Molecular Detection of the Seed-Borne Pathogen *Colletotrichum lupini* Targeting the Hyper-Variable IGS Region of the Ribosomal Cluster

Susanna Pecchia [1],* , **Benedetta Caggiano [1]**, **Daniele Da Lio [2]**, **Giovanni Cafà [3]** , **Gaetan Le Floch [2]** and **Riccardo Baroncelli [4],***

[1] Department of Agriculture, Food and Environment, University of Pisa, Via del Borghetto 80, 56124 Pisa, Italy
[2] Laboratoire Universitaire de Biodiversité et Ecologie Microbienne, EA 3882, IBSAM, ESIAB, Université de Brest, Technopôle Brest-Iroise, 29280 Plouzané, France
[3] CABI Europe-UK, Bakeham Lane, Egham, Surrey TW20 9TY, UK
[4] Instituto Hispano-Luso de Investigaciones Agrarias (CIALE), University of Salamanca, Calle del Duero 12, 37185 Villamayor (Salamanca), Spain
* Correspondence: susanna.pecchia@unipi.it (S.P.); riccardobaroncelli@gmail.com (R.B.)

Abstract: Lupins anthracnose is a destructive seed and airborne disease caused by *Colletotrichum lupini*, affecting stems and pods. Primary seed infections as low as 0.01–0.1% can cause very severe yield losses. One of the most effective management strategies is the development of a robust and sensitive seed detection assay to screen seed lots before planting. PCR-based detection systems exhibit higher levels of sensitivity than conventional techniques, but when applied to seed tests they require the extraction of PCR-quality DNA from target organisms in backgrounds of saprophytic organisms and inhibitory seed-derived compounds. To overcome these limitations, a new detection protocol for *C. lupini* based on a biological enrichment step followed by a PCR assay was developed. Several enrichment protocols were compared with Yeast Malt Broth amended with ampicillin, streptomycin, and lactic acid were the most efficient. A species-specific *C. lupini* primer pair was developed based on rDNA IGS sequences. The specificity was evaluated against 17 strains of *C. lupini*, 23 different *Colletotrichum* species, and 21 different organisms isolated from seeds of *Lupinus albus* cv. Multitalia, *L. luteus* cv. Mister, and *L. angustifolius* cv. Tango. The protocol described here enabled the detection of *C. lupini* in samples artificially infected with less than 1/10,000 infected seed.

1. Introduction

Lupinus spp. are important agronomic crops worldwide, with several species that are grown as ornamentals or as pioneer plants in soil maintenance programmes [1,2]. In natural ecosystems, lupin plants are key components of a wide range of soils and climates, mainly around the Mediterranean basin, East Africa, and the entire American continent. While the species of Africa, Asia, and Europe are few and well defined, the American species are many, morphologically intersecting, and intercrossing [3].

Lupin is an important crop mainly due to its elevated seed protein content, its adaptability to low fertility soils, and its valuable influence on crop rotation [1,4–6]. The seed composition of specific cultivars of white lupin (*Lupinus albus*) is very similar to soybean (35% proteins and 0.5% starch), with some species that are able to reach high levels of up to 18% of oil content. The vividly colored flowers of lupins contribute to their importance as ornamental plants (e.g., *L. polyphyllus*, also known as large-leaved lupine), and are amongst the most popular ornamental plants in temperate climate

areas [7]. For all these reasons, lupin production has rapidly increased in the last 10 years from 778,040 to 1,610,969 tonnes, [8]; nevertheless, effective lupin production is limited by diseases, which can lead to substantial yield and quality losses. In the past four decades, lupin production has encountered a new challenge: the devastating disease anthracnose affecting all plant tissues caused by *Colletotrichum lupini* [9–12].

Anthracnose has become a severe disease of lupin species worldwide, causing significant yield losses as high as 100%, representing a major limiting factor for lupin production [11–15]. Symptoms of anthracnose are similar for all *Lupinus* spp. [16], and are characterised by necrotic tissues with the presence of orange conidial masses and characteristic stems, petioles, and pods twisting [4]. Infected seeds become necrotic and wrinkled; when seeds are able to germinate, the seedlings show dark sunken necrosis in the cotyledons or in the hypocotyl [17]. The disease is mainly seed-borne [18], with primary seed infections as low as 0.01% that can cause extremely severe infections depending on climatic and agro ecological conditions [13,19]. While most lupin species are characterized by wide genetic diversity, it has been very difficult to find successful resistance sources. Recent achievements in this area are still limited by a very restricted number of known resistance genes, exposing lupin to the potential overcoming of such resistances [4].

Most species of the genus *Colletotrichum* (*Ascomycota*, *Sordariomycetes*, *Glomerellales*, and *Glomerellaceae*) are associated with plant diseases, and generally referred to as anthracnose. *Colletotrichum* spp. can infect a wide range of hosts and are distributed worldwide. Virtually every crop is susceptible to one or more species of *Colletotrichum*, which is considered one of the most important fungal plant pathogens based on scientific/economic importance [20]. Common *Colletotrichum* spp. hosts include many dicotyledonous plants and most important cereals. Species of *Colletotrichum* and *Magnaporthe* have a distinct hemibiotrophic lifestyle characterized by a brief biotrophic phase, associated with large intracellular primary hyphae and a subsequent switch to a necrotrophic phase in addition to narrow secondary hyphae that spread through the host tissue. In past decades, the genus *Colletotrichum* has been the centre of intense debate and frequent taxonomic changes [20], with recent multi-locus phylogenetics that has led to the identification of at least 14 major monophyletic clades known as "species complexes" [21,22]. *Colletotrichum* species identified within and among these complexes have shown significant differences in their host range. For example, the *C. acutatum* species complex includes *C. nymphaeae* and *C. fioriniae*, which display a broad host range, and *C. lupini*, which exhibits a host preference for lupin species. Due to this biological diversity, the *C. acutatum* species complex has been suggested as a suitable model to investigate genomic signatures associated with changes of key biological characters [4,23]. Recently, the intraspecific diversity of *Colletotrichum* associated with lupin crops in the western area of France and worldwide has been widely investigated [24]. All strains isolated from anthracnose-infected lupin have been identified as *C. lupini*, confirming that the disease is exclusively caused by this species. The genetic intraspecific diversity based on eight genomic loci with high resolution was low and revealed the presence of two distinct genetic groups [24].

The nuclear ribosomal cluster has been the most common DNA marker for PCR primer design of diagnostic assays for the detection of fungal plant pathogens. Moreover, this method allows for sensitive detection, as it is organized in units highly repeated within genomes. The cluster contains rRNA genes (18S, 5.8S, and 28S) separated by two internal transcribed spacers (ITS1 and ITS2) and one intergenic spacer (IGS) [25]. Recent phylogenetic studies have highlighted that the ITS region of the rDNA does not have sufficient variability to perform a correct taxonomic designation [26] or to develop specific primers for most *Colletotrichum* species. For this reason, other highly polymorphic single copy genes have been proposed to develop specific detection methods for *Colletotrichum* spp. [27]. In the present study, authors decided to focus their attention on the IGS region due its multicopy nature and high variability. The IGS region is highly polymorphic, and thus is a useful region for specific molecular diagnostic assays in fungal plant pathogens. These included *Botrytis cinerea* [28,29], *Fusarium circinatum* [30], *F. oxysporum* f. sp. *vasinfectum* [31], *Fusarium oxysporum* f. sp. *cubense* [32], *Phytophthora medicaginis* [33], *Verticillium dahliae* and *V. alboatrum* [34,35], and *Macrophomina phaseolina* [36].

Infected lupin seeds are the primary source of inoculum of *C. lupini*, and the development of a robust and sensitive diagnostic assay for early detection of contaminated seed lots able to screen before planting remains one of the most effective management strategies. The aim of this work is to provide a specific, sensitive, and simple molecular diagnostic assay for the early detection of *C. lupini* on seeds that could reduce the impact of anthracnose on lupin production.

2. Results

2.1. Evaluation of the Mycoflora Associated with Lupin Seeds

The most common fungi isolated from the three lupin species were *Alternaria alternata*, *Aspergillus* spp., *Cladosporium oxysporum*, *Mucor* sp., and *Penicillium* spp. Overall, fungi of 16 genera and 10 species were recovered from lupin seeds, in addition to one yeast and one sterile mycelium, as shown in Table 1. Fungal colonies developing around the seeds were transferred onto fresh Potato Dextrose Agar (PDA) plates amended with streptomycin before identification, and identification was based on morphological characteristics observed under stereoscopic and optical microscope. Fungal isolates with ambiguous taxonomic designation were analysed by molecular characterisation with the DNA regions of ITS rDNA, β-tubulin, or EF1-α.

Table 1. Percentage of contaminated seeds and mycoflora composition of the studied lupin varieties. Methods and accession number of genetic loci used for the characterization of main contaminants are also reported.

Lupinus spp. cultivar	*albus* Multitalia	*luteus* Mister	*angustifolius* Tango	Method [1]	Loci used for molecular characterization		
Contamination (%)	84	82	60		Accession of reference sequences		
Germination (%)	85.5	70.5	93.5		ITS	TUB	TEF
Alternaria alternata	42	4	2	M&G	MK560162	MK567923	-
Alternaria infectoria	2	-	-	M&G	MK560163	MK567924	-
Alternaria tenuissima	4	-	-	M&G	MK560164	MK567925	-
Arthrinium phaeospermum	2	-	-	M&G	MK560165	-	-
Aspergillus spp.	6	40	10	M	-	-	-
Botrytis cinerea	2	-	-	M&G	MK560166	-	-
Chaetomium sp.	-	-	2	M	-	-	-
Cladosporium oxysporum	14	28	22	M&G	MK560167	MK567926	-
Curvularia hawaiiensis	2	-	-	M&G	MK560168	MK567927	-
Diaporthe sp.	2	-	-	M	-	-	-
Eurotium sp.	-	-	2	M&G	MK560169	-	-
Fusarium incarnatum	2	-	-	M	-	-	MK567921
Fusarium tricinctum	2	-	-	M	-	-	MK567922
Lecythophora sp.	-	-	2	M&G	MK560170	MK567928	-
Mucor sp.	8	8	-	M	-	-	-
Penicillium spp.	12	22	22	M	-	-	-
Sordaria fimicola	-	2	-	M&G	MK560171	MK567929	-
Trichoderma spp.	2	-	2	M	-	-	-
Meyerozima caribbica	2	-	-	M&G	MK560172	-	-
Sterile mycelium	-	-	2	M	-	-	-

[1] Method used for the characterization: M indicates the strains were characterized morphologically, and M&G indicates morphologically and genetically.

Alternaria alternata, *Cladosporium oxysporum*, and *Penicillium* spp. were the main fungi in *L. albus* cv. Multitalia. *Aspergillus* spp., *Cladosporium oxysporum*, and *Penicillium* spp. were, instead, the main fungi isolated from seeds of *L. angustifolius* cv. Tango and *L. luteus* cv. Mister. Additional common fungal pathogens were isolated from the seeds such as *Fusarium tricinctum*, *F. incarnatum*, *Diaporthe* sp., *Alternaria alternata*, *Cladosporium oxysporum*, and *Botrytis cinerea*. However, such fungal strains are not known to be key pathogens of lupins. *L. albus* cv. Multitalia showed the greatest variability of fungal genera isolated (15/21), while *L. luteus* cv. Mister and *L. angustifolius* cv. Tango showed 6/21 and 9/21 fungal genera isolated, respectively (including 1 sterile mycelium). *C. lupini* was never isolated from the seeds of the three lupin species analysed, and therefore the same batch of seeds was used in the artificial inoculation experiments.

2.2. Artificial Inoculation of Lupin Seeds by the Water-Restriction Technique

The influence of substrate osmotic potential on the growth rate of *C. lupini* isolate IMI504893 and on seeds germination of three lupin species was investigated (Figure 1). *C. lupini* isolate IMI504893 was growing faster on substrate characterized by osmotic potentials lower than −0.3 MPa, corresponding to the osmotic potential of PDA without mannitol (Figure 1A). No significant differences were found on colony diameter at each time on PDA osmotically modified with mannitol (−0.8, −1.0, and −1.2 MPa). On the contrary, data on non-amended PDA (−0.3 MPa) were significantly different from corresponding data on PDA osmotically modified with mannitol (Figure 1). Sterilized seeds of lupin species were placed onto PDA medium at different osmotic potentials with the aim to evaluate the influence of the substrate on seed germination (Figure 1B–D). Seed germination was strongly reduced at all the osmotic potentials used; however, after 96 h of incubation on PDA with an osmotic potential of −1.2 MPa, seeds of *L. albus* cv. Multitalia (6.67%) and of *L. luteus* cv. Mister (33.33%) started to germinate (Figure S1).
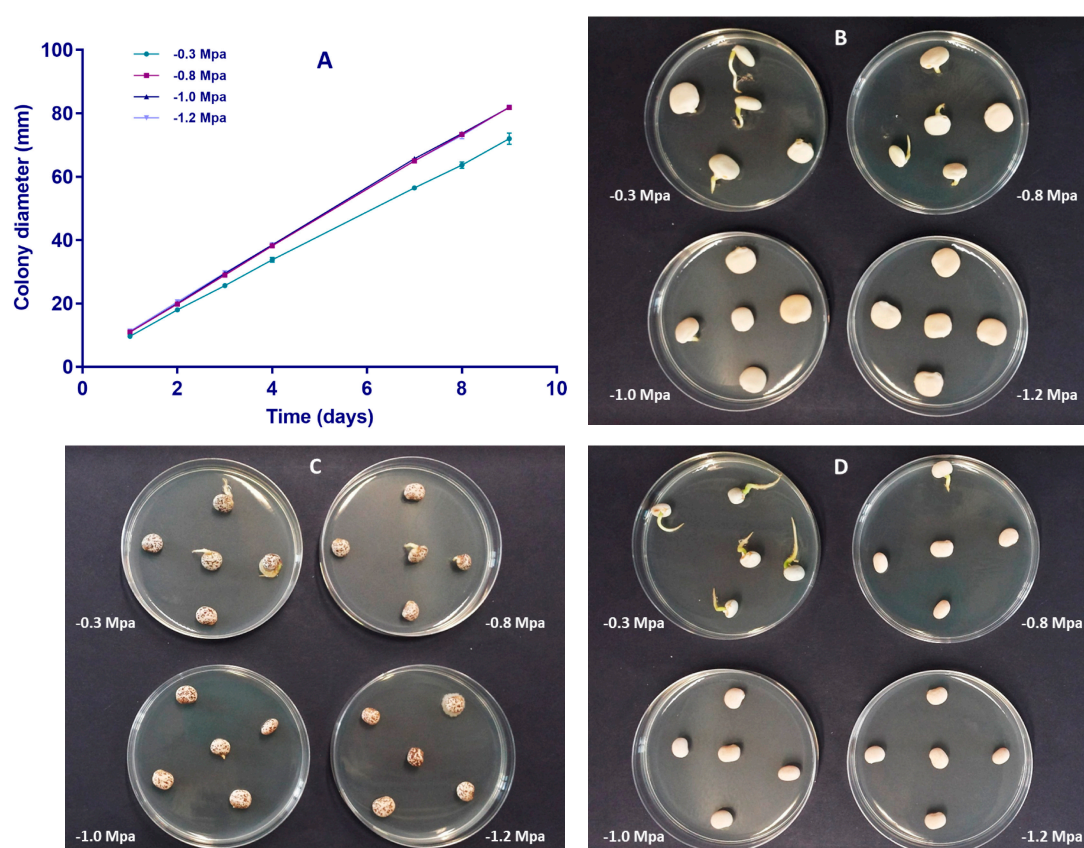
Figure 1. Mycelial growth of the isolate IMI504893 of *C. lupini* on Potato Dextrose Agar (PDA) osmotically modified with mannitol after 72 h of incubation (**A**). Lupin seed germination on PDA osmotically modified with mannitol: (**B**) = *L. albus* cv. Multitalia; (**C**) = *L. luteus* cv. Mister; (**D**) = *L. angustifolius* cv. Tango. −0.3 Mpa = PDA alone; −0.8 Mpa = PDA amended with 33.10 g L^{-1} mannitol; −1.0 MPa = PDA amended with 47.75 g L^{-1} mannitol; and −1.2 MPa = PDA amended with 62.46 g L^{-1} mannitol.

Given that the mycelial growth was stimulated at the osmotic potential of −1.2 MPa, and seed germination was inhibited at the same potential up to 72 h, the artificial inoculation of lupin seeds on PDA was adjusted at −1.2 MPa for 72 h. Inoculation was confirmed by plating 20 seeds of each cultivars on PDA amended with streptomycin. After 3 days, mycelium of *C. lupini* IMI504893 developed from all tested seeds.

2.3. Development of a Specific PCR Assay for C. lupini

Total genomic DNA was successfully extracted from all the fungi listed in Tables 1 and 2. PCR amplification of the ITS region with the primer pair ITS5/ITS4 was performed on non-target species of *Colletotrichum* spp. (Table 2) and on fungi isolated from the seeds (Table 1). The ITS region was amplified as positive control for the extracted DNA, thereby excluding false negative results. Amplicons were obtained from all the tested fungi and varied between 550 and 700 bp.

Table 2. *Colletotrichum* isolates used in this study and relative information.

Colletotrichum spp.	Isolate [1]	Host	Country [2]	GenBank Accession Numbers [3]		
				ITS	rDNA cluster	IGS
C. lupini	PT30, RB020	*Lupinus albus*	PT	MK463722	-	**MK567906**
C. lupini	CBS129944, RB042	*Cinnamomum* sp.	PT	MH865693	-	**MK567907**
C. lupini	CSL1294, RB116	*Lupinus polyphyllus*	GB	MK463723	-	**MK567908**
C. lupini	G52, RB119	*Lupinus albus*	DE	MK463724	-	**MK567909**
C. lupini	96A649, RB120	*Lupinus polyphyllus*	AU	MK463725	-	**MK567910**
C. lupini	IMI504884, RB121	*Lupinus albus*	CA	KJ018635	-	**MK567911**
C. lupini	C3, RB122	*Lupinus luteus*	PL	MK463726	-	**MK567912**
C. lupini	IMI504885, RB123	*Lupinus albus*	ZA	MK463727	-	**MK567913**
C. lupini	70555, RB124	*Lupinus albus*	CL	MK463728	-	**MK567914**
C. lupini	CBS109224, RB125	*Lupinus albus*	AT	JQ948172	-	**MK567915**
C. lupini	PT702, RB127	*Olea europea*	ES	MK463729	-	**MK567916**
C. lupini	IMI350308, RB147	*Lupinus* sp.	GB	MK463730	-	**MK567917**
C. lupini	CBS109221, RB172	*Lupinus albus*	DE	JQ948169	-	**MK567918**
C. lupini	CBS109225, RB173	*Lupinus albus*	US	JQ948155	**MK541036**	-
C. lupini	IMI375715, RB217	*Lupinus albus*	AU	JQ948161	-	**MK567919**
C. lupini	IMI504893, RB221	*Lupinus* sp.	FR	MK463733	**MK541037**	-
C. lupini	CBS509.97, RB235	*Lupinus albus*	FR	JQ948159	-	**MK567920**
C. abscissum	IMI504790, RB197	*Citrus x sinensis*	US	KT153558	**MK541030**	-
C. costaricense	CBS211.78, RB184	*Coffea* sp.	CR	JQ948181	**MK541033**	-
C. cuscutae	IMI304802, RB216	*Cuscuta* sp.	DM	JQ948195	-	-
C. melonis	CBS134730, RB237	*Malus domestica*	BR	KC204997	-	-
C. paranaense	IMI384185, RB218	*Caryocar brasiliense*	BR	JQ948191	-	-
C. tamarilloi	CBS129955, RB018	*Solanum betaceum*	CO	JQ948189	**MK541029**	-
Colletotrichum sp.	CBS101611, RB170	Fern	CR	JQ948196	-	-
C. nymphaeae	IMI 504889, RB190	*Fragaria x ananassa*	DK	KT153561	**MK541035**	-
C. simmondsii	CBS 122122, RB179	*Carica papaya*	AU	JQ948276	**MK541034**	-
C. fioriniae	IMI 504882, RB111	*Fragaria x ananassa*	NZ	KT153562	**MK541031**	-
C. acutatum	CBS 112980, RB175	*Pinus radiata*	ZA	JQ948356	-	-
C. godetiae	CBS 193.32, RB019	*Olea europaea*	IT	JQ948415	-	-
C. phormii	CBS 102054, RB171	*Phormium* sp.	NZ	JQ948448	-	-
C. salicis	CBS 607.94, RB157	*Salix* sp.	NL	JQ948460	**MK541032**	-
C. coccodes	RB302	*Solanum lycopersicum*	IT	**MK531998**	-	-
C. spinaciae	RB305	*Spinacia oleracea*	IT	**MK531997**	-	-
C. higginsianum	IMI 349063, RB300	*Brassica chinensis*	TT	JQ005760	-	-
C. graminicola	CBS130836, RB301	*Zea mays*	US	JQ005767	-	-
C. fructicola	CSL386, RB003	*Fragaria x ananassa*	US	KM246513	-	-
C. orchidophilum	IMI309357, RB209	*Phalaenopsis* sp.	GB	JQ948153	-	-
C. tofieldiae	IMI288810, RB164	*Dianthus* sp.	GB	GU227803	-	-
C. trichellum	RB306	*Hedera* sp.	IT	**MK532000**	-	-
C. truncatum	RB308	*Glycine max*	AR	**MK531999**	-	-

[1] CBS: culture collection of Centraalbureau voor Schimmecultures, Fungal Biodiversity Centre, Utrecht, The Netherlands; IMI: culture collection of CABI Europe UK Centre, Egham, UK; RB: personal collection of Riccardo Baroncelli. [2] ISO 3166-1 alpha-2 code related to the country of origin. [3] in bold are highlithed the sequences produced in this study.

The species specific primer pair CLF (5'-CCCGAGAAGGCTCCAAGTA-3')/CLR (5'-CATAAACGC CTAAGAACCGC-3') (Figure S2) was designed based on the multiple alignment of the IGS sequences of *C. lupini* (IMI504893, CBS109225), *C. tamarilloi* (CBS129955), *C. nymphaeae* (IMI 504889), *C. costaricense* (CBS211.78), *C. abscissum* (IMI504790), and *C. simmondsii* (CBS 122122). The species are taxonomically included in the *C. acutatum* species complex clades 1 and 2. The primers were designed from 100% sequence homology region of *C. lupini* isolates and from regions of the greatest sequence dissimilarity among other species.

The primer pair CLF/CLR was used in the PCR amplification with the DNA template of *C. lupini* and the *Colletotrichum* species indicated in Table 2, in addition to the DNA of the isolates of non-target species associated with lupin seeds (Table 1). Sensitivity of the PCR protocol using the primer pair CLF/CLR was performed using 10-fold dilutions ranging between 10 ng and 100 fg of mycelial DNA of *C. lupini* isolate 70555 (Figure S3).

PCR assay for *C. lupini* was optimized to increase primer specificity, avoiding primer mismatches and the probability of producing spurious bands or false positives. The reactions generated amplicons of the expected size of 700–750 bp in all the tested *C. lupini* isolates (Figure 2).
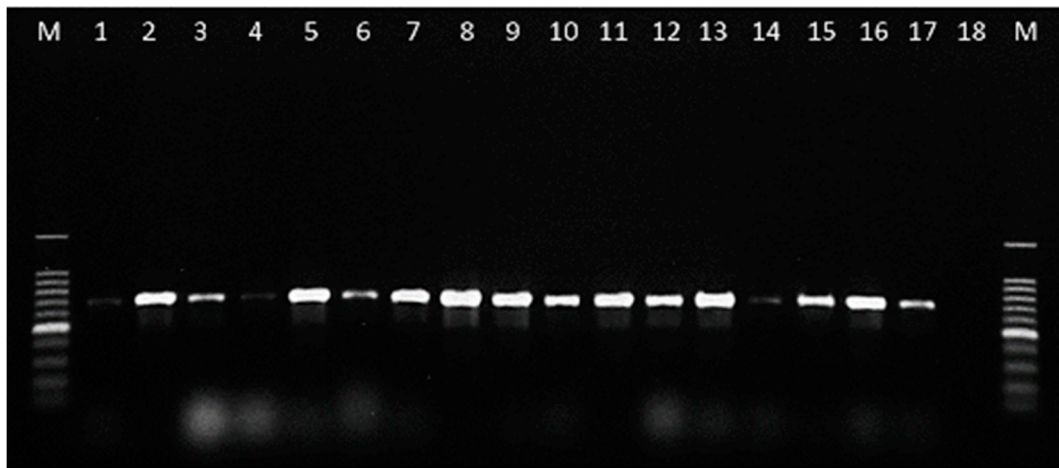
Figure 2. Specificity of PCR using the primer pair CLF (5′-CCCGAGAAGGCTCCAAGTA-3′)/CLR (5′-CATAAACGCCTAAGAACCGC-3′) on 17 isolates of *C. lupini* (Lane 1–17). Lane 18 = negative control (without DNA), Lane M = 100 bp DNA ladder.

The amplicons were sequenced in both directions and aligned to compare with the sequences used to design the primer pair, and to ensure the availability of the partial IGS region in all the isolates tested. Additional tests were carried out to confirm the specificity of the developed PCR assay by analysing the *Colletotrichum* species of Table 2 and the fungal strains isolated from lupin seeds of Table 1. The primer pair CLF/CLR proved specific to *C. lupini*, as no bands were obtained on all the non-target fungi tested (Figures 3 and 4).
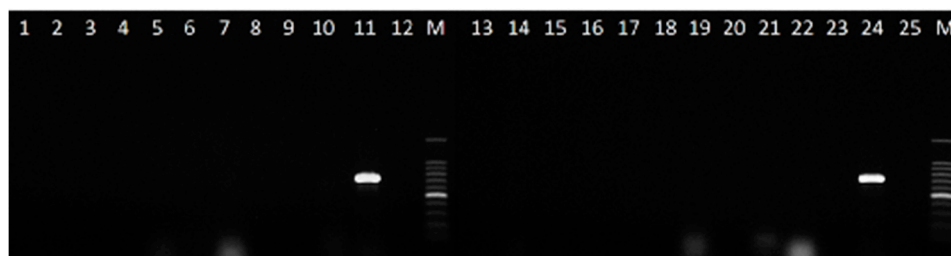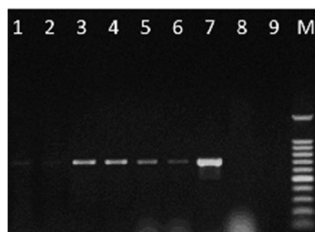

Figure 3. Specificity of PCR using the primer pair CLF/CLR for the detection of *C. lupini*. Lane 1 = *Alternaria alternata*, Lane 2 = *Alternaria infectoria*, Lane 3 = *Alternaria tenuissima*, Lane 4 = *Arthrinium phaeospermum*, Lane 5 = *Aspergillus* sp., Lane 6 = *Botrytis cinerea*, Lane 7 = *Chaetomium* sp., Lane 8 = *Cladosporium oxysporum*, Lane 9—*Curvularia hawaiiensis*, Lane 10 = *Diaporthe* sp., Lane 11 and 24 = *C. lupini* IMI 504893, Lane 12 and 25 = negative control (without DNA), Lane 13 = *Eurotium* sp., Lane 14 = *Fusarium incarnatum*, Lane 15 = *Fusarium tricinctum*, Lane 16 = *Lecytophora* sp., Lane 17 = *Mucor* sp., Lane 18 = *Penicillium* sp., Lane 19 = *Pleospora* sp., Lane 20 = *Sordaria fimicola*, Lane 21 = *Trichoderma* sp., Lane 22 = *Meyerozima caribbica*, Lane 23 = Sterile mycelium, and Lane M = 100 bp DNA ladder.
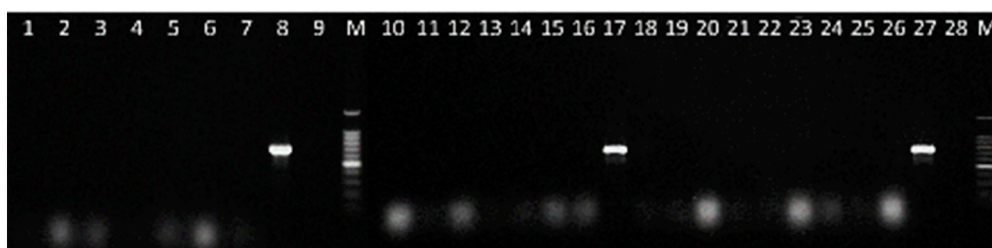
Figure 4. Specificity of PCR using the primer pair CLF/CLR for the detection of *C. lupini*. Lane 1 = *C. tamarilloi*, Lane 2 = *Colletotrichum* sp., Lane 3 = *C. costaricense*, Lane 4 = *C. abscissum*, Lane 5 = *C. cuscutae*, Lane 6 = *C. paranaense*, Lane 7 = *C. melonis*, Lane 8, 17 and 27 = *C. lupini* IMI 504893, Lane 9 and 28 = Negative control (without DNA), Lane 10 = *C. simmondsii*, Lane 11 = *C. nymphaeae*, Lane 12 = *C. fioriniae*, Lane 13 = *C. acutatum*, Lane 14 = *C. godetiae*, Lane 15 = *C. salicis*, Lane 16 = *C. phormii*, Lane 18 = *C. fructicola*, Lane 19 = *C. tofieldiae*, Lane 20 = *C. orchidophilum*, Lane 21 = *C. higginsianum*, Lane 22 = *C. graminicola*, Lane 23 = *C. coccodes*, Lane 24 = *C. spinaciae*, Lane 25 = *C. trichellum*, Lane 26 = *C. truncatum*, and Lane M = 100 bp DNA ladder.

2.4. Detection of C. lupini in Artificially Infected Seed Samples

The species-specific primer pair CLF/CLR confirmed the presence of *C. lupini* in all infected seed lots of *L. albus* cv. Multitalia, *L. luteus* cv. Mister, and *L. angustifolius* cv. Tango, even at contamination levels as low as 0.01% (Figure 5 and Figure S4; DNA concentrations and absorbance ratios are reported in Table S1).

Figure 5. Specificity of PCR using the primer pair CLF/CLR for the detection of *C. lupini* in seed samples artificially infected (1:10,000). Lane 1–2 = *L. albus* cv. Multitalia, Lane 3–4 = *L. luteus* cv. Mister, Lane 5–6 = *L. angustifolius* cv. Tango, Lane 7 = Positive control (*C. lupini* IMI 504893), Lane 8 = Seed control (100 seeds of *L. angustifolius* cv. Tango), Lane 9 = Negative control (without DNA), and Lane M = 100 bp DNA ladder.

The results were supported by the presence of a signal within the positive control (DNA of pure culture of *C. lupini* IMI504893), and no-signal within the negative control (not infected seed lots). The amplicons obtained from the 0.01% infection level of each species were sequenced (Table 2), with alignments carried out against the reference sequence of *C. lupini* IMI504893, showing 100% identity.

3. Discussion

Seed-borne fungi are one of the major biotic constraints in seed production worldwide. They are responsible for both pre- and post-emergence death of seeds reducing germination, seedling health, and plant morphology. Seed health tests include [37] visual examination and incubation methods, which require mycological skills; they are time consuming and their sensitivity/specificity is low or moderate [38,39].

In this work, a new protocol for early detection of *C. lupini* was developed based on a biological enrichment step, followed by a targeted PCR. The diagnostic assay included incubation of the seeds with amended Yeast Malt Broth to increase *C. lupini* biomass before DNA extraction. The seeds are incubated in the liquid culture, allowing the development of all culturable fungi, without the utilization of selective or semi selective media (BIO-PCR) that require specific knowledge of the target

organism [38]. The biological enrichment step allows for the detection only of viable cells, and reduces false positives, which constitute a limitation of PCR diagnostic assays [40]. It also allows for the development of all the fungi present on/in the seeds and promotes the development of the target fungus biomass before DNA extraction and amplification by PCR. This step is usually very useful in cases of low infection levels. The incubation of seeds in liquid fungal growth media has been previously undertaken with positive results for the detection of *Fusarium circinatum* in pine seeds [41], *Alternaria brassicae* in cruciferous seeds [42], *Ascochyta rabiei* in chickpea seeds [43], and *Magnaporthe grisea* in rice seeds [44]. The presence of bacteria may, however, reduce fungal growth though the addition of antibiotics to the media or the use of specific media, which can circumvent these problems [43,45].

The optimal osmotic potential and the incubation period were defined according to the germination process of the seeds and the growth of *C. lupini* at different osmotic potentials. No seeds of the three lupin species germinated on PDA adjusted at −1.2 MPa at 72 h and, in the same conditions, *C. lupini* growth was stimulated. For these reasons, it was decided to perform the seed inoculation on PDA adjusted with mannitol at −1.2 MPa for 72 h. Concerning the sensibility of the method, the PCR assay developed in this study was able to reveal infection levels below 0.01% (1:10,000) in the samples tested. Assuming a 100% DNA recovery from 1 infected seed (DNA resuspended in 100 µL), 1 µL of template DNA would contain the equivalent of 0.01 infected seed in a reaction.

The new CLF/CLR primer pair was used for amplification and sequencing of a target of about 736 bp in *C. lupini* of the IGS region of the ribosomal cluster. Bioinformatic analyses were performed on the entire sequence of the clusters, and more specifically the complete IGS region of species belonging to the *Colletotrichum acutatum* species complex clades 1 and 2 (*C. lupini*, *C. tamarilloi*, *C. nymphaeae*, *C. costaricense*, *C. abscissum*, and *C. simmondsii*). PCR-based detection systems exhibit higher levels of sensitivity than conventional techniques. However, when applied to seed tests they require the isolation of high-quality DNA of target organisms for detection above the background noise of other organisms and inhibitory seed-derived compounds. Niepold (2003) designed a primer pair from an already sequenced ITS1 region in order to detect *C. lupini*. The sequences, when subjected to a BLASTn search, showed 100% identity with many other *Colletotrichum* species (e.g., *C. acutatum*, *C. scovillei*, *C. simmondsii*, *C. salicis*, *C. godetiae*, *C. phormii*, and *C. nymphaeae*). More recently, Szuszkiewicz (2016), during a study on the molecular detection of *C. lupini* in lupin seeds, developed a species-specific primer pair based on ITS region that was specific to the *C. acutatum* species complex. Dubrulle et al. (2019) used specific primers targeting a single copy gene to detect *C. lupini* DNA in winter and spring lupin fields in France. Of the 47 samples collected, *C. lupini* was quantified in six samples above the limit of quantification (LOQ) (10^{-3} ng DNA), although all at a very low level (between 10^{-3} and 5×10^{-3} ng DNA) [24]. IGS region could be an attractive alternative to the ITS region when closely related taxa or even different species need to be investigated. The IGS region tends to accumulate more variability than the ITS region of the ribosomal cluster and, therefore, contains more sequence polymorphisms. It occurs in multiple copies, increasing the sensitivity of PCR-based diagnostics compared to single-copy target sequences and allowing its amplification using generic PCR primers [46]. The greatest amount of sequence variation in the ribosomal cluster exists within the IGS region, even if it poses more difficulties for amplification and sequencing, as it is long (2000–5000 bp) and rich in GC [47]. To our knowledge, besides the potential of the target, no published studies reported the use of the IGS region for the molecular diagnosis of *Colletotrichum*.

Seed-borne mycoflora of the three lupin species used in this study *L. albus* cv. Multitalia, *L. luteus* cv. Mister, and *L. angustifolius* cv. Tango were evaluated for three main reasons: (i) to assess the level of seed contamination/infection by cultivable fungi, (ii) to collect a data set of fungi associated with lupin seeds to be used as non-target fungi in the development of the species specific PCR assay, and (iii) to investigate the presence of *C. lupini* in the seed lots used. *L. albus*, *L. luteus*, and *L. angustifolius* showed 84, 82, and 60% seed contamination, respectively. Most of the fungi recovered on seeds of the three lupin species were also reported in several published studies on the mycoflora associated with white, yellow, and blue lupin [48–52].

In this study, *C. lupini* was never isolated from seeds of the three lupin species analysed, and therefore seeds were used in the artificial inoculation experiments and in the preparation of seed batches by mixing artificially infected seeds and healthy seeds in different proportions. Seeds were kept in direct contact with developing colonies of the fungal pathogen on agar media amended with an osmotic compound that inhibited seed germination. 2,4-D (sodium salt of 2,4-dichlorophenoxyacetic acid), mannitol, sodium, and potassium chlorides can be used to modify the osmotic potential of PDA [53]. In this study, mannitol was used to obtain PDA with different osmotic potentials. Mycelial growth of *C. lupini* was stimulated at all the osmotic potentials higher than −0.3 MPa (PDA without mannitol). In general, development of fungi is not reduced by osmotic potential lower than −2.0 MPa, and in the range between −0.3 and −1.0 MPa growth of some fungi are stimulated. Similar results were reported for the inoculation of *Phaseolus vulgaris* seeds with *Colletotrichum lindemutianum* and cotton seeds with *Colletotrichum gossypii* var. *cephalosporioides* [54,55]. Germination rate of each lupin cultivar used in this study decreased as osmotic potential increased. Similar results were observed in cotton, rice, sunflower, and common bean seeds [53,56,57]. Several studies reported that the incubation period to obtain inoculated seeds ranged between 24 and 144 h [58–61].

DNA concentration has been taken into consideration by Cullen et al. [62] to exploit the sensitivity of PCR for the specific detection of *Helminthosporium solani* in seeded soils. The molecular detection of fungi on seeds can be influenced by factors such as low concentration of target pathogen, the presence of other organisms, and other natural compounds of the seeds. Gondran and Pacault [17] recommended that seed testing for *L. albus* should detect as little as one anthracnose-infected lupin seed in 10,000 (0.01%). Shea et al. [63] reported that in Western Australia, a PCR test, based on research carried out at the Centre for Legumes in Mediterranean Agriculture and the State Agricultural Biotechnology Centre, was able to detect one infected seed in 10,000. The test was improved over time to become quantitative, providing an estimation of the seed infection level based on the amount of *C. lupini* DNA present in the sample. However, the PCR protocol has not been published but only described as a useful seed-testing service for lupin growers in high-risk areas.

The protocol described in this work is a useful diagnostic tool for the routine detection of *C. lupini* in seed lots: the assay is simple, reliable, economic, and sensitive. Over time, with a greater understanding of the anthracnose disease, seed testing should be widely utilised as a tool for seed certification, for local seed supply, and for international seed biosecurity. The use of pathogen-free seeds is an essential strategy for the integrated and sustainable management of plant diseases.

4. Materials and Methods

4.1. Fungal Cultures

Isolates used in this study and relative information are summarized in Table 2. Purified fungal cultures were routinely grown on Potato Dextrose Agar (PDA, Difco Laboratories, Detroit, MI, USA) and were maintained on PDA slants covered with mineral oil at 4 °C for long-term storage.

4.2. DNA Extraction

Fungal mycelium for DNA extraction was grown in 125 mL of Yeast Malt Broth (YMB—0.3% yeast extract, 0.3% mal extract, 0.5% peptone, and 1% dextrose) at 150 rpm for 2–4 days at 24 ± 1 °C in the dark. Mycelium was harvested by filtration through sterile Miracloth (Calbiochem, San Diego, CA, USA), washed thoroughly using sterile distilled water, and pressed dry between sterile paper towels. The harvested mycelium was either used immediately for DNA extraction or stored at −20 °C until use.

Total genomic DNA was extracted using the SDS-CTAB method described by Kim et al. [63] with some modifications. Mycelium (200 mg) was placed into a 2 mL extraction tube prefilled with 0.5 mm Silica glass beads (acid washed) (Benchmarck Scientific Inc., Sayreville, NJ, USA), 50 mg of PVP40 (Sigma-Aldrich, Saint Louis, MS, USA), and 400 μL of ice-cold lysis buffer (150 mM NaCl, 50 mM EDTA, 10 mM Tris-HCl pH 7.4, 30 μg mL^{-1} Proteinase K). The mycelium was homogenized by a bead-beating

method using the BeadBug™ Microtube homogenizer (Benchmarck Scientific Inc., Sayreville, NJ, USA). Tubes were subjected to three beating cycles of 30 s at 4000 rpm followed by a 30 s interval. During the interval and after the cycle, the samples were cooled down in ice. SDS was added to a final concentration of 2% (w/v), and the mixture was incubated at 65 °C for 40 min. The lysed suspension was centrifuged for 10 min at 4 °C and 2500 g, the volume of supernatant was measured, and the NaCl concentration was then adjusted to 1.4 M and 1/10 volume of 10% CTAB buffer (10% CTAB, 500 mM Tris-HC1, 100 mM EDTA, and pH 8.0) and added. After thorough mixing, the solution was incubated at 65 °C for 10 min. After cooling at 15 °C for 2 min, extraction with chloroform-isoamyl alcohol (24:1 v/v) was conducted for 10 min at 4 °C and 6700 *g*. DNA was precipitated with two volumes of 95% cold ethanol. Samples were stored at −20 °C (for a minimum of 1 h) and centrifuged for 1 min at 4 °C and 11,600 g, and the resulting pellet was rinsed once with 70% cold ethanol, vacuum-dried, and dissolved in sterile nuclease-free water. DNA solutions were stored at −20 °C until use.

DNA concentration was estimated with a GeneQuant II spectrophotometer (Pharmacia Biotech, Cambridge, UK), whereas its integrity was examined visually by gel electrophoresis on 0.8% (w/v) agarose gels run in 0.5× TBE buffer followed by GelRed™ staining (Biotium Inc., Fremont, CA, USA), according to the manufacturer's instructions. Following quantification, the genomic DNA was diluted to a final concentration of 25–50 ng μL^{-1}.

4.3. Evaluation of Mycoflora Associated with Lupin Seeds

A hundred non-sterilized seeds of *Lupinus albus* cv. Multitalia, *L. luteus* cv. Mister, and *L. angustifolius* cv. Tango were plated on PDA amended with streptomycin (300 mg L^{-1}), five seeds per Petri plate, and incubated at 24 ± 1 °C under a 12 h near-ultraviolet light/12 light cycle for 7 days.

Plates were examined under stereoscopic and compound microscopes to identify the developing fungal colonies. Hyphal-tip and/or single-spore isolation techniques were used to obtain pure cultures. Fungi were identified according to their morphological properties and, when necessary, by DNA sequencing.

The ITS and 5.8 S region of rDNA was amplified with the primer pair ITS5 (5′-GGAAGTAA AAGTCGTAACAAGG-3′), and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) [25]. The partial β-tubulin gene region was amplified with the primer pair BT2A (5′-GGTAACCAAATCGGTGCTGCTTTC-3′) and BT2B (5′ACCCTCAGTGTAGTGACCCTTGGC-3′) [64]. The partial sequences of the elongation factor-1 alpha gene (*tef1*) were amplified with the primer pair EF1-728F (5′-CATCGAGAAGTTCGAGAAGG-3′) and EF1-986R (5′-TACTTGAAGGAACCCTTACC-3′) [65]. Sequence chromatograms obtained by direct sequencing were visualised and analysed using Chromas Lite (v. 2.1.1) and BioEdit (v. 7.2.5) programs.

Each sequence was used to perform individual nucleotide-nucleotide searches with the BLASTn algorithm at the NCBI website (https://blast.ncbi.nlm.nih.gov/). Sequence-based identities with a cutoff of 97% or greater were considered significant in this study, and the best hit was defined as the sequence with the highest maximum identity to the query sequence.

Seed germination was determined according to the procedure described by the International Seed Testing Association (ISTA, 2019).

4.4. Genome Data and Analysis of Ribosomal IGS Region

Based on whole genome sequencing project available on GenBank, a set of four raw sequencing data information sets from high-throughput sequencing platforms stored in the sequences reads archive (SRA) database were downloaded: accession number for *C. simmondsii* SRP074810, for *C. nymphaeae* SRP074816, for *C. fioriniae* SRP074685, and for *C. salicis* SRP074780 [66]. Raw data reads were assembled using SPAdes v 3.11.1 [67], and high-coverage scaffolds were extracted with a homemade script. Scaffolds corresponding to the ribosomal RNA encoding gene cluster were identified by BLASTN searches based on available references. When the software failed to assemble the region in one unique scaffold, a manual approach based on local alignment of raw reads to assembled scaffolds with Bowtie2 [68] was used to increase the size of the scaffolds and to reach an overlap of at least 50 bp.

Annotated genome sequences of *C. lupini* IMI504884 and CBS109225, *C. abscissum* IMI504890, *C. costaricense* IMI309622, and *C. tamarilloi* CBS129955 were downloaded from the MycoCosm portal [69] and the ribosomal cluster sequence retrieved.

All sequences were analysed and compared. The sequences starting with the forward primer CNL12 (5′CTGAACGCCTCTAAGTCAG3′) and ending with the reverse primer CNS1 (5′GAGA CAAGCATATGACTACTG3′), which are universal primers, located at the 3′ end of the 28S gene and the 5′ end of the 18S gene, respectively, were used for the amplification of complete IGS region in many fungi [25,70].

The sequences were aligned using the MAFFT v. 7 [71] and were visually checked for regions having homologies among isolates of *C. lupini* but not among other *C. acutatum* species.

4.5. Development of Specific Oligonucleotide PCR Primers

Conserved regions among the isolates that were specific to *C. lupini* were selected to design species-specific oligonucleotides. Two primers, forward and reverse, were designed using Primer3Plus online software with default options [72]. The forward (CLF: 5′CCCGAGAAGGCTCCAAGTA3′) and reverse (CLR: 5′CATAAACGCCTAAGAACCGC3′) primers yielded a product of 736 bp. The theoretical specificity of the primer set was checked with the sequences from the other fungi in the GenBank by using BLASTn analysis.

4.6. Development of a Specific PCR Assay for C. lupini

The specific primer pair CLF/CLR designed during this study was used for amplification. The optimized PCR amplification protocol was performed in a total volume of 25 μL containing 25–50 ng of template DNA, 0.2 μM of each oligonucleotide primer, and 12.5 μL of GoTaq® Green Master Mix (Promega, Madison, WI, USA). An initial denaturation step of 94 °C for 2 min was followed by 25 cycles of a 45 s at 94 °C (denaturation step), 15 s at 63 °C (annealing step), and 45 s at 72 °C (extension step). After 25 cycles, samples were incubated for 7 min at 72 °C (final extension step). Negative controls (no DNA) were included for each set of reactions.

Templates were represented by (i) DNA of *C. lupini* and other *Colletotrichum* species (Table 2) and (ii) DNA of isolates of non-target fungi associated with lupin seeds and isolated in this work (Table 1). When more than one species was isolated (e.g., *Aspergillus* spp.), the most representative was chosen. PCR products were analysed by electrophoresis in 0.5× TBE buffer with 2% (w/v) agarose gels and detected by UV fluorescence after GelRed™ staining (Biotium Inc., Fremont, CA, USA), according to the manufacturer's instructions. The 100 bp DNA ladder (Promega, Madison, WI, USA) was used as molecular size marker. PCR products of 17 *C. lupini* isolates were purified using the QIAquick PCR purification Kit (Qiagen, Milano, Italy) and sequenced in both directions to confirm the nucleotide sequence. Sequence chromatograms were visualised and edited using Chromas Lite (v. 2.1.1) and BioEdit v.7.2.5 [73] and deposited in GenBank (Table 2).

4.7. Artificial Inoculation of Lupin Seeds by Water Restriction Technique

The water restriction technique [56], also known as 'osmo-priming' process [74] is based on the control of the osmotic potential of the substrate to inhibit or reduce the seed germination process, which allows for the inoculation of seeds without producing any radicle protrusion during the incubation period. This technique, at the same time, does not affect microorganisms associated with seeds.

Before the artificial inoculations of lupin seeds, in order to choose the best conditions, we evaluated the effect of water restriction on the mycelial growth of the pathogen and on lupin seed germination. The effect of water restriction on *C. lupini*, and mycelial growth on osmotically modified PDA medium containing mannitol, was evaluated. Osmotic potential of −0.8 (33.10 g L^{-1}), −1.0 (47.75 g L^{-1}), and −1.2 (62.46 g L^{-1}) MPa was used for the incorporation of the osmotic solute into the PDA medium. The osmotic potential of PDA without the solute was estimated to be −0.35 MPa [57]. Agar plugs (6 mm diameter) were removed from the edge of an actively growing colony of *C. lupini* (isolate IMI504893)

and placed at the center of PDA plates (9 cm) amended with mannitol. Plates were incubated at 24 ± 1 °C under a 12 h near-ultraviolet light/12 light cycle for 9 days. Mycelial growth was measured taking the average of two diameters of colonies at appropriate angles to each other at different day intervals. The experiment was performed in triplicate.

Two-way analysis of variance (ANOVA) and Tukey's multiple-comparison post-test were conducted with colony diameter data obtained from the experiment using GraphPad Prism version 7.00 for Windows (GraphPad Software, La Jolla, CA, USA). Differences were considered significant at a p value < 0.05.

To evaluate the effect of water restriction in the absence of the fungus, seeds of the three lupin species were placed on Petri plates containing PDA osmotically modified with mannitol for 7 days, as described above. Seeds of *L. albus* cv. Multitalia, *L. luteus* cv. Mister, and *L. angustifolius* cv. Tango were disinfected with 1% NaOCl (v/v) for 2 min, rinsed twice in sterile distilled water, and dried at room temperature on sterile filter paper before plating. Percentage of germinated seeds was recorded.

At the end of these experiments, according to the results obtained, lupin seeds were inoculated with the isolate IMI504893 of *C. lupini*, using the 'osmo-priming' technique with the following conditions: osmotic potential adjusted to −1.2 MPa with mannitol over a 72 h incubation period. Lupin seeds of each species were disinfected and placed in a single layer on the fungal colonies under the same conditions previously described. The seeds were then shade-dried and kept at 4 °C for future mixing with healthy seeds. Inoculation accuracy was confirmed at the end of the experiments, and after six months by placing 20 seeds on the surface of PDA plates amended with streptomycin (300 mg L^{-1}) for 1 week at 24 ± 1 °C.

4.8. Detection of C. lupini in Seed Samples with Different Fungal Incidence

To determine the efficacy and the sensibility of the PCR technique in the detection of *C. lupini* in lupin seed samples, seed batches were prepared by mixing infected seeds and healthy seeds in the following proportions to produce seed lots with the following infection levels: 1:10 (10%); 1:100 (1%); 1:1000 (0.1%); 1:10,000 (0.01%), and 0.00%. One thousand seed batches' weights were 281.42 g for *L. albus* cv. Multitalia, 135.46 g for *L. luteus* cv. Mister, and 146.85 g for *L. angustifolius* cv. Tango. The artificially infected seeds, before mixing, were disinfected with 1% NaOCl (v/v) for 2 min, rinsed twice in sterile distilled water, and dried at room temperature on sterile filter paper.

Seed batches were placed in sterile Erlenmeyer flasks (between 250 and 6000 mL depending on the cultivar and the batch), covered with liquid culture YMB medium amended with streptomycin (0.03%), ampicillin (0.0025%) and lactic acid (0.1%), and incubated for 72 h at 24 ± 1 °C with continuous shaking (150 rev min^{-1}). This incubation step is an enrichment phase that allows an optimal increase of the fungal biomass from seed.

At the end of the incubation period, the content of each flask was harvested by filtration through sterile Miracloth (Calbiochem, San Diego, CA, USA). The seeds were discarded after harvesting the mycelium. After thoroughly washing with deionized sterile water, the resulting mycelium was pressed dry between sterile paper towels and was either used immediately for DNA extraction or stored at −20 °C until use.

DNA extraction, PCR, and agarose electrophoresis were undertaken as described above. The universal primer pair ITS5/ITS4 [25] was used as a positive control to assess the quality of the extracted DNA. DNA from pure cultures of *C. lupini* isolate IMI504893 was used as a positive control in the assays. Furthermore, the specific PCR product obtained was eluted by gel extraction, and DNA sequencing was carried out to confirm that the designed primers correctly amplified the expected consensus region of the target organism. The molecular assay was carried out with two replicates, and the experiments were repeated twice for each level of seed infection.

References

1. Gladstones, J.S. Lupins as crop plants. *Field Crop Abstr.* **1970**, *23*, 123–148.

2. Herridge, D.F. The narrow-leafed lupin (*Lupinus angustifolius* L.) as a nitrogen-fixing rotation crop for cereal production. I. Indices of nitrogen fixation. *Aust. J. Agric. Res.* **1988**, *39*, 1003–1015. [CrossRef]

3. Cowling, W.; Buirchell, B.; Tapia, M.E. *Lupin. Lupinus spp. Promoting the Conservation and Use of Underutilized and Neglected Crops. 23*; Institute of Plant Genetics and Crop Plant Research, Gatersleben, and International Plant Genetic Resources Institute: Rome, Italy, 1998.

4. Talhinhas, P.; Baroncelli, R.; Le Floch, G. Anthracnose of lupins caused by *Colletotrichum lupini*: A recent disease and a successful worldwide pathogen. *J. Plant Pathol.* **2016**, *98*, 5–14.

5. Fernández-Pascual, M.; Pueyo, J.J.; Felipe, M.; Golvano, M.P.; Lucas, M.M. Singular features of the Bradyrhizobium-Lupinus symbiosis. *Singul. Featur. Bradyrhizobium-Lupinus Symbiosis* **2007**, *1*, 1–16.

6. Carof, M.; Godinot, O.; Ridier, A. Diversity of protein-crop management in western France. *Agron. Sustain. Dev.* **2019**, *39*, 15. [CrossRef]

7. Elmer, W.H.; Yang, H.A.; Sweetingham, M.W. Characterization of *Colletotrichum gloeosporioides* isolates from ornamental lupines in Connecticut. *Plant Dis.* **2001**, *85*, 216–219. [CrossRef]

8. Food and Agriculture Organization of the United Nations. FAOSTAT Statistics Database. Available online: http://www.fao.org/faostat/en/ (accessed on 1 January 2019).

9. Gondran, J. Les maladies du lupin blanc doux en France. *Perspect. Agric.* **1984**, *77*, 31–41.

10. Baier, A.C.; Linhares, A.G. Breeding for anthracnose tolerance in lupins. In Proceedings of the 6th International Lupin Conference, Temuco-Pucon, Chile, 25–30 November 1990; p. 127.

11. Nirenberg, H.I.; Feiler, U.; Hagedorn, G. Description of *Colletotrichum lupini* comb. nov. in modern terms. *Mycologia* **2002**, *94*, 307–320. [CrossRef] [PubMed]

12. Lotter, H.C.; Berger, D.K. Anthracnose of lupins in South Africa is caused by *Colletotrichum lupini* var. *setosum*. *Australas. Plant Pathol.* **2005**, *34*, 385–392. [CrossRef]

13. Thomas, G.J.; Sweetingham, M.W. Cultivar and environment influence the development of lupin anthracnose caused by *Colletotrichum lupini*. *Australas. Plant Pathol.* **2004**, *33*, 571–577. [CrossRef]

14. Thomas, G.J.; Sweetingham, M.W.; Yang, H.A.; Speijers, J. Effect of temperature on growth of Colletotrichum lupini and on anthracnose infection and resistance in lupins. *Australas. Plant Pathol.* **2008**, *37*, 35–39. [CrossRef]

15. Riegel, R.; Véliz, D.; von Baer, I.; Quitral, Y.; Muñoz, M. Genetic diversity and virulence of *Colletotrichum lupini* isolates collected in Chile. *Trop. Plant Pathol.* **2010**, *35*, 144–152. [CrossRef]

16. Thomas, T.H. Lupins as crop plants: Biology, production and utilization. Edited by J. S. Gladstones, C. Atkins and J. Hamblin. 1998. *Plant Growth Regul.* **2001**, *33*, 246. [CrossRef]

17. Gondran, J.; Pacault, D. L'anthracnose du lupin blanc. *Phytoma-Déf. Végétaux* **1997**, 28–31.

18. Decker, P. Anthracnose of blue lupin is seed-borne. *Plant Dis. Rep.* **1947**, *31*, 270–271.

19. Sreenivasaprasad, S.; Talhinhas, P. Genotypic and phenotypic diversity in *Colletotrichum acutatum*, a cosmopolitan pathogen causing anthracnose on a wide range of hosts. *Mol. Plant Pathol.* **2005**, *6*, 361–378. [CrossRef]

20. Dean, R.; Van Kan, J.A.L.; Pretorius, Z.A.; Hammond-Kosack, K.E.; Di Pietro, A.; Spanu, P.D.; Rudd, J.J.; Dickman, M.; Kahmann, R.; Ellis, J.; et al. The Top 10 fungal pathogens in molecular plant pathology. *Mol. Plant Pathol.* **2012**, *13*, 414–430. [CrossRef]

21. Marin-Felix, Y.; Groenewald, J.Z.; Cai, L.; Chen, Q.; Marincowitz, S.; Barnes, I.; Bensch, K.; Braun, U.; Camporesi, E.; Damm, U.; et al. Genera of phytopathogenic fungi: GOPHY 1. *Stud. Mycol.* **2017**, *86*, 99–216. [CrossRef]

22. Damm, U.; Sato, T.; Alizadeh, A.; Groenewald, J.Z.; Crous, P.W. The *Colletotrichum dracaenophilum, C. magnum* and *C. orchidearum* species complexes. *Stud. Mycol.* **2019**, *92*, 1–46. [CrossRef] [PubMed]

23. Baroncelli, R.; Talhinhas, P.; Pensec, F.; Sukno, S.A.; Le Floch, G.; Thon, M.R. The *Colletotrichum acutatum* species complex as a model system to study evolution and host specialization in plant pathogens. *Front. Microbiol.* **2017**, *8*, 2001. [CrossRef]

24. Dubrulle, G.; Pensec, F.; Picot, A.; Rigalma, K.; Pawtowski, A.; Gironde, S.; Harzic, N.; Nodet, P.; Baroncelli, R.; Le Floch, G. Phylogenetic diversity and temperature effect on growth and pathogenicity of *Colletotrichum lupini*. *Plant Dis.* **2019**, in press.

25. White, T.J.; Bruns, T.; Lee, S.; Taylor, J. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In *PCR Protocols: A Guide to Methods and Applications*; Innis, M.A., Gelfand, D., Shinsky, J., White, T., Eds.; Academic Press: New York, NY, USA, 1990; pp. 315–322.

26. Lio, D.D.; Cobo-Díaz, J.F.; Masson, C.; Chalopin, M.; Kebe, D.; Giraud, M.; Verhaeghe, A.; Nodet, P.; Sarrocco, S.; Floch, G.L.; et al. Combined Metabarcoding and Multi-locus approach for Genetic characterization of *Colletotrichum* species associated with common walnut (*Juglans regia*) anthracnose in France. *Sci. Rep.* **2018**, *8*, 10765. [CrossRef] [PubMed]

27. Schena, L.; Abdelfattah, A.; Mosca, S.; Li Destri Nicosia, M.G.; Agosteo, G.E.; Cacciola, S.O. Quantitative detection of *Colletotrichum godetiae* and C. acutatum sensu stricto in the phyllosphere and carposphere of olive during four phenological phases. *Eur. J. Plant Pathol.* **2017**, *149*, 337–347. [CrossRef]

28. Suarez, M.B.; Walsh, K.; Boonham, N.; O'Neill, T.; Pearson, S.; Barker, I. Development of real-time PCR (TaqMan®) assays for the detection and quantification of *Botrytis cinerea* in planta. *Plant Physiol. Biochem.* **2005**, *43*, 890–899. [CrossRef]

29. Sanzani, S.M.; Schena, L.; De Cicco, V.; Ippolito, A. Early detection of *Botrytis cinerea* latent infections as a tool to improve postharvest quality of table grapes. *Postharvest Biol. Technol.* **2012**, *68*, 64–71. [CrossRef]

30. Schweigkofler, W.; O'Donnell, K.; Garbelotto, M. Detection and quantification of airborne conidia of *Fusarium circinatum*, the causal agent of pine pitch canker, from two California sites by using a real-time PCR approach combined with a simple spore trapping method. *Appl Env. Microbiol* **2004**, *70*, 3512–3520. [CrossRef] [PubMed]

31. Zambounis, A.G.; Paplomatas, E.; Tsaftaris, A.S. Intergenic spacer–RFLP analysis and direct quantification of australian *Fusarium oxysporum* f. sp. *vasinfectum* isolates from soil and infected cotton tissues. *Plant Dis.* **2007**, *91*, 1564–1573.

32. Dita, M.A.; Waalwijk, C.; Buddenhagen, I.W.; Souza, M.T., Jr.; Kema, G.H.J. A molecular diagnostic for tropical race 4 of the banana fusarium wilt pathogen. *Plant Pathol.* **2010**, *59*, 348–357. [CrossRef]

33. Liew, E.C.Y.; Maclean, D.J.; Irwin, J.A.G. Specific PCR based detection of *Phytophthora medicaginis* using the intergenic spacer region of the ribosomal DNA. *Mycol. Res.* **1998**, *102*, 73–80. [CrossRef]

34. Schena, L.; Nigro, F.; Ippolito, A. Real-Time PCR detection and quantification of soilborne fungal pathogens: The case of *Rosellinia necatrix, Phytophthora nicotianae, P. citrophthora* and *Verticillium dahliae*. *Phytopathol. Mediterr.* **2004**, *43*, 273–280.

35. Bilodeau, G.J.; Koike, S.T.; Uribe, P.; Martin, F.N. Development of an assay for rapid detection and quantification of *Verticillium dahliae* in soil. *Phytopathology* **2011**, *102*, 331–343. [CrossRef] [PubMed]

36. Pecchia, S.; Da Lio, D. Development of a rapid PCR-Nucleic Acid Lateral Flow Immunoassay (PCR-NALFIA) based on rDNA IGS sequence analysis for the detection of *Macrophomina phaseolina* in soil. *J. Microbiol. Methods* **2018**, *151*, 118–128. [CrossRef] [PubMed]

37. ISTA International Seed Testing Association. Available online: https://www.seedtest.org/ (accessed on 15 January 2019).

38. Walcott, R.R. Detection of seedborne pathogens. *HortTechnology* **2003**, *13*, 40–47. [CrossRef]

39. Mancini, V.; Murolo, S.; Romanazzi, G. Diagnostic methods for detecting fungal pathogens on vegetable seeds. *Plant Pathol.* **2016**, *65*, 691–703. [CrossRef]

40. Marcinkowska, J.Z. Methods of finding and identification of pathogens in seeds. *Plant Breed. Seed Sci.* **2002**, *46*, 31–48.

41. Ioos, R.; Fourrier, C.; Iancu, G.; Gordon, T.R. Sensitive detection of *Fusarium circinatum* in pine seed by combining an enrichment procedure with a real-time polymerase chain reaction using dual-labeled probe chemistry. *Phytopathology* **2009**, *99*, 582–590. [CrossRef]

42. Guillemette, T.; Iacomi-Vasilescu, B.; Simoneau, P. Conventional and real-time PCR-based assay for detecting pathogenic *Alternaria brassicae* in cruciferous seed. *Plant Dis.* **2004**, *88*, 490–496. [CrossRef]

43. Phan, H.T.T.; Ford, R.; Bretag, T.; Taylor, P.W.J. A rapid and sensitive polymerase chain reaction (PCR) assay for detection of *Ascochyta rabiei*, the cause of ascochyta blight of chickpea. *Australas. Plant Pathol.* **2002**, *31*, 31–39. [CrossRef]

44. Chadha, S.; Gopalakrishna, T. Detection of *Magnaporthe grisea* in infested rice seeds using polymerase chain reaction. *J. Appl. Microbiol.* **2006**, *100*, 1147–1153. [CrossRef]

45. Taylor, J.L. A simple, sensitive, and rapid method for detecting seed contaminated with highly virulent *Leptosphaeria maculans*. *Appl Env. Microbiol* **1993**, *59*, 3681–3685.

46. Kumar, P.; Akhtar, J.; Kandan, A.; Kumar, S.; Batra, R.; Dubey, S.C. Advance Detection Techniques of Phytopathogenic Fungi: Current Trends and Future Perspectives. In *Current Trends in Plant Disease Diagnostics and Management Practices*; Springer: Berlin, Germany, 2016; pp. 265–298.

47. Schena, L.; Nigro, F.; Ippolito, A.; Gallitelli, D. Real-time quantitative PCR: A new technology to detect and study phytopathogenic and antagonistic fungi. *Eur. J. Plant Pathol.* **2004**, *110*, 893–908. [CrossRef]

48. Abdel-Hafez, S.I.I. Mycoflora of bean, broad bean, lentil, lupine and pea seeds in Saudi Arabia. *Mycopathologia* **1984**, *88*, 45–49. [CrossRef]

49. El-Nagerabi, S.A.F.; Elshafie, A.E. Composition of mycoflora and aflatoxins in lupine seeds from the Sudan. *Phytopathol. Mediterr.* **2000**, *39*, 257–262.

50. Nedzinskiene, T.L.; Asakaviciute, R. Development of fungi on *Lupinus angustifolius* L. and *Lupinus luteus* L. *Res. Plant Biol.* **2011**, *1*, 20–29.

51. Alomran, M.M.; Lupien, S.L.; Dugan, F.M. Mycobiota of *Lupinus albus* seeds from a public germplasm collection. *North Am. Fungi* **2013**, *8*, 1–15. [CrossRef]

52. Pszczolkowska, A.; Okorski, A.; Kotecki, A.; Gas, M.; Kulik, T.; Reczek, A. Incidence of seed-borne fungi on *Lupinus mutabilis* depending on a plant morphotype, sowing date and plant density. *J. Elem.* **2016**, *21*, 501–512. [CrossRef]

53. Machado, J.C.; Coutinho, W.M.; Guimarães, R.M.; Vieira, M.; Ferreira, D.F. Use of osmotic solutes to control seed germination of rice and common bean in seed health blotter tests. *Seed Sci. Technol.* **2008**, *36*, 66–75. [CrossRef]

54. de Carvalho, J.C.B. *Uso da restrição hídrica na inoculação de Colletotrichum lindemuthianum em sementes de feijoeiro (Phaseolus vulgaris L.)*; Universidade Federal de Lavras: Lavras, Brazil, 1999.

55. Machado, A.Q. *Uso da restrição hídrica em testes de sanidade de sementes de algodoeiro*; Universidade Federal de Lavras: Lavras, Brazil, 2002.

56. Machado, J.C.; Guimaraes, R.M.; Vieira, M.; Souza, R.M.; Pozza, E.A. Use of water restriction technique in seed pathology. *Seed Test. Int.* **2004**, *128*, 14–18.

57. de Falleiro, B.A.S.; de Almeida, P.B.A.; Coutinho, W.M.; Suassuna, N.D.; Kobayasti, L. Use of osmotic solutions for inhibition of sunflower seed germination in blotter test. *Trop. Plant Pathol.* **2010**, *35*, 343–350. [CrossRef]

58. Farias, C.R.J.; Del Ponte, E.M.; Corrêa, C.L.; Afonso, A.P.S.; Pierobom, C.R. Infecção de sementes de trigo com Bipolaris sorokiniana pela técnica de restrição hídrica. *Trop. Plant Pathol.* **2010**, *35*, 253–257. [CrossRef]

59. da Machado, J.C.; Machado, A.Q.; Pozza, E.A.; Machado, C.F.; Zancan, W.L.A. Inoculum potential of *Fusarium verticillioides* and performance of maize seeds. *Trop. Plant Pathol.* **2013**, *38*, 213–217. [CrossRef]

60. da Botelho, L.S.; Barrocas, E.N.; da Machado, J.C.; de Martins, R.S. Detection of *Sclerotinia sclerotiorum* in soybean seeds by conventional and quantitative PCR techniques. *J. Seed Sci.* **2015**, *37*, 55–62. [CrossRef]

61. da Siqueira, C.S.; Barrocas, E.N.; da Machado, J.C.; Corrêa, C.L. Transmission of *Stenocarpella maydis* by maize seeds. *Rev. Ciênc. Agronômica* **2016**, *47*, 393–400.

62. Cullen, D.W.; Lees, A.K.; Toth, I.K.; Duncan, J.M. Conventional PCR and real-time quantitative PCR detection of *Helminthosporium solani* in soil and on potato tubers. *Eur. J. Plant Pathol.* **2001**, *107*, 387–398. [CrossRef]

63. Shea, G.; Thomas, G.; Buirchell, B.; Salam, M.; McKirdy, S.; Sweetingham, M. Case study: Industry response to the lupin anthracnose incursion in Western Australia. In *Lupins for Health and Wealth (Fremantle, Western Australia ed.)*; Palta, J.A., Berger, J.B., Eds.; International Lupin Association: Canterbury, New Zealand, 2008; Volume 1, pp. 546–551.

64. Kim, W.K.; Mauthe, W.; Hausner, G.; Klassen, G.R. Isolation of high molecular weight DNA and double-stranded RNAs from fungi. *Can. J. Bot.* **1990**, *68*, 1898–1902. [CrossRef]

65. Carbone, I.; Kohn, L.M. A Method for Designing Primer Sets for Speciation Studies in Filamentous Ascomycetes. *Mycologia* **1999**, *91*, 553–556. [CrossRef]

66. Baroncelli, R.; Amby, D.B.; Zapparata, A.; Sarrocco, S.; Vannacci, G.; Le Floch, G.; Harrison, R.J.; Holub, E.; Sukno, S.A.; Sreenivasaprasad, S.; et al. Gene family expansions and contractions are associated with host range in plant pathogens of the genus *Colletotrichum*. *BMC Genomics* **2016**, *17*, 555. [CrossRef]

67. Bankevich, A.; Nurk, S.; Antipov, D.; Gurevich, A.A.; Dvorkin, M.; Kulikov, A.S.; Lesin, V.M.; Nikolenko, S.I.; Pham, S.; Prjibelski, A.D.; et al. SPAdes: A new genome assembly algorithm and its applications to single-cell sequencing. *J. Comput. Biol.* **2012**, *19*, 455–477. [CrossRef]

68. Langmead, B.; Salzberg, S.L. Fast gapped-read alignment with Bowtie 2. *Nat. Methods* **2012**, *9*, 357–359. [CrossRef]

69. Grigoriev, I.V.; Nikitin, R.; Haridas, S.; Kuo, A.; Ohm, R.; Otillar, R.; Riley, R.; Salamov, A.; Zhao, X.; Korzeniewski, F.; et al. MycoCosm portal: Gearing up for 1000 fungal genomes. *Nucleic Acids Res.* **2014**, *42*, D699–D704. [CrossRef]

70. Anderson, J.B.; Stasovski, E. Molecular phylogeny of northern hemisphere species of *Armillaria*. *Mycologia* **1992**, *84*, 505–516. [CrossRef]

71. Katoh, K.; Standley, D.M. MAFFT multiple sequence alignment software version 7: Improvements in performance and usability. *Mol. Biol. Evol.* **2013**, *30*, 772–780. [CrossRef] [PubMed]

72. Untergasser, A.; Nijveen, H.; Rao, X.; Bisseling, T.; Geurts, R.; Leunissen, J.A. Primer3Plus, an enhanced web interface to Primer3. *Nucleic Acids Res.* **2007**, *35*, W71–W74. [CrossRef] [PubMed]

73. Hall, T.A. BioEdit: A user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. In *Proceedings of the Nucleic Acids Symposium Series*; Oxford University Press: London, UK, 1999; pp. 95–98.

74. Heydecker, W.; Higgins, J.; Turner, Y.J. Invigoration of seeds. *Seed Sci. Technol.* **1975**, *3*, 881–888.