<i>C. simmondsii</i>	CBS 122122	BRIP28519	Australia	1987	<i>Carica papaya</i>	fr
RB183 ^a	<i>C. salicis</i>	CBS 192.56		Germany		<i>Salix</i> sp.	
RB184 ^a	<i>C. costaricensis</i>	CBS 211.78; IMI 309622		Costa Rica		<i>Coffea</i> sp.	tw
RB186 ^a	<i>C. simmondsii</i>	CBS 294.67	DPI 13483	Australia	1959	<i>Carica papaya</i>	
RB187 ^a	<i>C. acutatum</i>	CBS 370.73	NRCC 10088	New Zealand	1965	<i>Pinus radiata</i>	
RB190 ^a	<i>C. nymphaeae</i>	IMI 504889	SA-01	Denmark		<i>Fragaria × ananassa</i>	
RB196 ^a	<i>C. abscissum</i>		OCO-ARC-4	U.S.A.		<i>Citrus × sinensis</i>	
RB197 ^a	<i>C. abscissum</i>	IMI 504890	STF-FTP-10	U.S.A.		<i>Citrus × sinensis</i>	
RB209 ^a	<i>C. orchidophilum</i>	IMI 309357	CPC 16815	United Kingdom		<i>Phalaenopsis</i> sp.	
RB210 ^a	<i>C. chrysanthemi</i>	IMI 364540	CPC 18930	China		<i>Chrysanthemum coronarium</i>	l
RB216 ^a	<i>C. cuscuteae</i>	IMI 304802	CPC 18873	Dominica		<i>Cuscuta</i> sp.	
RB217 ^{a,b,c}	<i>C. lupini</i>	IMI 375715	CPC 19390	Australia	1997	<i>L. albus</i>	
RB218 ^a	<i>C. paranaense</i>	IMI 384185	CPC 18937	Brazil		<i>Caryocar brasiliense</i>	
RB235 ^{a,b,c}	<i>C. lupini</i>	CBS 509.97; UBOCC-A-117276	LARS 178	France		<i>L. albus</i>	
RB237 ^a	<i>C. melonis</i>	CBS 134730	CPC20912	Brazil		<i>Malus</i> sp.	
RB238 ^a	<i>C. paranaense</i>	CBS 134729	CPC20901	Brazil		<i>Malus</i> sp.	
RB239 ^a	<i>C. abscissum</i>	CBS 134727	CPC20894	Brazil		<i>Psidium</i> sp.	
RB240 ^a	<i>C. paranaense</i>	CBS 134728	CPC20928	Brazil		<i>Prunus</i> sp.	
AB024 ^a	<i>C. lupini</i>	UBOCC-A-118079		Brittany, France	2016	<i>L. albus</i> var. Orus	st-l
AB025 ^a	<i>C. lupini</i>	UBOCC-A-118080		Brittany, France	2016	<i>L. albus</i> var. Orus	st-l
AB026 ^a	<i>C. lupini</i>	UBOCC-A-118081		Brittany, France	2016	<i>L. albus</i> var. Orus	st-l
AB027 ^a	<i>C. lupini</i>	UBOCC-A-118082		Brittany, France	2016	<i>Lupinus</i> sp.	st-l
AB028 ^a	<i>C. lupini</i>	UBOCC-A-118083		Brittany, France	2016	<i>Lupinus</i> sp.	st-l
AB029 ^a	<i>C. lupini</i>	UBOCC-A-118084		Brittany, France	2016	<i>Lupinus</i> sp.	st-l
AB030 ^a	<i>C. lupini</i>	UBOCC-A-118085		Brittany, France	2016	<i>Lupinus</i> sp.	st-l
AB031 ^a	<i>C. lupini</i>	UBOCC-A-118086		Brittany, France	2016	<i>Lupinus</i> sp.	st-l
AB037 ^a	<i>C. lupini</i>	UBOCC-A-118087		Brittany, France	2016	<i>Lupinus</i> sp.	st-l
AB042 ^a	<i>C. lupini</i>	UBOCC-A-118088		Brittany, France	2016	<i>Lupinus</i> sp.	st-l
AB043 ^a	<i>C. lupini</i>	UBOCC-A-118089		Brittany, France	2016	<i>Lupinus</i> sp.	st-l
AB044 ^a	<i>C. lupini</i>	UBOCC-A-118090		Brittany, France	2016	<i>L. albus</i> var. Orus	st

(Continued on next page)

^a Strains used for phylogenetic tree study underlying for reference strains.

^b Strains used for morphological diversity study.

^c Strains used for in vitro growth modeling.

^d Strains used for aggressiveness evaluation under optimal and suboptimal temperature study.

Table 1. (Continued from previous page)

Strain	Species	Collection code	Isolate	Origin	Isolation date	Host	Organ
AB045 ^a	<i>C. lupini</i>	UBOCC-A-118091		Brittany, France	2016	<i>Lupinus</i> sp.	st-l
AB046 ^a	<i>C. lupini</i>	UBOCC-A-118092		Brittany, France	2016	<i>Lupinus</i> sp.	st-l
AB047 ^a	<i>C. lupini</i>	UBOCC-A-118093		Brittany, France	2016	<i>L. albus</i> var. Orus	st-l
AB048 ^a	<i>C. lupini</i>	UBOCC-A-118094		Brittany, France	2016	<i>Lupinus</i> sp.	st-l
AB049 ^a	<i>C. lupini</i>	UBOCC-A-118095		Brittany, France	2016	<i>Lupinus</i> sp.	st-l
AB055 ^a	<i>C. lupini</i>	UBOCC-A-118097		Brittany, France	2016	<i>Lupinus</i> sp.—spring variety	st-l
AB056 ^a	<i>C. lupini</i>	UBOCC-A-118098		Brittany, France	2016	<i>L. albus</i> var. Energy	st-l
AB057 ^a	<i>C. lupini</i>	UBOCC-A-118099		Brittany, France	2016	<i>Lupinus</i> sp.	st
AB058 ^a	<i>C. lupini</i>	UBOCC-A-118100		Brittany, France	2016	<i>L. albus</i> var. Amiga	st-l
AB059 ^a	<i>C. lupini</i>	UBOCC-A-118101		Brittany, France	2016	<i>L. albus</i> spring variety	st-l
AB060 ^a	<i>C. lupini</i>	UBOCC-A-118102		Brittany, France	2016	<i>L. albus</i> var. Amiga	st-l
AB061 ^a	<i>C. lupini</i>	UBOCC-A-118103		Brittany, France	2016	<i>L. albus</i> var. Amiga	st-l
AB062 ^a	<i>C. lupini</i>	UBOCC-A-118104		Brittany, France	2016	<i>Lupinus</i> sp.—spring variety	st-l
AB063 ^a	<i>C. lupini</i>	UBOCC-A-118105		Brittany, France	2016	<i>L. albus</i> var. Amiga	st-l
AB064 ^a	<i>C. lupini</i>	UBOCC-A-118106		Brittany, France	2016	<i>L. luteus</i> var. Mister	st-l
AB065 ^a	<i>C. lupini</i>	UBOCC-A-118107		Brittany, France	2016	<i>Lupinus</i> sp.	st-l-po
AB066 ^a	<i>C. lupini</i>	UBOCC-A-118108		Brittany, France	2016	<i>L. angustifolius</i>	st-l-po
AB067 ^a	<i>C. lupini</i>	UBOCC-A-118109		Brittany, France	2016	<i>L. albus</i> var. Feodora	st-l-po
AB068 ^a	<i>C. lupini</i>	UBOCC-A-118110		Brittany, France	2016	<i>L. albus</i> var. Orus	st
AB069 ^a	<i>C. lupini</i>	UBOCC-A-118111		Brittany, France	2016	<i>L. albus</i> var. Orus	st
AB070 ^a	<i>C. lupini</i>	UBOCC-A-118112		Brittany, France	2016	<i>L. albus</i> var. Orus	st
AB071 ^a	<i>C. lupini</i>	UBOCC-A-118113		Brittany, France	2016	<i>L. albus</i> var. Orus	st
AB072 ^a	<i>C. lupini</i>	UBOCC-A-118114		Brittany, France	2016	<i>L. albus</i> var. Orus	st
AB073 ^a	<i>C. lupini</i>	UBOCC-A-118115		Brittany, France	2016	<i>L. albus</i> var. Clovis	st
AB074 ^a	<i>C. lupini</i>	UBOCC-A-118116		Brittany, France	2016	<i>L. albus</i> var. Orus	st
AB075 ^a	<i>C. lupini</i>	UBOCC-A-118117		Brittany, France	2016	<i>L. albus</i> var. Orus	st
AB076 ^a	<i>C. lupini</i>	UBOCC-A-118118		Brittany, France	2016	<i>Lupinus</i> sp.—spring variety	st
AB077 ^a	<i>C. lupini</i>	UBOCC-A-118119		Brittany, France	2016	<i>L. albus</i> var. Orus	st
AB078 ^a	<i>C. lupini</i>	UBOCC-A-118120		Brittany, France	2016	<i>L. albus</i> var. Orus	st
AB079 ^a	<i>C. lupini</i>	UBOCC-A-118121		Brittany, France	2016	<i>Lupinus</i> sp.—spring variety	st
AB080 ^a	<i>C. lupini</i>	UBOCC-A-118122		Brittany, France	2016	<i>L. albus</i> var. Orus	st
AB081 ^a	<i>C. lupini</i>	UBOCC-A-118123		Brittany, France	2016	<i>L. albus</i> var. Orus	st
AB082 ^a	<i>C. lupini</i>	UBOCC-A-118124		Brittany, France	2016	<i>L. albus</i> var. Orus	st
AB083 ^a	<i>C. lupini</i>	UBOCC-A-118125		Brittany, France	2016	<i>L. albus</i> var. Orus	st
AB084 ^a	<i>C. lupini</i>	UBOCC-A-118126		Brittany, France	2016	<i>Lupinus</i> sp.	st
AB085 ^a	<i>C. lupini</i>	UBOCC-A-118127		Brittany, France	2016	<i>L. albus</i> var. Orus	st
AB086 ^a	<i>C. lupini</i>	UBOCC-A-118128		Brittany, France	2016	<i>L. albus</i> var. Orus	st
AB087 ^a	<i>C. lupini</i>	UBOCC-A-118129		Brittany, France	2016	<i>L. albus</i> var. Orus	st
AB088 ^a	<i>C. lupini</i>	UBOCC-A-118130		Brittany, France	2016	<i>L. albus</i> var. Orus	st
AB089 ^a	<i>C. lupini</i>	UBOCC-A-118131		Brittany, France	2016	<i>L. albus</i> var. Orus	st
PN178 ^a	<i>C. lupini</i>	UBOCC-A-117294		France	2017	<i>L. albus</i> var. Orus	st
PN179 ^{a,b}	<i>C. lupini</i>	UBOCC-A-118132		France	2017	<i>L. albus</i> var. Orus	st
PN190 ^a	<i>C. lupini</i>	UBOCC-A-118133		France	2017	<i>Lupinus</i> sp.	st
PN191 ^a	<i>C. lupini</i>	UBOCC-A-118134		France	2017	<i>Lupinus</i> sp.	st
RB219 ^a	<i>C. lupini</i>	UBOCC-A-118136		Brittany, France	2014	<i>Lupinus</i> sp.	se-l
RB220 ^a	<i>C. lupini</i>	UBOCC-A-118137		Brittany, France	2014	<i>L. albus</i> var. Orus	st
RB221 ^{b,c,d}	<i>C. lupini</i>	IMI 504893; UBOCC-A-117274		Brittany, France	2014	<i>Lupinus</i> sp.	se-st
RB222 ^a	<i>C. lupini</i>	UBOCC-A-118138		Brittany, France	2014	<i>Lupinus</i> sp.	st-l
RB223 ^a	<i>C. lupini</i>	UBOCC-A-118139		Brittany, France	2014		
RB224 ^a	<i>C. lupini</i>	UBOCC-A-118140		Brittany, France	2014	<i>L. albus</i> var. Orus	st
RB225 ^a	<i>C. lupini</i>	UBOCC-A-118141		Brittany, France	2014	<i>Lupinus</i> sp.	po
RB226 ^{b,c,d}	<i>C. lupini</i>	UBOCC-A-117275		Brittany, France	2014	<i>L. albus</i> var. Lumen	po
RB227 ^a	<i>C. lupini</i>	UBOCC-A-118142		Brittany, France	2014	<i>L. albus</i> var. Orus	st
RB228 ^{b,c}	<i>C. lupini</i>	UBOCC-A-118143		Brittany, France	2014	<i>Lupinus</i> sp.	st-l
RB229 ^a	<i>C. lupini</i>	UBOCC-A-118144		Brittany, France	2014	<i>Lupinus</i> sp.	st-l
RB230 ^a	<i>C. lupini</i>	UBOCC-A-118145		Brittany, France	2013	<i>L. albus</i> var. Lumen	st-l
RB236 ^a	<i>C. lupini</i>	UBOCC-A-118146		Brittany, France	2014	<i>Lupinus</i> sp.	
RB241 ^a	<i>C. lupini</i>	UBOCC-A-118147		Brittany, France	2015	<i>Lupinus</i> sp.	
RB242 ^a	<i>C. lupini</i>	UBOCC-A-118148		France	2016	<i>Lupinus</i> sp.	

The nucleotide sequences for ITS, ACT, CHS, GAPDH, HIS, HMG, TUB, and APN/MAT1 have been deposited at the NCBI website and are available with the accession numbers listed in Table 3.

Effect of temperature in vitro: Radial growth rate assessment and model fitting. Mycelial plugs from the margin of actively growing cultures of six isolates (Table 1) were placed in the center of PDA plates. The plates were then incubated at 5, 7, 10, 15, 17, 20, 25, 27, 30, and 37°C in temperature-controlled incubators (KB 240; Binder GmbH) (Thomas et al. 2008). Colony radius was measured daily in two perpendicular directions for a maximum of 40 days (Morin-Sardin et al. 2016; Nguyen Van Long et al. 2017). Three biological replicates were made for each temperature × isolate combination.

A two-step modeling approach was adopted. In the first step, radial growth as a function of incubation time was modeled with a two-phase primary model with latency for each strain and incubation temperature (Dagnas and Membré 2013) (equation 1):

$$r(t) = \begin{cases} r_0, & t \leq \lambda \\ r_0 + \mu \cdot (t - \lambda), & t > \lambda \end{cases} \quad (1)$$

where $r(t)$ is the radius of the mycelium (millimeters) at the time of incubation t (days) and λ is the latency (days) before growth characterized by μ , the radial growth rate (millimeters per day). In this study, the radius $r(0)$ was fixed at 2.5 mm.

In the second step, radial growth rate μ (millimeters per day) was modeled as a function of incubation temperature (T) with a cardinal model (adapted from Rosso 1995) (equation 2):

$$\mu = \mu_{opt}(T) \cdot CM_N(T)$$

$$CM_N(T) = \begin{cases} \frac{(T - T_{max}) \cdot (T - T_{min})^n}{(T_{opt} - T_{min})^{n-1} \cdot \{(T_{opt} - T) \cdot (T - T_{opt}) - (T_{opt} - T_{max}) \cdot [(n-1) \cdot T_{opt} + T_{min} - n \cdot T]\}}, & T_{min} < T < T_{max} \\ 0, & T \leq T_{min} \\ 1, & T \geq T_{max} \end{cases} \quad (2)$$

where T_{min} , T_{opt} , and T_{max} (cardinal T values) are the minimum, optimum, and maximum T values, respectively; n is a shape parameter ($n = 2$); μ is the radial growth rate; and μ_{opt} is the value of μ when $T = T_{opt}$. Modeling was conducted with Matlab software (R2017a; Mathworks). To evaluate the model fitting performances, the coefficient of determination (r^2) and the root mean square error (RMSE) were computed (Ratkowsky 2004).

Evaluation of in planta aggressiveness. The variety of lupin used was a spring variety named Feodora (Jouffray-Drillaud). Lupin seeds were disinfected for 1 min into a solution of 70% ethanol and then, rinsed three times into distilled sterile water before being placed on water agar media for 3 days in Jiffy pots (peat pellets, 41 mm diameter). Plants were grown at 25°C during day and night with 16 h of light, 8 h of darkness, and 60% humidity for 2 weeks before inoculation. Plants were watered every 2 days during the entire experiment.

Spores of RB221 and RB226, harvested from 2-week-old PDA cultures at 25°C, were suspended into 1.5 ml of distilled sterile water, and the concentration was adjusted to 1×10^4 spores per milliliter. Five microliters of the spore suspension was used to inoculate sterilized lupin boiled seeds (adapted from Saubeau et al. 2014) placed on plates of 1.3% (wt/vol) water agar (Biomérieux). After a week at 25°C, the final spore inoculum was prepared by shaking infected lupin seeds into 3 to 4 ml of distilled water and adjusted to 1×10^7 spores per milliliter.

The pathogenicity assay at suboptimal temperature was performed using two isolates with contrasted behavior at low temperatures in vitro (i.e., RB221 and RB226 displaying different T_{min} and T_{opt}) (Table 4). These inoculations were performed with

2-week-old plantlets; at that time, plants were developed enough to observe symptoms, especially at low temperatures. At this stage, we previously noticed that a wound of the stem with a surgical needle before injection of the inoculum was needed to ensure the observation of disease symptoms (G. Dubrulle, unpublished data). Five microliters of inoculum (1×10^7 spores per milliliter) was directly injected with a needle into the middle of the stem. For each strain tested, 10 plants were inoculated, and three biological repetitions were performed. Plantlets inoculated with water were used as negative control. Inoculated plants were grown under the same parameters as described previously and exposed to 5, 10, or 25°C.

Symptoms were quantified and plants were collected at 0 and 7 days postinoculation (dpi) at 25°C; at 0, 7, 14, and 21 dpi at 10°C; and at 0, 7, 14, 21, and 28 dpi at 5°C. Aggressiveness was determined by measuring the size of the necrotic lesions and quantifying the amount of *C. lupini* DNA by qPCR using species-specific primers. One hundred milligrams of fresh stem of each plant was sampled around the inoculated or necrotic area. For each condition, 10 plants were pooled together, frozen in liquid nitrogen, and lyophilized for 48 h before DNA extraction. In addition, the area under the disease progress curve (AUDPC) of necrosis length or *C. lupini* DNA was also calculated. To calculate AUDPC, lesion lengths of 10 plants measured at each time were averaged for each of the three biological repetitions.

Statistical analyses were performed using RStudio software (version 3.2.5). For each studied variable (necrosis length, amount of *C. lupini* DNA, or AUDPC), an analysis of variance (ANOVA) was performed to study the influence of temperature, isolate, and time-point postinoculation on both lesion length and fungal DNA quantification.

ANOVAs were performed to identify the effect of each variable on lesion length or fungal DNA quantification. Then, a multiple comparisons test with posthoc Tukey honestly significant difference (HSD) method was performed to identify statistical groups within each variable. The level of significance was set at $\alpha = 0.05$. The impact of the strain on the two measures was tested with an ANOVA at each temperature or each time point postinoculation, and the temperature effect was evaluated with an ANOVA for each strain and time postinoculation. The lesion length and fungal DNA quantification were compared by pairwise condition.

Extraction of *C. lupini* DNA in planta. Dried stem samples were ground in a Mixer mill (Retsch; MM400) three times at 30-Hz frequency for 30 s either in a 50-ml stainless steel jar containing one 15-mm-diameter stainless steel ball for field samples or in 2-ml Eppendorf tubes containing a ceramic ball for inoculated plants. DNA was extracted from approximately 100 mg of ground lupin samples from fields and 30 mg of inoculated plants with the FastDNA Spin kit (MP Biomedicals) following the manufacturer's instructions. Quality and concentration of the DNA were determined using a UV spectrophotometer (Nanodrop1000; Thermo Scientific), and DNA was stored at -20°C until use. Total DNA extracted from samples was diluted to 10 ng/μl final concentration for qPCR analysis.

qPCR conditions for *C. lupini* quantification in planta. A set of primers was designed in a conserved species-specific region of the *C. lupini* genome. The species-specific primers CLUP01-F 5'-ATGATGCTTTCTTTGGTGGCG-3' and CLUP01-R 5'-CCGGCCTGAAGACACAACG-3' (R. Baroncelli, personal communication) were tested in PCR or qPCR assays with nine *C. lupini* isolates covering the intraspecific diversity observed in this study. In addition, we checked that primers CLUP01-F and CLUP01-R did not amplify sequences from 10 closely related *Colletotrichum* species belonging to the Acutatum complex (namely *C. fioriniae*, *C. godetiae*, *C. nymphaeae*, *C. rhombiforme*, *C. phormii*, *C. kinghornii*, *C. laticiphila*, *C. acutatum*, *C. simmondsii*, and *C. brisbanense*) and 3 fungal isolates not belonging to the *Colletotrichum* genus but isolated from lupin (*Phoma pinodella*, *Truncatella angustata*, and *Botrytis cinerea*). The 25-μl PCR mix consisted of 1× GoTaq buffer, 0.2 mmol of dNTP, 0.2 μM each primer, 2 mM MgCl₂, 1.25 U of GoTaq polymerase (Promega), and 1 μl

of template DNA. The PCR assay started by initial denaturation at 95°C for 5 min followed by 35 cycles at 95°C for 1 min, 68°C for 1 min, and 72°C for 30 s and a final extension step at 72°C for 3 min. PCR products were verified on a 2% (wt/vol) agarose gel in 1× TAE (tris, acetate, EDTA) buffer (40 mM Tris-acetate and 1 mM EDTA, pH 8.0) and visualized after staining with a GelRed solution under UV light.

All qPCR assays were performed on a CFX96 Real-Time PCR System (Bio-Rad) in a 20-μl reaction volume containing 10 μl of SsoFast EvaGreen Supermix (Bio-Rad), 500 nM species-specific primers CLUP01-F and CLUP01-R, 7 μl of DNase-free water, and 1 μl of total DNA sample diluted to 10 ng/μl. The following thermal program was applied: an initial denaturation step of 98°C for 2 min followed by 40 amplification cycles of a 5-s denaturing step (98°C) and 3-s annealing-extension step (68°C). The analysis of the melting curve was used to check the absence of nonspecific PCR amplification and dimer formation. The melting curve was generated with a temperature gradient of 0.5°C/s from 65 to 95°C. The melting temperature obtained with *C. lupini* amplicons was 81°C. In addition, each run included no template controls, which never amplified or at cycle threshold (C_T) >39. Quantification of *C. lupini* was expressed in nanograms of fungal DNA in 10 ng of total DNA. Negative controls were not shown in the analysis.

The fungal DNA extracted from pure cultures of *C. lupini* isolate RB221 was used to prepare a 10-fold dilution series of five

concentration points starting with 10 ng/μl. These dilutions series are hereafter referred to as “fungal DNA series.” To take into account the presence of plant DNA, additional dilution series were prepared by mixing the fungal DNA series in a lupin background DNA concentration fixed at 10 ng/μl (hereafter referred to as “background DNA series”). The standard curve used to quantify fungal DNA in the lupin matrices corresponded to the 10- to 10⁻³-ng/μl range of fungal DNA all mixed with 10 ng/μl of lupin DNA. The C_T values were obtained by the CFX Manager Software version 1.6 (Bio-Rad). To calculate the amplification efficacy (AE) of each standard curve, C_T values were plotted against log₁₀ (fungal DNA), and the slope of the linear curves was determined using the following equation:

$$AE = \left(10^{(-1/\text{slope})}\right) - 1$$

The average PCR efficacy of the standard curves always ranged between 90 and 105%, whereas the R^2 of standard curves was systematically >0.98. The C_T values of our standard curves ranged between 23.5 ± 0.4 and 36.8 ± 0.5.

Results

Phylogenetic study among *C. lupini* isolates. In this study, 116 isolates were used, including 71 isolates collected in western France between 2014 and 2017, 16 reference strains of *C. lupini*, and 28

Table 2. List of primer pairs and PCR conditions used for this study

Locus	Name	Primers	Primer sequences	Step	Time	Temp. (°C)	Cycles	Reference
ITS	Internal transcribed spacer	ITS5 ITS4	GGAAGTAAAAGTCGTAACAAGG TCCGCTTATTGATATGC	Denaturation	5 min	95	1	White et al. (1990)
				Amplification	1 min	95	30	
					1 min	55		
					1 min	72		
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	GDF1 GDR1	GCCGTCACGACCCCTTCATTGA GGGTGGAGTCGTAAGTGGAGCATGT	Final extension	10 min	72	1	Guerber et al. (2003)
				Denaturation	5 min	94	1	
				Amplification	30 s	94	35	
					30 s	60		
TUB2	β-tubulin 2	BT2Fd BT4R	GTBCACCTYCARACCGGYCARTG CCRGAYTGRCRAARACRAAGTTGTC		30 s	72		Woudenberg et al. (2009)
				Final extension	7 min	72	1	
				Denaturation	2 min	95	1	
				Amplification	1 min	94	30	
CHS-1	Chitin synthase 1	CHS-79F CHS-354R	TGGGGCAAGGATGCCTGGAAGAAG TGGAAGAACCATCTGTGGGAGTTG		1 min	67		Modified from Carbone and Kohn (1999)
					1 min	72		
				Final extension	5 min	72	1	
				Denaturation	2 min	95	1	
ACT	Actin	ACT-512F ACT-783R	ATGTGCAAGGCCGGTTTTCGC TAGGAGTCTTCTGACCCAT	Amplification	1 min	95	40	Modified from Carbone and Kohn (1999)
					30 s	57		
					25 s	72		
				Final extension	5 min	72	1	
HIS3	Histone H3	CYLH3Fext CYLH3R	AGTCCACTGGTGGCAAGGC AGCTGGATGTCCTTGGACTG	Denaturation	2 min	95	1	Crous et al. (2006)
				Amplification	1 min	95	40	
					30 s	57		
					25 s	72		
HMG	HMG box region	HMGacuF2 HMGacuR	TACCGCAGCGACTACCAAGC TCTTGTGGCGCTCCCTTG	Final extension	5 min	72	1	Baroncelli et al. (2015b)
				Denaturation	5 min	95	1	
				Amplification	1 min	95	40	
					1 min	55		
APN/ MAT1	Apn2-Mat1-2-1 intergenic	Apnmat1F Apnmat1R	GCGAAAGCCTACAAGGAGCG TGGTCAAAGTTCAACCTGGG		30 s	72		This study
				Final extension	20 min	72	1	
				Denaturation	5 min	95	1	
				Amplification	1 min	95	40	
					1 min	55		
					20 s	72		
					20 min	72	1	
				Final extension	20 min	72	1	

Table 3. GenBank accession numbers for internal transcribed spacer (ITS), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), chitin synthase 1 (CHS-1), histone H3 (HIS3), actin (ACT), β -tubulin 2 (TUB2), HMG box region (HMG), and Apn2-Mat1-2-1 intergenic (APN/MAT1)^a

Strain	ITS	GAPDH	CHS-1	HIS3	ACT	TUB2	HMG	APN/MAT1	References
RB017	JQ948188	JQ948518	JQ948849	JQ949179	JQ949509	JQ949839	KM251976	MK478306	Damm et al. (2012); Baroncelli et al. (2015b)
RB018	JQ948189	JQ948519	JQ948850	JQ949180	JQ949510	JQ949840	KM251977	MK478307	Damm et al. (2012); Baroncelli et al. (2015b)
RB020	MK463722	KM252117	MK463771	MK463798	MK478159	MK478186	KM251979	MK478308	Baroncelli et al. (2015b)
RB021	JQ948342	JQ948672	JQ949003	JQ949333	JQ949663	JQ949993	MK478211		Damm et al. (2012)
RB022	JQ948343	JQ948673	JQ949004	JQ949334	JQ949664	JQ949994	KM251980	MK478309	Damm et al. (2012); Baroncelli et al. (2015b)
RB042	MH865693	JQ948508	JQ948839	JQ949169	JQ949499	JQ949829	MK478212	MK478310	Damm et al. (2012); Vu et al. (2019)
RB096	JQ948466	JQ948797	JQ949127	JQ949457	JQ949787	JQ950117	KM252052	MK478311	Damm et al. (2012); Baroncelli et al. (2015b)
RB111	KT153562	KT153552	KT153547	KT153557	KT153542	KT153567	KM252057	MK478312	Damm et al. (2012); Baroncelli et al. (2015b)
RB116	MK463723	KM252194	MK463772	MK463799	MK478160	KM251944	KM252059	MK478313	Baroncelli et al. (2015b)
RB119	MK463724	MK463748	MK463773	MK463800	MK478161	MK478187	MK478213	MK478314	
RB120	MK463725	MK463749	MK463774	MK463801	MK478162	MK478188	MK478214	MK478315	
RB121	KJ018635	KJ018659	MK463775	MK463802	MK478163	MK478189	KM252062	MK478316	Baroncelli et al. (2015b)
RB122	MK463726	MK463750	MK463776	MK463803	MK478164	MK478190	MK478215	MK478317	
RB123	MK463727	MK463751	MK463777	MK463804	MK478165	MK478191	MK478216	MK478318	
RB124	MK463728	MK463752	MK463778	MK463805	MK478166	MK478192	MK478217	MK478319	
RB125	JQ948172	JQ948502	JQ948833	JQ949163	JQ949493	JQ949823	MK478218	MK478320	Damm et al. (2012)
RB127	MK463729	MK463753	MK463779	MK463806	MK478167	MK478193	MK478219	MK478321	
RB147	MK463730	KM252203	MK463780	MK463807	MK478168	KM251951	KM252079	MK478322	Baroncelli et al. (2015b)
RB151	JQ948453	JQ948784	JQ949114	JQ949444	JQ949774	JQ950104	KM252082	MK478323	Damm et al. (2012); Baroncelli et al. (2015b)
RB156	JQ948476	JQ948807	JQ949137	JQ949467	JQ949797	JQ950127	KM252084	MK478324	Damm et al. (2012); Baroncelli et al. (2015b)
RB157	JQ948460	JQ948791	JQ949121	JQ949451	JQ949781	JQ950111	KM252085	MK478325	Damm et al. (2012); Baroncelli et al. (2015b)
RB163	JQ948243	JQ948573	JQ948904	JQ949234	JQ949564	JQ949894	KM252091	MK478326	Damm et al. (2012); Baroncelli et al. (2015b)
RB170	JQ948196	JQ948526	JQ948857	JQ949187	JQ949517	JQ949847	KM252095	MK478327	Damm et al. (2012); Baroncelli et al. (2015b)
RB172	JQ948169	JQ948499	JQ948830	JQ949160	JQ949490	JQ949820	MK478220	MK478328	Damm et al. (2012)
RB173	JQ948155	JQ948485	JQ948816	JQ949146	JQ949476	JQ949806	KM252096	MK478329	Damm et al. (2012); Baroncelli et al. (2015b)
RB177	JQ005776	JQ948677	JQ005797	JQ005818	JQ005839	JQ005860	MK478221	MK478330	Damm et al. (2012)
RB178	JQ948396	JQ948727	JQ949057	JQ949387	JQ949717	JQ950047	MK478222	MK478331	Damm et al. (2012)
RB179	JQ948276	JQ948606	JQ948937	JQ949267	JQ949597	JQ949927	KM252099	MK478332	Damm et al. (2012); Baroncelli et al. (2015b)
RB183	JQ948462	JQ948793	JQ949123	JQ949453	JQ949783	JQ950113	MK478223		Damm et al. (2012)
RB184	JQ948181	JQ948511	JQ948842	JQ949172	JQ949502	JQ949832	KM252102	MK478333	Damm et al. (2012); Baroncelli et al. (2015b)
RB186	JQ948277	JQ948607	JQ948938	JQ949268	JQ949598	JQ949928	KM252104	MK478334	Damm et al. (2012); Baroncelli et al. (2015b)
RB187	JQ948351	JQ948682	JQ949012	JQ949342	JQ949672	JQ950002	MK478224	MK478335	Damm et al. (2012)
RB190	KT153561	KT153551	KT153546	KT153556	KT153541	KT153566	MK478225	MK478336	Bragança et al. (2016)
RB196	KT153559	KT153549	KT153544	KT153554	KT153539	KT153564	KM252107	MK478337	Baroncelli et al. (2015b); Bragança et al. (2016)
RB197	KT153558	KT153548	KT153543	KT153553	KT153538	KT153563	KM252108	MK478338	Baroncelli et al. (2015b); Bragança et al. (2016)
RB209	JQ948153	JQ948483	JQ948814	JQ949144	JQ949474	JQ949804	MK478226	MK478339	Damm et al. (2012)
RB210	JQ948273	JQ948603	JQ948934	JQ949264	JQ949594	JQ949924	MK478227		Damm et al. (2012)
RB216	JQ948195	JQ948525	JQ948856	JQ949186	JQ949516	JQ949846	MK478228	MK478340	Damm et al. (2012)
RB217	JQ948161	JQ948491	JQ948822	JQ949152	JQ949482	JQ949812	MK478229	MK478341	Damm et al. (2012)
RB218	JQ948191	JQ948521	JQ948852	JQ949182	JQ949512	JQ949842	MK478230	MK478342	Damm et al. (2012)
RB235	JQ948159	JQ948489	JQ948820	JQ949150	JQ949480	JQ949810	MK478243	MK478355	Damm et al. (2012)
RB237	KC204997	KC205031	KC205048	KC205007	KC205082	KC205065	MK478245	MK478357	Bragança et al. (2016)
RB238	KC204992	KC205026	KC205043	KC205004	KC205077	KC205060	MK478246	MK478358	Bragança et al. (2016)
RB239	KC204988	KC205022	KC205039	KC205003	KC205073	KC205056	MK478247	MK478359	Bragança et al. (2016)
RB240	KC205002	KC205036	KC205053	KC205019	KC205087	KC205070	MK478248	MK478360	Bragança et al. (2016)
AB024								MK478250	
AB025								MK478251	
AB026								MK478252	
AB027								MK478253	
AB028	MK463719	MK463745	MK463768	MK463795	MK478156	MK478183	MK478208	MK478254	

(Continued on next page)

^a Those in bold type refer to accession numbers newly determined in this study.

Table 3. (Continued from previous page)

Strain	ITS	GAPDH	CHS-1	HIS3	ACT	TUB2	HMG	APN/MAT1	References
AB029	MK463720	MK463746	MK463769	MK463796	MK478157	MK478184	MK478209	MK478255	
AB030								MK478256	
AB031								MK478257	
AB037								MK478258	
AB042								MK478259	
AB043								MK478260	
AB044								MK478261	
AB045								MK478262	
AB046								MK478263	
AB047								MK478264	
AB048								MK478265	
AB049								MK478266	
AB055								MK478267	
AB056	MK463721	MK463747	MK463770	MK463797	MK478158	MK478185	MK478210	MK478268	
AB057								MK478269	
AB058								MK478270	
AB059								MK478271	
AB060								MK478272	
AB061								MK478273	
AB062								MK478274	
AB063								MK478275	
AB064								MK478276	
AB065								MK478277	
AB066								MK478278	
AB067								MK478279	
AB068								MK478280	
AB069								MK478281	
AB070								MK478282	
AB071								MK478283	
AB072								MK478284	
AB073								MK478285	
AB074								MK478286	
AB075								MK478287	
AB076								MK478288	
AB077								MK478289	
AB078								MK478290	
AB079								MK478291	
AB080								MK478292	
AB081								MK478293	
AB082								MK478294	
AB083								MK478295	
AB084								MK478296	
AB085								MK478297	
AB086								MK478298	
AB087								MK478299	
AB088								MK478300	
AB089								MK478301	
PN178								MK478302	
PN179								MK478303	
PN190								MK478304	
PN191								MK478305	
RB219	MK463731	MK463754	MK463781	MK463808	MK478169	MK478194	MK478231	MK478343	
RB220								MK478344	
RB221								MK478345	
RB222								MK478346	
RB223								MK478347	
RB224								MK478348	
RB225								MK478349	
RB226								MK478350	
RB227								MK478351	
RB228								MK478352	
RB229								MK478353	
RB230								MK478354	
RB236								MK478356	
RB241								MK478361	
RB242								MK478362	

reference strains belonging to other species of the CAsc and an outgroup *C. orchidophilum* (Table 1). Phylogenetic analyses based on eight different loci was first performed on 63 isolates (ITS, ACT, CHS, GAPDH, HIS, HMG, TUB, and APN/MAT1). Because APN/MAT1 showed the highest variability, it was the only locus used to characterize the additional 54 strains. All isolates collected in western France between 2014 and 2017 from infected lupin plants belonged solely to the *C. lupini* species. The molecular analysis of 87 *C. lupini* isolates (28 strains from 14 species belonging to the CAsc and using *C. orchidophilum* as an outgroup) was performed on a sequence alignment with 2,124 characters, of which 1,591 were conserved, 303 were parsimony informative, and 208 were singleton. The loci boundaries were ITS: 1 to 500, CHS-1: 501 to 763, TUB2: 764 to 1,255, ACT: 1,256 to 1,496, HIS3: 1,497 to 1,875, and GAPDH: 1,876 to 2,124. Based on the multilocus molecular analysis, the 87 *C. lupini* isolates belonged to two different groups: isolates RB121, RB173, and RB235 belonged to group I, whereas the other isolates were clustered together (Fig. 1).

Morphological diversity. Morphological diversity was analyzed on a subset of nine strains, including strains of group I and group II as well as isolates from both our collection and reference collections (Table 1).

On PDA, all isolates produced more or less dark flat mycelia with an entire margin; surface was buff to gray covered with felty to woolly pale gray aerial mycelium, and reverse was buff to dark brown with a pale regular margin for some strains (RB173, RB235, RB121, and PN179). Radial sulcate appeared on RB173, PN179, and RB235 (Fig. 2A).

Melanized appressorial structures were observed on PDA for RB221, PN179, and RB228 (Fig. 2B).

In freshly isolated strains, conidia were produced in small orange masses. However, conidiomata were no longer observed along subcultures, and conidia production was substantially reduced except for RB235, which continued to produce a prolific amount of conidia compared with the other strains. For all strains, conidia were typical of *C. acutatum* described previously (Nirenberg et al. 2002). Most conidia showed a biguttulate aspect with a rounded extremity while the other was slightly pointed (Fig. 2C and D). For all strains, the width and length of conidia were examined, ranging from 3.5 to 4.5 μm (mean 4.1) and from 9 to 15 μm (mean 12.5), respectively.

Interestingly, after 35 days, microsclerotia were observed for RB121 when challenged to either itself, PN179, RB226, or RB228; for RB228 when confronted to either itself or RB121; or for RB217 and PN179 when confronted to RB173 (Fig. 2E).

Physiological diversity in vitro: Growth kinetics and effect of temperature. Growth kinetics of six *C. lupini* strains could be divided into two distinct phases: a lag phase followed by a constant radial growth of the mycelium. In optimal conditions for growth of each fungus, lag phases were relatively short, whereas growth rates were high at the low temperature tested (data not shown). Between minimal and optimal growth temperature, lag phases were longer, and growth rates were lower.

For all of the tested strains, the μ parameter increased as the temperature increased from 5 to 25°C and then, decreased at higher temperatures.

The minimal growth temperatures were 5°C for RB221, RB226, RB228, and RB235 and 7°C for RB173 and RB217. At

5°C, the observed growth rate was too low and could be modeled only for the strain RB228. For all the strains tested, the maximum temperature for which growth was observed was 30°C. The secondary model (equation 2) was fitted to describe growth rate dependence as a function of temperature (ranging from 5 to 37°C) for all strains (Fig. 3). Residual variability between predictive and observed values of growth was low (RMSE values ranged from 1.9 to 2.7 mm/day), and the coefficient of determination (r^2) values ranged from 0.90 to 0.95 for each strain. The estimated values of the parameters from the model were T_{min} varying from -4.2 to 1.3°C, T_{opt} from 23.7 to 25.6°C, and T_{max} from 30.6 to 31.8°C (Table 4). In addition, μ_{opt} ranged between 3.09 $\pm$ 0.06 mm/day (strain RB042) and 3.80 $\pm$ 0.13 mm/day (strain RB228) (Table 4).

Evaluation of in planta aggressiveness under optimal and suboptimal growth conditions. The effect of repetitions or two-way interactions between repetitions and any other factor (temperature, strain, or time postinoculation) was not significant for both necrotic lesion length and *C. lupini* DNA. When comparing lesion length of plants inoculated with either RB221 or RB226 and incubated at either 5, 10, or 25°C for 7 days, a significant temperature effect was found ($P < 0.001$), whereas no significant strain effect was noticed ($P = 0.582$). Lesion length was thus significantly higher at 25°C (mean lesion length: 29.9 $\pm$ 3.2 mm) than at both 5 and 10°C (mean lesion length: 0.08 $\pm$ 0.1 mm). At 7 dpi at 10°C ($P = 0.005$), the amount of DNA from RB226 (mean: 3.07 $\pm$ 0.30 ng) was significantly higher than the amount of DNA from RB221 (mean: 1.17 $\pm$ 0.52 ng). In addition, the level of fungal DNA was significantly higher at 10°C than at 5°C independently of the strain ($P = 0.049$) (Fig. 4B).

When comparing data obtained at 5 and 10°C over a 21-day period, lesion length significantly varied according to the temperature ($P < 0.001$) and the strain ($P < 0.001$). At both 14 and 21 dpi, a bigger necrosis was measured at 10°C or when inoculated with RB226 (Fig. 4A).

Field detection of *C. lupini*. *C. lupini* DNA was quantified in winter and spring lupin fields over the plant growth (Table 5). Of the 47 samples collected, *C. lupini* was quantified in six samples above the limit of quantification (10^{-3} ng of DNA), although all at a very low level (between 10^{-3} and 5.10^{-3} ng of DNA). Interestingly, *C. lupini* DNA was detected in four out of six asymptomatic samples, which suggested that the primers tested may enable the early detection of *C. lupini* in the field, before the appearance of symptoms.

Discussion

C. lupini has been frequently misidentified as *G. lupines* (Bondar 1912), *G. lupinicola* (Seymour 1929), or *G. cingulata* as the teleomorph of *C. gloeosporioides* (Weimer 1943, 1951, 1952; Wells and Bell 1969; Wells and Forbes 1967) or *C. acutatum*. The study we conducted first aimed at determining taxonomic diversity among 71 isolated strains from lupin with anthracnose symptoms using a multilocus sequencing method of eight loci, including locus APN/MAT1, which was used for the first time to characterize CAsc phylogeny. We clearly confirmed that our collection of 71 isolates from infected lupin plants belonged to the *C. lupini* species, suggesting that lupin anthracnose was exclusively associated with *C. lupini*. However, *C. lupini* strains are not exclusively isolated from the *Lupinus* genus. Among the strains used as reference to build the phylogenetic tree, RB042

Table 4. Cardinal value of the eight strains of *Colletotrichum lupini* obtained by the secondary modeling^a

Strains	T_{min} (°C)	T_{opt} (°C)	T_{max} (°C)	μ_{opt} (mm/day)	r^2	RMSE
RB173	1.29 $\pm$ 0.52	24.41 $\pm$ 0.25	31.22 $\pm$ 0.16	3.10 $\pm$ 0.06	0.95	2.03
RB217	-0.91 $\pm$ 0.55	25.31 $\pm$ 0.24	30.62 $\pm$ 0.08	3.79 $\pm$ 0.06	0.91	2.65
RB221	-4.15 $\pm$ 0.64	25.24 $\pm$ 0.22	30.90 $\pm$ 0.10	3.55 $\pm$ 0.04	0.93	2.34
RB226	0.69 $\pm$ 0.50	24.63 $\pm$ 0.23	30.86 $\pm$ 0.10	3.31 $\pm$ 0.06	0.93	2.22
RB228	0.74 $\pm$ 0.64	23.86 $\pm$ 0.40	31.78 $\pm$ 0.38	3.79 $\pm$ 0.12	0.90	2.68
RB235	-1.92 $\pm$ 0.63	23.68 $\pm$ 0.22	30.96 $\pm$ 0.10	3.32 $\pm$ 0.05	0.94	2.14

^a r^2 , determination coefficient; RMSE, root mean square error; T_{max} , maximum growth temperature; T_{min} , minimum growth temperature; T_{opt} , optimum growth temperature; μ_{opt} , radial growth rate at optimum temperature.

(CBS 129944) was isolated from Ceylon cinnamon tree (*Cinnamomum zeylanicum*) and identified as *C. lupini* species (Damm et al. 2012). The two ITS accession numbers of strain RB042, MH865693 and JQ948178, were submitted on the NCBI by Damm et al. (2012) and Vu et al. (2019). Based on the alignment of the RB042 sequence obtained in this study, we noticed that one nucleotide differed from the closest accession number MH865693 (Vu et al. 2019). The two TUB2 accession numbers of strain RB121, KJ018647 and AJ748629, were submitted on the NCBI by Baroncelli et al. (2015a) and Talhinhos et al. (2002). Based on the alignment of the RB121 sequence obtained in this study, we noticed that three nucleotides differed from the closest accession.

Morphological, physiological, and molecular studies enabled Nirenberg et al. (2002) to describe *C. lupini* as a separate species within the CAsc and characterized this species in two populations: *C. lupini* var. *lupini* and *C. lupini* var. *setosum*. A study in 2012 identified a higher degree of differences between strains based on the sequencing of six genes (Damm et al. 2012). In this study, two groups were observed based on eight sequence loci of 87 *C. lupini* strains and are qualified as group I and group II. These two groups belong to the A1 group described by Talhinhos et al. (2002) accepted as *C. lupini* (Damm et al. 2012). The smallest (group I) contains three strains, including the neotype strain RB173 (CBS 109225, UBOCC-A-117263), RB235, and RB121 (IMI 504884, HY09), two of which were identified as *C. lupini* var. *lupini* by Nirenberg et al. (2002). All the other strains belonged to the largest group (group II), including RB172 identified as *C. lupini* var. *setosum* by Nirenberg et al. (2002) and RB020 (PT30) belonging to the most numerous subgroup of A1 described by Talhinhos et al. (2002). In our study, the two groups were not distributed by geographical origin or specific morphological characteristics. The three strains of group I were isolated from Ukraine, France, and Canada. All isolates from our collections were included in the second group. Furthermore, the eight loci used were sufficient to discriminate the strains within each group. Additional markers, including single-nucleotide polymorphism markers or microsatellites, must be devised to better characterize genetic diversity at the intraspecific level and determine to which extent isolates within each group may originate from a clonal population.

All studied strains shared common morphological characteristics of *C. lupini*, such as the color of the mycelium. However, we noticed that none of our strains produced pink colonies with abundant orange spore masses, unlike some strains described by Talhinhos et al. (2002). Another morphological characteristic, also described for Australian lupin isolates (Yang and Sweetingham 1998), was the presence of a pale regular margin, which was present only for strains RB121, RB173, RB235, and PN179.

Microsclerotia were observed for RB121, RB228, RB217, and PN179 by the development of black mass structures on mycelium only under cocultures with itself or other isolates. RB121 was previously described in the literature (Nirenberg et al. 2002; Talhinhos et al. 2002), but these structures were never mentioned in its description. To our knowledge, microsclerotia have never been described within species belonging to the CAsc but are exclusively within the *Colletotrichum truncatum* s.l. species (Buchwaldt et al. 1996; Freeman et al. 2012). Microsclerotia allow the fungus to lie dormant in the soil during winter or under stressed conditions. Because microsclerotia were exclusively observed under cocultures, confrontation may induce the development of such conservation structures.

C. lupini has been identified as the only pathogenic agent for spring and winter lupin crops. Apparition of symptoms on winter lupins at spring raises the need to understand how *C. lupini* colonizes the plant during winter.

Radial growth modeling showed that T_{min} amplitude between strains was 5.45 versus 1.89°C for T_{opt} and 0.92°C for T_{max} , suggesting that most variability was observed at low temperatures. *C. lupini* was not able to grow at 5°C for RB173 and RB217 and at 37°C for all strains. The same observation was made by other studies, which demonstrated that *C. lupini* was able to grow at temperatures ranging from 5 to 35°C (Baroncelli et al. 2015a; Thomas and Sweetingham 2004). Some strains, particularly those isolated during this study, were able to grow slowly at 5°C. Estimated values of T_{min} suggested the potential for these strains to grow under 2°C, but this needs validation. Interestingly, after 40 days at 37°C, mycelium was no longer able to grow when placed 4 more weeks at 25°C, irrespective of strain (data not shown). This result indicates that a moderate thermal treatment may be used as a seed sanitization treatment. On *L. angustifolius*, seed infection was undetectable on 1,000 seeds after exposure for 1 to 4 days at 70°C or 8 days at 65°C (Thomas and Adcock 2004). Exposure of *L. albus* seeds for <2 weeks at <70°C reduced germination <15%, which increased significantly with higher temperatures (Thomas and Adcock 2004).

Although no symptoms were observed 7 dpi at 5 and 10°C, even with detectable amounts of fungal DNA in the plant at 10°C, symptoms appeared the day after the plant was placed at 25°C (data not shown). DNA quantification at 5°C at 21 dpi for RB221 and RB226 suggests that *C. lupini* is able to grow in planta at low temperature (Fig. 4). Optimal growth temperatures were significantly lower for RB226 ($T_{opt} = 24.63 \pm 0.23$) than for RB221 ($T_{opt} = 25.24 \pm 0.22$) based on our in vitro model. In planta,

Fig. 1. Phylogenetic tree of *Colletotrichum lupini* isolates. Group I contains three *C. lupini* strains (RB121, RB235, and RB173), and group II contains all the other *C. lupini* strains studied.

we also found that RB226 grew faster than RB221 under suboptimal conditions at 10°C with higher levels of necrosis and DNA. At 5 and 25°C, growth was too slow or too fast, respectively, to differentiate the strains. Interestingly, whatever the strain, necrosis symptoms appeared on plants between 7 and 14 dpi at 10°C, whereas fungal DNA was quantifiable earlier at 7 dpi, indicating that early detection of the pathogen in plants was

possible before symptoms appeared. The molecular detection of *C. lupini* was also possible in spring and winter lupin fields, even in the absence of symptoms on the plants (Table 5). The design of primers specific to *C. lupini* developed in this study is the first step to develop an efficient molecular detection tool, which will enable producers to perform early detection of *C. lupini* in the field.

Fig. 2. *Colletotrichum lupini* strains for morphological diversity. **A**, Morphology of the mycelium of the nine strains on potato dextrose agar media plates viewed on forward (Fw) and reverse (Rv) sides. **B**, Appressoria of RB221. Conidia of **C**, RB226 and **D**, RB221. **E**, Microsclerotia of RB121.

Fig. 3. Secondary models obtained for the six studied strains for temperature on potato dextrose agar medium. Radial growth (millimeters per day) of RB173, RB217, RB221, RB226, RB228, and RB235. Experimental data are indicated by $\circ$, and calculated values according to equation 2 are indicated by —.

Fig. 4. Box plots of **A**, lesion length and **B**, fungal DNA quantification inoculated with either RB221 or RB226 on two leaf-stage plants and incubated between 7 and 21 days postinoculation (dpi) at low temperature (5 and 10°C). *Significant differences between strains at each condition (analysis of variance, Tukey post hoc, $P < 0.05$).

Table 5. Quantification of *Colletotrichum lupini* DNA from lupin samples collected from winter and spring lupin fields over the plant growth

	Number of fields	Number of fields with visual symptoms	Number of total samples	Number of samples with DNA levels above LOQ/number of fields ^a	Number of samples with DNA above LOQ and without symptoms
Winter lupin	10	2	39	5 (0.004 ng DNA)/4 fields	3 (0.001 ng DNA)
Spring lupin	2	0	8	1 (0.001 ng DNA)/1 field	1

^a LOQ, limit of quantification.

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