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CHARACTERIZATION OF WHITE LUPIN (*LUPINUS ALBUS*) CULTIVARS' RESPONSE TO PRIMARY INFECTION BY DIVERSE *COLLETOTRICHUM LUPINI* STRAINS FROM A SINGLE RUSSIAN FIELD

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ABSTRACT

Anthracnose, induced by *Colletotrichum lupini*, significantly threatens the white lupin industry, primarily originating from infections in asymptomatic seeds. This study evaluated the aggressiveness of six *C. lupini* strains reisolated from symptomatic pods in the same field in 2021. The assessment targeted Russian cultivars ('Aly Parus,' 'Michurinskij,' 'SN 5-19') and the Australian cultivar 'Andromeda.'

Employing a seed infection assay, seeds were directly inoculated with *C. lupini* mycelia, revealing morphological variations and distinct aggressiveness of the strains at different plant developmental stages. While none impeded plant emergence, four strains caused significant early mortality across all cultivars, with no discernible differences in susceptibility.

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This study briefly discusses utilizing survival analysis to comprehensively understand the fungus's effects on plants. By exploring diverse tolerance strategies employed by white lupins during different developmental stages, it is suggested that the observed diversity in pathogenicity highlights the need for assembling diverse strains for effective screening and resistance breeding.

Keywords: Anthracnose, Legume, Pathological assays, Seed inoculation assay, Survival analysis

1. INTRODUCTION

The growing global demand for protein products has emphasized the need for high-quality protein sources. Soybean has traditionally been the most commonly utilized high-protein crop (Glebovna Gataulina *et al.*, 2021). However, challenges such as the limited shelf-life of soy cakes (Yagovenko *et al.*, 2022), heavy reliance on soybean imports (Lucas *et al.*, 2015), and high production costs for non-GMO soybeans in particular in Eurasia, have created a demand for alternative plant protein sources. As a result, decision-makers are increasingly exploring alternative crops within the Fabaceae family, emphasizing cultivation in higher latitudes unsuitable for soybean.

White lupin (*Lupinusalbus* L.), yellow lupin (*L. luteus* L.), and narrow-leafed lupin (*L. angustifolius* L.) are legumes native to Europe and offer a promising alternative to soybeans (Lucas *et al.*, 2015). Lupin seeds share a comparable chemical composition with soybeans, characterized by high protein content and low starch content, making them suitable as animal feed. Additionally, lupin seeds are rich in valuable antioxidants, rendering them a source of nutritious and healthy food for human consumption (Tirdil'ová *et al.*, 2020). Lupins, being of significant agricultural importance, are cultivated in diverse regions across the globe (Talhinhas *et al.*, 2016) and are grown as ornamental plants, pioneer species for soil remediation, and in crop rotation. Lupins are particularly valuable for their ability to fix atmospheric nitrogen and enhance soil phosphorus levels (Lucas *et al.*, 2015; Talhinhas *et al.*, 2016). In 2020, global lupin cultivation encompassed a total harvested area of 888,507 hectares, with Australia accounting for approximately 55% and Europe for 32% of this production, as reported by FAO (2022). In Russia, lupin is highly regarded for its remarkable protein content (Alkemade *et al.*, 2022b) and its cost-effective production methods. Furthermore, lupin exhibits adaptability to various regions, particularly thriving in low-nutrient soils found in Russia (Glebovna Gataulina *et al.*, 2021).

Lupin, despite its agricultural significance, is highly vulnerable to pests and diseases, with anthracnose being a prominent and widespread threat caused primarily by the fungus *Colletotrichum lupini* (Dean *et al.*, 2012; Talhinhas *et al.*, 2016; Dubrulle *et al.*, 2020b). Anthracnose poses a significant risk to various host plants, including cereals, legumes, fruits,

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vegetables, and trees, leading to substantial yield losses (Dean *et al.*, 2012; de Silva *et al.*, 2017; Dubrulle *et al.*, 2020a; da Silva *et al.*, 2020; Talhinhas and Baroncelli, 2021). It has been observed in both humid and dry climates (Talhinhas *et al.*, 2016). The *Colletotrichum* genus is recognized as one of the top ten phytopathogens of significant scientific and economic importance (Dean *et al.*, 2012). It encompasses species that exhibit various lifestyles, including necrotrophic, hemibiotrophic, latent, and endophytic, with several species capable of switching between these lifestyles (de Silva *et al.*, 2017). As a result, these fungi can infect plants at different stages of development, from seedling to flowering and even post-harvest (de Silva *et al.*, 2017; Talhinhas and Baroncelli, 2021).

Primary infection caused by asymptomatic seeds is the usual source of the spread of the disease. Seedlings arising from infected seeds typically exhibit visible symptoms, such as necrosis on aerial parts like hypocotyls, cotyledons, petioles, stems, and pods (Semaškienė *et al.*, 2008; Alkemade *et al.*, 2022b). These lesions produce dark necrotic spots with orange conidial masses (acervuli) (White *et al.*, 2008; Alkemade *et al.*, 2022b). The spores released from the acervuli are dispersed by raindrop splashing, leading to secondary infections (Thomas and Sweetingham, 2004; Semaškienė *et al.*, 2008). Other commonly observed symptoms in affected plants include twisting of stems, petioles, and pods, with severe stem twisting often resulting in plant collapse and death (NSW Dept. of Industry & Investment, 2011). Plant response to anthracnose is dependent on the developmental stage, with young stems being susceptible while older stem tissue shows resistance (Thomas and Sweetingham, 2004). Thomas and Sweetingham (2004) reported that even a small percentage of infected seeds, ranging from 0.01% to 0.05%, led to a significant reduction in white lupin yield by 48% to 70%, prompting the development of guidelines for "pathogen-free seeds" and the use of fungicides (White *et al.*, 2008). The white lupin industry in Australia faced a severe threat from anthracnose, resulting in a ban on white lupin cultivation in 1997 due to its high susceptibility to the disease (Shea *et al.*, 1999). To address this challenge, several alternative strategies to control anthracnose have been proposed (Alkemade *et al.*, 2022a), and advances in genomics tools have facilitated efforts to identify resistance genetic sources in white lupin (Hufnagel *et al.*, 2020; Rychel-Bielska *et al.*, 2020; Baroncelli *et al.*, 2021; Hufnagel *et al.*, 2021; Alkemade *et al.*, 2022a). Although Alkemade *et al.* (2022a) reported that a full resistance was not observed in a genome-wide association study (GWAS) screening a large germplasm collection, they identified 15 accessions that exhibited higher resistance compared to the overall mean of 181 tested accessions.

Developing anthracnose-resistant varieties is the most promising solution to solve this critical problem. Nevertheless, the identification of partial resistance against anthracnose is a difficult task. Understanding and accounting for possible variations in virulence and aggressiveness of the *C. lupine* stains is a critical step to achieve towards selective breeding of most promising

breeding lines. We therefore address, for the first time, the question of possible heterogeneity of pathogenic stains within a same field and possible significance for breeding programs. The aim of the study was to characterize the response of four different white lupin cultivars to primary infection caused by six *C. lupini* strains isolated from a single Russian field, in order to provide guidelines for elaborating breeding programs for more partially resistant varieties. In this work, the question on the type of initial inoculum is addressed and we reveal that phenotypic and pathogenic variability between pathogen strains exist in a same field.

2. MATERIALS AND METHODS

2.1 Plant material and seed sterilisation

Four white lupin cultivars were provided by the All-Russian Lupin Scientific Research Institute (Bryansk, Russia). The cultivars included a commercial Australian cultivar *Andromeda*, two Russian cultivars *Aly Parus*and *Michurinskij* that are registered and authorized for production purposes in Russia (Kurlovich and Lukashevich, 2021; Yagovenko *et al.*, 2022), while *SN 5-19* is an early maturing, high yielding breeding line (Table 1).

Table 1: Properties of the white lupin cultivars used in this study, as evaluated in the Bryansk region of the Russian Federation in 2019

Cultivar (code)	Andromeda (AN)	Aly Parus (AL)	Michurinskij (MI)	SN 5-19 (SN)
Origin	Australia	Russia	Russia	Russia
Ripening information	118 days (personal communication)	120-125 days (Kurlovich and Lukashevich, 2021; Yagovenko *et al.*, 2022)	110-113 days (Yagovenko *et al.*, 2022)	120 days (personal communication)
Protein content in seeds	37.7% (Adhikari*et al.* 2009)	34.5% (Kurlovich and Lukashevich, 2021; Yagovenko *et al.*, 2022)	36.0%(Kurlovich and Lukashevich, 2021; Yagovenko *et al.*, 2022)	19-32% (personal communication)
Resistance against disease	Moderately susceptible to anthracnose	Moderately resistant to Fusarium, susceptibility to anthracnose	Moderately resistant to Fusarium, susceptibility to anthracnose.	Resistant to Fusarium, moderately affected by anthracnose.
Degree of damage by Anthracnose	69.3%	73.7%	75.2%	50.0%

Source: All-Russian Lupin Scientific Research Institute.

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Seeds were sterilized for 48 h with chlorine gas, using the protocol from Cheng, *et al.* (2021). To produce chlorine gas, 8 ml of 12 N HCl was mixed with 100 ml commercial bleach (5.25% sodium hypochlorite) in a tightly closed desiccator (Cheng *et al.*, 2021). After the sterilization process, the seeds were exposed to UV in a clean laminar hood (Lamsystems, Germany) for 10 min. The seeds were then stored at +4°C until used.

2.2 Isolation, molecular and morphological characterization of *Colletotrichum lupini* isolates

Colletotrichum lupini strains were isolated from infected plants. The infected plant material was sterilized for 20 min with 75% ethanol followed by a 30 min wash with 10% sodium hypochlorite and finally washed three times with sterile distilled water (ddH_2O). The seeds were incubated on Petri dishes (Ø= 9 cm, Polyefir, Belarus) with potato dextrose agar (39 gL^{-1}PDA, Scharlab, Spain) and antibiotics (ampicillin - 50 µg/L , streptomycin - 50 µg/ml, tetracycline - 50 µg/ml) in a controlled environment (Binder, KB240, Germany: 22.5°C, 70% RH, darkness).Mycelium from a single colony was collected using a cut pipette tip, transferred to a fresh PDA plate and grown under the same conditions. This was repeated twice to ensure monosporal cultures whose purity was further verified by microscopic observations. For long-term storage, spore solutions for each strain were stored in 20% glycerol at −80°C.

The strains were molecularly characterized using three sets of previously published primers (Table 2) that differentiated the species within the *Colletorichum acutatum* complex (Dubrulle *et al.*, 2020b; Kamber *et al.*, 2021). Same protocols as those initially described by the authors were carried out. Only strains that were positive for all three markers were included in further analysis. Six strains were characterized at three temperatures (22°C, 24°C and 28 °C). The chosen temperatures correspond to the full range of day length mean temperature that can be found for this part of Russia in summer. To do so, 15 µl of spore solution (50 spores/ µl) from the -80°C glycerol stock, were plated on small Petri dishes (Ø= 6 cm, Kirgen Bioscience, China) with PDA and incubated for 48 hours, upon which small colonies were observed. For each strain, the mycelial plugs were cut by perforating the two-days old agar of each strain with the wide end of a sterile 1000 µL pipette tip and transferred to new PDA plates (Ø = 9 cm) (with four replicates). The plates were then incubated at three different s as mentioned above. The plates were periodically scanned (HP LaserJet Enterprise MFP, M725 series) from the top and the bottom, until the mycelia filled the whole plate on day 16. The scans were done on days 2, 5, 9, 12 and 16 and the images were analysed using ImageJ software (Schindelin *et al.*, 2012). To understand the colony growth, area under the growth progression curve (AUGPC) was calculated using the measurement for the diameter (two orthogonal measurements) and area of the colonies extracted from ImageJ.

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Table 2: Primers used to characterize selected *Colletotrichum lupini* fungal strains

Primer name	Forward	Reverse	Reference
Apnmat1	GCGAAAGCCTACAAGGAGCG	TGGTCAAAGTTCAACCTGGG	(Dubrulle *et al.*, 2020b)
CLUP01	ATGATGCTTTCTTTGGTGGCG	CCGGCCTGAAGACACAACG	(Dubrulle *et al.*, 2020b)
Clup_GAPDH	CCCACGGCAAAAGAGTCAGA	CGGCTGTTTCGGCATGATTG	(Kamber *et al.*, 2021)

The sporulation capacity for each strain was evaluated after 16 days of cultivation using a conidial suspension. This suspension was prepared by flooding each Petri dish with 3 ml of sterile distilled water, and the spores were counted using a Goryaev chamber ("MiniMed", Russia).

2.3 Seed inoculation and phenotyping for plant disease response

Seed inoculation assay was conducted to assess the response of the white lupin cultivars to primary infection with the six *C. lupini* strains. To do so, 30 µl of spore solution (50 spores/ µl) from the -80°C glycerol stock of each isolate were spread on a PDA plate (Ø= 9 cm) and incubated under the same controlled environment as previously described. After two weeks, five seeds from each of the four cultivars were placed directly on the mycelia of the fungi in different Petri dishes that were sealed using parafilm and inoculated for two days at 22 °C. The protocol was adapted from the seed inoculation protocol used by Wirtz, *et al.*, (2021), where PDA-mannitol plates were used instead. The seeds were then potted in commercial soil premix ("Peter Peat Pro for seedlings", Russia), watered with filtered tap water with fertilizer (600 µS or 0.03 % (w/v): Universol Blue, 18-11-18+2,5MgO+Te) on alternative days and grown in a phytotron (KrässGlasCon GmbH, Neu-Ulm, Germany: 24 ± 2°C, 16 h light, and ~70% relative humidity).

A split-plot design was set up due to the typical experimental constraint of infecting one tray containing all cultivar using the same pathogenic strain. These constraints for inoculation thus create a split-plot design with 'fungal strain' as the whole plot factor with levels being the six *C. lupini* strains and mock-inoculated control, and 'lupin cultivar' as the split plot factor with four levels being the four studied cultivars. The split-plot was organized with three replicates (blocks) at the physical whole plot level corresponding to independent biological repeats, where each replicate was started about ten days apart. Plant development and disease symptoms were recorded daily for each plant over a period of 21 days. The response measured were the dates of emergence, opening of the cotyledons, 2- (stage 1.2), 4- (stage 1.4) and 6- (stage 1.6) leaf stages and death(NSW Dept. of Industry & Investment, 2011). The date of emergence was the date

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when the hypocotyl emerged out of the soil or stage 0.9 from the 'lupin growth stages and numeral system' (NSW Dept. of Industry & Investment, 2011).

For each seedling, two sets of independent time-to-event data was recorded, the time to emergence from the soil and the time to death in days post-sowing. These were coded as "1" for emergence and "0" for non-germinated seeds, while for the time of death "1" corresponds to dead plants and "0" to those still alive (right censoring). At the end of the experiment, the total number of plants emerged and those that died during the experiment were also recorded and analysed. Only right censoring was considered for both emergence and survival data. In addition to this, visual symptoms of anthracnose, characterized by stem twisting, leaf curling necrosis were also scored. For ease of calculations in survival analysis, scores were reduced to a binary system, where any symptom observed was denoted as "1" and symptomless as "0".

2.4 Statistical analysis

Morphological traits among the fungal strains such as the sporulation capacity (log-10 transformation) and the area under the growth progression curve (AUGPC) was analysed using Linear Mixed-effects Models (LMMs). The models were adapted to split-plot design to test for the effect of Temperature (whole-plot factor) on Strain (split-plot factor) and their interaction on colony growth and sporulation capacity, with Strain and Temperature as fixed effect and Replicate (whole-plot) as random effects. The following model was used,

$$Y_{ijk} = \mu + \alpha_j + \beta_k + \gamma_{jk} + b_i + \varepsilon_{ijk} \quad (1)$$

With Y_{ijk} response variable at temperature *k* infecting strain *j* in replicate *i*, μ the mean (fixed effect), α_j Strain (fixed effect), β_k Temperature (fixed effect), γ_{jk} Interaction of Strain x Temperature (fixed effect), b_i the random effect of whole plots (and error level for Temperature factor) and εijk the error term. Multiple mean comparisons were performed using Tukey pairwise comparisons tests at p-value ≤ 0.05.

For evaluation of the percentage of emergence following infection and the percentage of dead plants, data were transformed using the classical $Asin(\sqrt{proportion})$ transformation. The LMM models were adapted to split-plot design to test for the effect of Strain (whole-plot factor) on lupin Cultivar (split-plot factor) and their interaction; with Strain and Cultivar as fixed effect and Replicate and Tray (whole-plot) as random effects. The following model was used,

$$Y_{ijkl} = \mu + \alpha_j + \beta_k + \gamma_{jk} + b_i + c_{l(i)} + \varepsilon_{ijkl} \quad (2)$$

with Y_{ijkl} the response variable of cultivar *k* infected with strain *j* in tray *l* of replicate *i*, μ the mean (fixed effect), α_j Strain (fixed effect), β_k Cultivar (fixed effect), γ_{jk} Interaction of Strain x

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Cultivar (fixed effect), b_i the random effect of Replicate, $c_{l(i)}$ the random effect of the Tray (whole plot and error level for Strain factor)and ε_{ijkl} the error term. Multiple mean comparisons were performed using Tukey pairwise comparisons tests at p-value ≤ 0.05. Multiple comparisons to compare means in *C. lupini* infected conditions to the mock-inoculated control were performed using the Dunnett test at p-value ≤ 0.05.Statistical analysis was performed in R (R 4.3.1) using packages 'lmerTest', 'emmeans', 'multcomp' and 'agricolae'.

The temporal patterns of plant emergence and death of the four white lupin cultivars were assessed in response to the six different *C. lupini* strains and mock-inoculated control using the Kaplan-Meier survivor function. The non-parametric Kaplan-Meier survival estimator is used to calculate the probability of an event (emergence and death) in a given length of time and to estimate the survival function from data that are censored, truncated or have missing values. The curves show the probability of achieving an event at time "t". The parameters assessed the emergence (the number of plants that emerged over a period of 21 days), and death (the number of plants that died due to the disease). The survival distributions of the cultivars in response to the different strains were compared using the log-rank test. Analyses were performed using the R package 'survival'.

3. RESULTS

3.1 Morphological characterization and fitness assessment of six *Colletotrichum lupini* strains reveals phenotypic diversity in isolates originating from the same field level.

Six fungal strains were selected among a collection of 150 strains isolated from symptomatic seeds and pods of white lupin cultivar (data not shown). Infected plants were harvested in 2021 a same field located in Bryansk region, village Michurinskij (53.276813°N, 34.249736°E, Russian Federation) and provided by the All-Russian Lupin Scientific Research Institute. The strains were molecularly characterized using previously published primers and assigned to the *C. lupini* species (Fig. 1).

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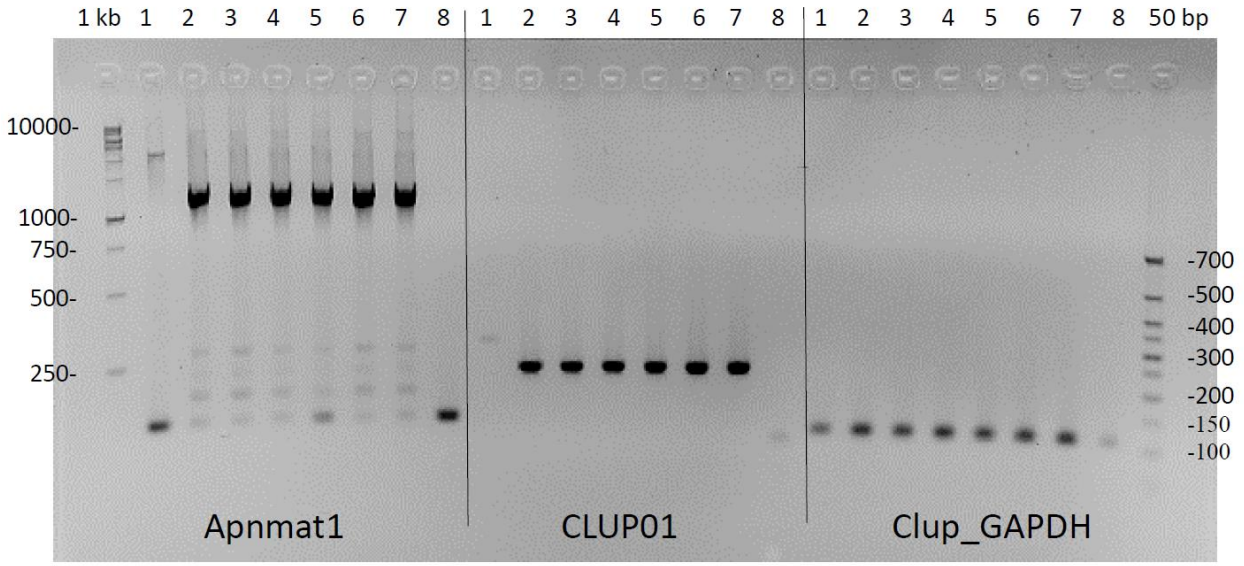

Fig. 1: Molecular characterization of six *Colletotrichum lupini* strains isolated from several infected plants of the same field in Russia

2% Gel stained with ethidium bromide showing PCR amplicons using primer pairs used to characterize the fungal strains (Table 1). The first and last lane have 1kb ladder and a 50 bp ladder, respectively, with sizes (bp) written on the sides. Ultra Pure water was used as negative controls in lanes number 1 and 8, respectively. The lanes 2-7 represent the six *Colletotrichum lupini* strains numbered 18, 73, 107, 139, 3 and 5.

The morphological characteristics for the six strains of *C. lupine* were assessed using three criteria: the colour of the mycelium, the growth on PDA medium and the sporulation capacity at different temperatures. The chosen temperatures correspond to the full range of day length mean temperature that can be found for this part of Russia in summer. The colour, the growth diameter and the area of the mycelium were followed for 16 days. The strains 3 and 139 formed white mycelia from the start, while strains 18, 73 and 107 were pink coloured until day 9 and after day 12, all strains turned a darker shade to a dark grey shade (Fig. 2).

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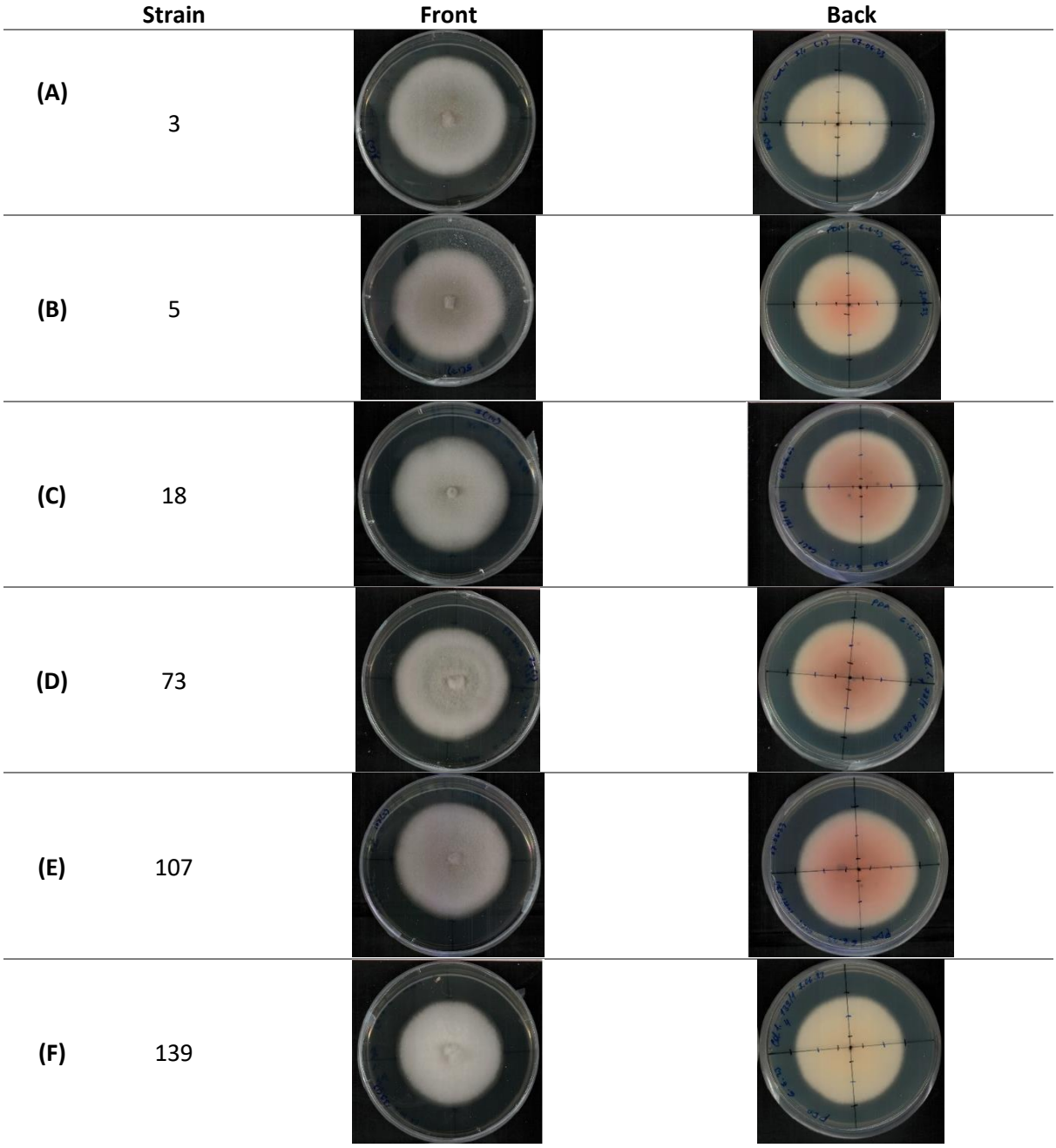

Fig. 2: Photos of typical culture of *Colletotrichum lupini* strains isolated from the same cultivation field

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A-F: Strains numbered 3, 5, 18, 73,107, 139 respectively. Pictures are view from the front and the reverse of Petri dishes, showing the differences in colony colour after 9 days of growth on PDA medium at 24 °C.

The *in vitro* sporulation capacity of the strains changed with ranging temperature from 22 °C to 28 °C (Fig.3A and Table 3). Strain 139 exhibits the most stable and not significantly changing sporulation capacity with temperature, with the highest sporulation ability at 22 °C. Strains 3, 5, 73 and 107 showed a significant increase in sporulation with increasing temperature. 28 °C is the optimal temperature for sporulation for strains 3 and 107, and strains 5 and 73 perform equally well at 24°C and 28°C. Except for strain 139, 22°C was the most unfavourable temperature, with the lowest sporulation ability for strain 5. In summary, we demonstrate contrasted responses for *in vitro* sporulation capacity at different temperatures, suggesting heterogeneity of fitness capacity of strains isolated from the same field.

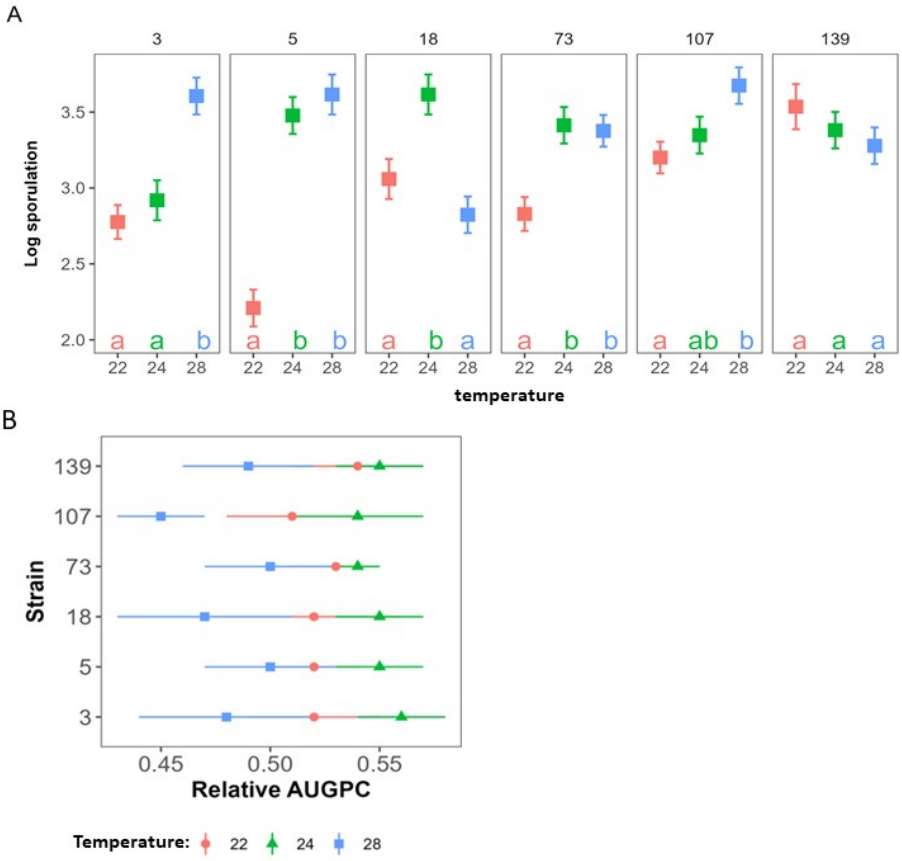

Fig. 3: Effect of temperature on (A) sporulation capacity of fungal strain. (B) The Area Under the Growth Progression Curve (AUGPC) for six different strains of *Colletotrichum lupini* isolated from the same cultivation field

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Strains were grown on PDA at 22°C, 24°C and 28°C. The mean values and confidence intervals were calculated for two independent experiments, with four Petri dishes each. Different letters indicate significant differences between the temperatures using a Tukey test

Table 3: Analysis of Variance of the effect of temperature and fungal strain on sporulation capacity (log-10 transformed) after 16 days of culture on PDA medium.

	Mean Sq	DF	F value	Pr(>F)
Strain	0.34251	5	4.0384	0.002**
Temperature	2.30277	2	27.151	< 2e-16***
Strain: Temperature	0.8215	10	9.686	< 2e-16***

The AUGPC was calculated based onmycelium growth of the fungal strains observed during 16 days of culture. The AUGPC was significantly different depending on the strain and the temperature; however, there was no strain x temperature interaction (Table 4). The results showed all the strains grew slower at the higher temperature of 28°C with an optimal growth at 24 °C **(**Figure 3B**)**.

Table 4: Analysis of variance of the effect of temperature and fungal strain on the fungal growth measured as the Area Under Growth Progression Curve (AUGPC) during 16 days

	Mean Sq	DF	F value	Pr(>F)
Strain	20.24	5	2.79	0.02*
Temperature	403.6	2	55.72	< 2e-16***
Strain: Temperature	7.65	10	1.06	0.40

Figure 4 displays the duration, in days post-primary infection, required for the infested plants to reach specific stages such as emergence, opening of the cotyledons, and the development of 2, 4, and 6 leaves. We were interested in studying the effect of *C. lupini* seed infection on two keys stages: emergence from the soil and death of the plants.

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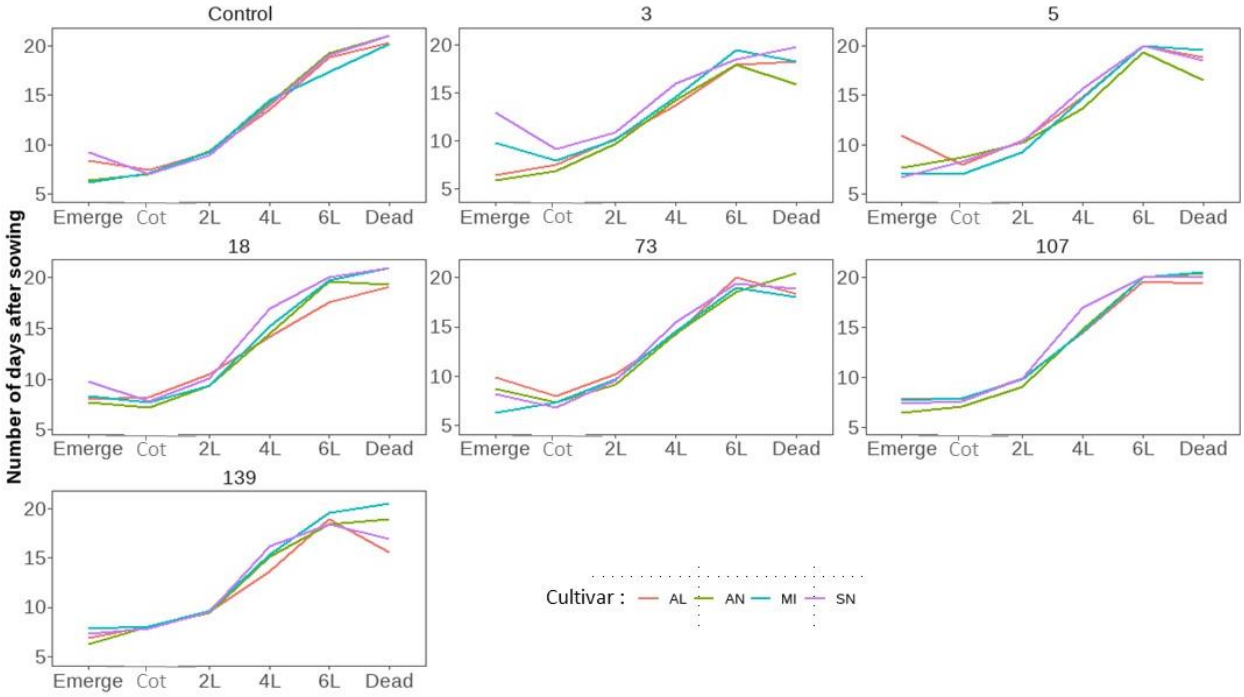

Fig. 4: Developmental curves of four cultivars of white lupin after inoculation with different *C. lupini* strains from the same field.

The average time in number of days to reach each of the developmental stages is plotted after infection by each of the six strains (3, 5, 18, 73, 107 and 139) and in mock-inoculated control condition. Emerge = seedling emergence, Cot = opening of the cotyledons, 2L = 2-leaf stage, 4L= 4-leaf stage, 6L = 6-leaf stage. The coloured curves represent the four lupin cultivars, where AL = 'Aly Parus', AN = 'Andromeda', MI = 'Michurinskij' and SN = 'SN 5-19'. The plot is an average of three blocks and five plants per block, which were set up about 10 days apart.

3.2 *Colletotrichum lupine* strains do not affect the germination capacities of white lupine seedlings, however modify time to seedling emergence.

The percentage of seedlings that emerged after 21 days was recorded in all combinations of white lupin cultivar and pathogen strains. The effect of the fungal strains on the different cultivars was assessed using LMM for split-plots, followed by Dunnett test to compare to the mock-inoculated corresponding controls (Fig.5A and Table 5).The results showed that there is no significant reduction in emergence rate of particular cultivars compared to non-inoculated condition. This suggests that primary infection of plantlets through contaminated seeds does not affect the germination capacity. This trait is therefore likely not useful to consider as a breeding target.

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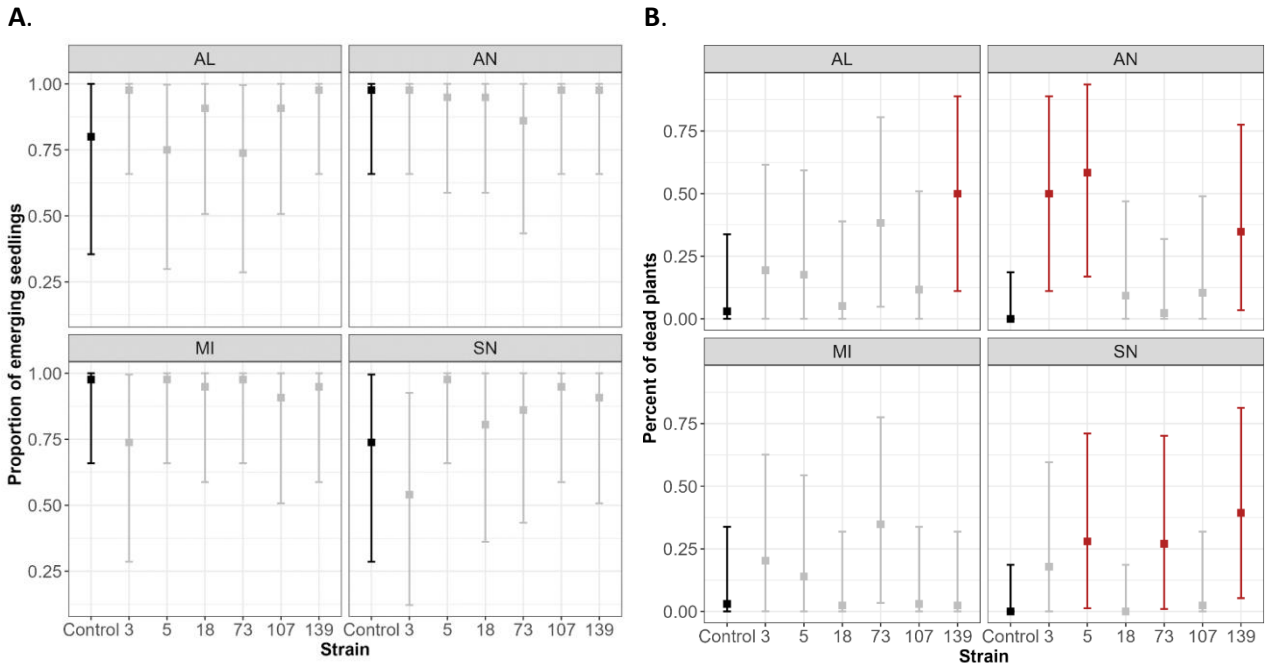

Fig. 5: Percentage of seedling emergence of four cultivars of white lupin after inoculation with different *C. lupini* strains from the same field.

(A) Proportion of seedling emergence. Data were obtained over three biological independent replicates, with five seeds per cultivar inoculated with each fungal strain for each replicate. **(B) Percentage of dead plants after 21 days post-infection.** Data were obtained over three biological independent replicates, after emergence from five seeds per cultivar inoculated with each fungal strain for each replicate.

For each lupin cultivar, a Dunnett test was performed to test the response of each strain with non inoculated control. Control is shown in black, non-significant differences in grey and significant differences at p = 0.10 are shown in red. Lupin cultivars are AL = 'Aly Parus', AN = 'Andromeda', MI = 'Michurinskij' and SN = 'SN 5-19'. 'Control' corresponds to mock-inoculated plants, while the numbers at the bottom denote the strains.

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Table 5: Analysis of variance of the effect of fungal strain (and control) and cultivar on the percentage of seeds emergence after 21 days

	Mean Sq	DF	F value	Pr(>F)
Strain	0.0518	6	0.579	0.745
Cultivar	0.1650	3	1.842	0.150
Strain x Cultivar	0.0751	18	0.838	0.649

A Cox Proportional Hazards regression based on log-rank test (data not shown) showed that strains 5 and 18 significantly slowed time to emergence ($p<0.05$), regardless the white lupin cultivar. The analysis also showed that both 'Andromeda' and 'SN5-19' cultivars emerged statistically faster than 'AliyParus' and 'Michurinsky', irrespective of the inoculation conditions.

3.3 *Colletotrichum lupini* strains collected in a same cultivation field have different aggressiveness towards several lupincultivars.

White lupin cultivars show contrasted responses to the *C. lupini* strains in terms of death occurrence after primary infection**.** The date of symptom onset, characterized by the curling or necrosis of the stems, was duly recorded. Growth retardation was also noted when the seedling did not progress to the next stage of development. Finally, death was reported when severely necrotic seedlings collapsed due to curling or thinning of stems and when plant development was halted for about two days.

The analysis reveals a significant effect of the pathogen strain on the proportion of dead plants (Fig. 5B and Table 6). The least aggressive strains were strain 18 followed by strain 107, with estimated marginal means for proportion of dead plants to be 0.030 and 0.062, respectively. The two most aggressive strains were strain 5 and 139, with estimated marginal means for proportion of dead plants to be 0.283 and 0.284, respectively. This also masks strong differences in aggressiveness with regard to the different cultivars, even if the Strain x Cultivar interaction is not formally significant likely due to too small number of scored plants. For example, the strain 139 significantly increased the percentage of plants that died compared to mock-inoculated condition for cultivar 'AliyParus'. For cultivar Andromeda and SN 5-19, three strains significantly increased the percentage of dead plants that are strains 3,5, 139 and strains 5, 73 and 139, respectively. Only for 'Michurinskij', no strains produced significant increase of dead plants compared to mock-inoculated conditions. Even if the Cultivar effect is not significant, the 'Michurinskij' cultivar is the less susceptible (estimated marginal means for proportion of dead plants to be 9%) and the cultivar 'Aly Parus' is the most susceptible (estimated marginal means for proportion of dead plants to be 18.3%).

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Table 6: Analysis of variance of the effect of fungal strain (and control) and cultivar on the proportion of dead plants after 21 days post-infection

	Mean Sq	DF	F value	Pr(>F)
Strain	0.36584	6	5.0998	0.0081 ***
Cultivar	0.10251	3	1.4290	0.2478
Strain x Cultivar	0.10634	18	1.4824	0.1455

The Kaplan-Meier curves of the time to death showed the percentage of plants that are alive decreased over time (Fig.6). The median number of days of survival for each strain per cultivar was obtained from the Kaplan-Meier curve and is presented in Table 7. The results showed not only the final proportion of dead plants at 21 dpi is different between the strain of *C. lupini* from the same cultivation field, but also that some plants started to die as early as 4- and 6-days post sowing. This was observed for cultivars 'Andromeda' and ''Michurinskij' that were infected with strain 3.In the case ''Michurinskij', this early time to death was however not correlated with final strong aggressiveness of strain 3. As a whole, the Cox (Proportional Hazards) regression based on the Wald test to differentiate the curves evidenced that considering the other variables, such as cultivar, replicates and also disease symptoms, plant death caused by strains 3, 5, 73 and 139 were significantly earlier than the control (p=0.05), regardless the white lupin cultivar (data not shown). This is consistent with the finding that these strains were described as the most aggressive strains using proportion of dead plants at 21 dpi.

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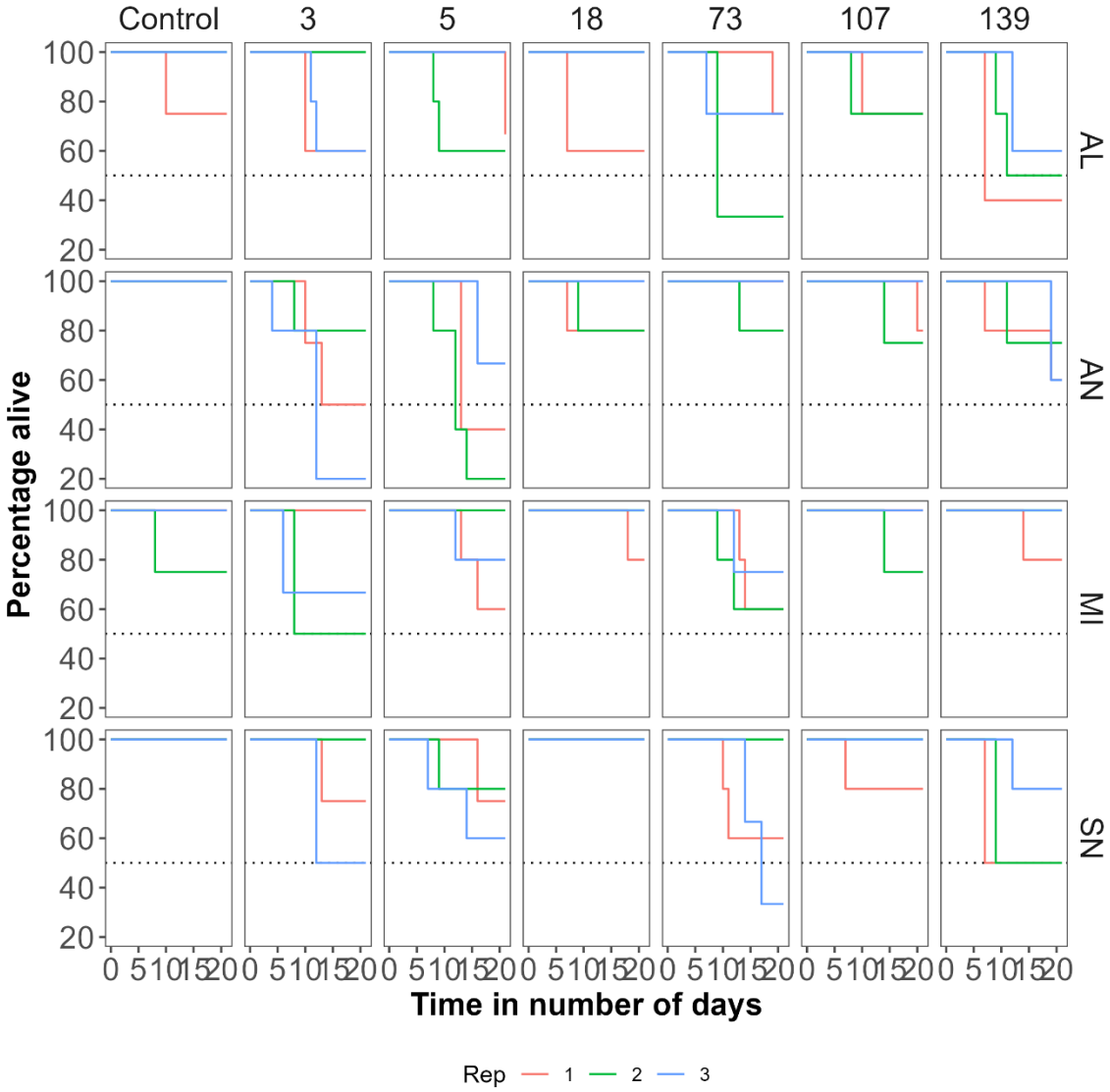

Fig. 6: Probability of surviving seedlings for the four cultivars in response to six *C. lupini* strains from the same cultivation field, fitted by Kaplan-Meier survival curves.

Breaks in the curves indicate death events and the curve length between two breaks represents the percentage of alive seedlings in the same day. The cultivars are AL = Aly Parus, AN = Andromeda, MI = Michurinskij and SN = SN 5-19. 'Control' corresponds to mock-inoculated plant, the strains are named according to their ID-number (i.e. 3, 5, 18, 73, 107, 139).

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Table 7: The median time (in days post-infection) taken for the plants to die per cultivar and per strain, using Kaplan-Meier survival model.

Strain	3	5	18	73	107	139
Cultivar						
AL	10	8	7	7	8	7
AN	4	8	7	13	14	7
MI	6	12	18	9	14	14
SN	12	7	-	10	7	7

AL = 'Aly Parus', AN = 'Andromeda', MI = 'Michurinskij' and SN = 'SN 5-19'

4. DISCUSSION

Anthracnose is a devastating disease for lupins around the world (Talhinhas *et al.*, 2016).To date, there is no complete resistance in white lupin. Instead prevention and control measures have been recommended by sowing pathogen-free seeds, including possible disinfection of seeds using dry heat, UV and thermal radiation (White *et al.*, 2008), carrying out early pathogen detection *via* molecular PCR-based methods (Pecchia *et al.*, 2019; Kamber *et al.*, 2021), using fungicides, which are prohibited for use in European Union(Talhinhas *et al.*, 2016). Other alternative methods such as biological treatments have become popular strategies for the management of anthracnose in the Andean lupin (Alkemade *et al.*, 2022b; Falconí and Yánez-Mendizábal, 2022). It was also suggested that *C. lupini* could be colonizing the seed coat rather than the embryo or endosperm (Alkemade *et al.* 2022a) so removing the seed coat prior to sowing, could help reduce the chance of causing large scale infections. Nevertheless, the development of resistant varieties through breeding appears to be the most promising solution to meet this significant challenge faced by the lupin industry. It is therefore important to understand the response of lupin cultivars against *C. lupini*, in order to find some genetic sources of resistance among the cultivars.

We studied the effect of the primary infection with six *C. lupini* strains re-isolated from a single field in Russia on four white lupin cultivars common to Russia.

We inoculated the seeds *via* direct contact with mycelium and the inoculated seeds were sown in the phytotron under a controlled environment. Our results show that the number of emerged seedlings alone is not enough to reveal the contrasting effects of the strains on the response of the different cultivars, but instead the time to seedling emergence may also vary among the cultivars in response to the different strains. Infected seeds may still germinate (Semaškienė *et*

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al., 2008), though slower than the control, as seen from our data. The infected plants from infected seeds would eventually lead to secondary infection as already reported before (Thomas and Sweetingham, 2004). Up to now, substantial research efforts have been directed towards comprehending the pathogenic fungus and its lifestyle, rather than the response of the lupin. This includes in-depth investigations through proteomic analysis, as indicated by Dubrulle *et al.* (2020a), and the exploration of pathogenic compounds, including toxins released during infection (Wojakowska *et al.*, 2013). A better understanding of the plant host response to infection and tolerance at different plant development stages is important for disease control and risk management.

Our results evidenced that different isolates can have a different effect on the development, from late seedling emergence to early plant death, where cultivars are more susceptible at a specific developmental stage. We showed that the number of emerged seedlings alone is not enough to reveal the contrasting effects of the strains on the response of the different cultivars, but instead the time to seedling emergence may also vary among the cultivars in response to the different strains. This confirms the infected seeds may still germinate (Semaškienė *et al.*, 2008), though slower than the control, as seen from our data, and contribute to initial inoculum in the fields.

This suggests that genetic mechanisms for plant response to a specific strain may depend on plant developmental stage and underlying physiology and may be controlled by different genomic loci and regulatory pathway (Thomas and Sweetingham, 2004).

In this study, we examined the impact of primary infection using six *C. lupini* strains, which were re-isolated from a single field in Russia, on four white lupin cultivars. Results revealed a significant phenotypic diversity in morphological and aggressiveness criteria (reduction of plant death time and proportion of dead plants) regardless of white lupine cultivar. This is remarkable, given the low number of strain assessed. These findings are consistent with recent data showing that *C. lupini* reproduces clonally, exhibiting distinct morphologies and virulence patterns both between and within clonal lineages (Alkemade *et al.* 2023). Given the inherent variability in pathogenic aggressiveness, this underscores the importance of formulating breeding guidelines. It is proposed that an essential prerequisite for an effective breeding program involves the assembly of diverse strains sourced from multiple locations, both across various sites and within each specific site. This approach is instrumental in achieving robust screening outcomes for partial resistances.

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REFERENCES

[1] Alkemade, J.A. *et al.* (2021) 'A High-Throughput Phenotyping Tool to Identify Field-Relevant Anthracnose Resistance in White Lupin'. *Plant Disease* 105(6), pp. 1719–1727. Available at: https://apsjournals.apsnet.org/doi/10.1094/PDIS-07-20-1531-RE.

[2] Alkemade, J.A. *et al.* (2022a) 'Genome-wide association study reveals white lupin candidate gene involved in anthracnose resistance'. *Theoretical and Applied Genetics* 135(3), pp. 1011–1024. doi: 10.1007/s00122-021-04014-7.

[3] Alkemade, J.A. *et al.* (2022b) 'The potential of alternative seed treatments to control anthracnose disease in white lupin'. *Crop Protection* 158, p. 106009. Available at: https://linkinghub.elsevier.com/retrieve/pii/S0261219422001053.

[4] Alkemade JA, *et al.* (2023) Attack of the clones: Population genetics reveals clonality of *Colletotrichumlupini*, the causal agent of lupin anthracnose. *Mol Plant Pathol*. 2023 Jun;24(6):616-627. doi: 10.1111/mpp.13332.

[5] Baroncelli, R. *et al.* (2021) 'Complete Genome Sequence of the Plant-Pathogenic Fungus *Colletotrichumlupini*'. *Molecular Plant-Microbe Interactions* 34(12), pp. 1461–1464. doi: 10.1094/MPMI-07-21-0173-A.

[6] Cheng, Y., Wang, X., Cao, L., Ji, J., Liu, T. and Duan, K. (2021) 'Highly efficient Agrobacterium rhizogenes-mediated hairy root transformation for gene functional and gene editing analysis in soybean'. *Plant Methods* 17(1). doi: 10.1186/s13007-021-00778-7.

[7] Dean, R. *et al.* (2012) 'The Top 10 fungal pathogens in molecular plant pathology'. *Molecular Plant Pathology* 13(4), pp. 414–430. doi: 10.1111/j.1364-3703.2011.00783.x.

[8] Dubrulle, G. *et al.* (2020a) 'Deciphering the Infectious Process of *Colletotrichumlupini*in Lupin through Transcriptomic and Proteomic Analysis'. *Microorganisms* 8(10), p. 1621. Available at: https://www.mdpi.com/2076-2607/8/10/1621.

[9] Dubrulle, G. *et al.* (2020b) 'Phylogenetic diversity and effect of temperature on pathogenicity of *Colletotrichumlupini*'. *Plant Disease* 104(3), pp. 938–950. doi: 10.1094/PDIS-02-19-0273-RE.

[10] Falconí, C.E. and Yánez-Mendizábal, V. (2022) 'Available Strategies for the Management of Andean Lupin Anthracnose'. *Plants* 11(5). doi: 10.3390/plants11050654.

[11] Gallardo, C. *et al.* (2019) 'Anatomical and hormonal description of rootlet primordium development along white lupin cluster root'. *PhysiologiaPlantarum* 165(1), pp. 4–16. doi: 10.1111/ppl.12714.

[12] Glebovna Gataulina, G., Medvedeva, N.V., Pilipenko, S.E. and Shitikova, A.V. (2021) 'Formation of seedlings of white lupin, Lupinusalbus L. and blue lupin, Lupinusangustifolius L. in laboratory and field experiments'. *Caspian Journal of Environmental Sciences* 19(4), pp. 771–776.

[13] Hufnagel, B. *et al.* (2020) 'High-quality genome sequence of white lupin provides insight into soil exploration and seed quality'. *Nature Communications* 11(1). doi: 10.1038/s41467-019-14197-9.

[14] Hufnagel, B. *et al.* (2021) 'Pangenome of white lupin provides insights into the diversity of the species'. *Plant Biotechnology Journal* 19(12), pp. 2532–2543. Available at: https://onlinelibrary.wiley.com/doi/10.1111/pbi.13678 (Accessed: 24 August 2022).

[15] Kamber, T., Malpica-López, N., Messmer, M.M., Oberhänsli, T., Arncken, C., Alkemade, J.A. and Hohmann, P. (2021) 'A qpcr assay for the fast detection and quantification of *Colletotrichumlupini*'. *Plants* 10(8), pp. 2–9. doi: 10.3390/plants10081548.

[16] Kurlovich, B.S. and Lukashevich, M.I. (2021) 'Creation of New Lupin's Forms with Economically Useful Traits'. *Agri Res & Tech: Open Access J* 44(2). Available at: https://juniperpublishers.com/online-submission.php.

[17] Lucas, M.M. *et al.* (2015) 'The future of lupin as a protein crop in Europe'. *Frontiers in Plant Science* 6(September). doi: 10.3389/fpls.2015.00705.

[18] McNair, J.N., Sunkara, A. and Frobish, D. (2012) 'How to analyse seed germination data using statistical time-to-event analysis: non-parametric and semi-parametric methods'. *Seed Science Research* 22(2), pp. 77–95. Available at: https://www.cambridge.org/core/product/identifier/S0960258511000547/type/journal_article.

[19] NSW Dept. of Industry & Investment (2011) *Lupin growth & development*. Edwards, J., Walker, J., and McIntosh, G. (eds.). NSW government: NSW Department of Industry and Investment.

[20] Pecchia, S., Caggiano, B., da Lio, D., Cafà, G., le Floch, G. and Baroncelli, R. (2019) 'Molecular detection of the seed-borne pathogen colletotrichumlupini targeting the hyper-variable IGS region of the ribosomal cluster'. *Plants* 8(7), pp. 4–6. doi: 10.3390/plants8070222.

[21] Romano, A. and Stevanato, P. (2020) 'Germination data analysis by time-to-event approaches'. *Plants* 9(5), pp. 2–5. doi: 10.3390/plants9050617.

[22] Rychel-Bielska, S., Nazzicari, N., Plewiński, P., Bielski, W., Annicchiarico, P. and Książkiewicz, M. (2020) 'Development of PCR-based markers and whole-genome selection model for anthracnose resistance in white lupin (Lupinusalbus L.)'. *Journal of Applied Genetics* 61(4), pp. 531–545. Available at: https://link.springer.com/10.1007/s13353-020-00585-1.

[23] Schandry, N. (2017) 'A practical guide to visualization and statistical analysis of R. Solanacearum infection data using R'. *Frontiers in Plant Science* 8. doi: 10.3389/fpls.2017.00623.

[24] Schindelin, J. *et al.* (2012) 'Fiji: An open-source platform for biological-image analysis'. *Nature Methods* 9(7), pp. 676–682. doi: 10.1038/nmeth.2019.

[25] Semaškienė, R., Brazauskienė, I., Lisova, R., Liepienė, N. and Maknickienė, Z. (2008) 'The incidence of anthracnose (Colletotrichum spp.) on lupine seed'. *Zemdirbyste-Agriculture*, pp. 144–150. Available at: https://www.researchgate.net/publication/267831184.

[26] Shea, G., Thomas, G. and Western Australia Bill, A.O. (1999) *Crop Updates 1999 - Lupins*. Available at: https://researchlibrary.agric.wa.gov.au/crop_up.

[27] de Silva, D.D., Crous, P.W., Ades, P.K., Hyde, K.D. and Taylor, P.W.J. (2017) 'Life styles of Colletotrichum species and implications for plant biosecurity'. *Fungal Biology Reviews* 31(3), pp. 155–168. Available at: https://linkinghub.elsevier.com/retrieve/pii/S174946131630094X.

[28] da Silva, L.L., Moreno, H.L.A., Correia, H.L.N., Santana, M.F. and de Queiroz, M.V. (2020) '*Colletotrichum*: species complexes, lifestyle, and peculiarities of some sources of genetic variability'. *Applied Microbiology and Biotechnology* 104(5), pp. 1891–1904. Available at: http://link.springer.com/10.1007/s00253-020-10363-y.

[29] Talhinhas, P. and Baroncelli, R. (2021) Colletotrichum*species and complexes: geographic distribution, host range and conservation status*. Springer Netherlands. Available at: https://doi.org/10.1007/s13225-021-00491-9.

[30] Talhinhas, P., Baroncelli, R. and le Floch, G. (2016) 'Anthracnose of lupins caused by *Colletotrichumlupini* : A recent disease and a successful worldwide pathogen'. *Journal of Plant Pathology* 98(1), pp. 5–14. doi: 10.4454/JPP.V98I1.040.

[31] Talhinhas, P., Sreenivasaprasad, S., Neves-Martins, J. and Oliveira, H. (2002) 'Genetic and Morphological Characterization of *Colletotrichumacutatum* Causing Anthracnose of Lupins'. *Phytopathology*® 92(9), pp. 986–996. Available at: https://apsjournals.apsnet.org/doi/10.1094/PHYTO.2002.92.9.986.

[32] Thomas, G.J. and Sweetingham, M.W. (2004) 'Cultivar and environment influence the development of lupin anthracnose caused by *Colletotrichumlupini*'. *Australasian Plant Pathology* 33(4), pp. 571–577. doi: 10.1071/AP04060.

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[33] Tirdiľová, I., Vollmannová, A., Siekel, P., Zetochová, E., Čéryová, S. and Trebichalský, P. (2020) 'Selected legumes as a source of valuable substances in human nutrition.' *Journal of Food and Nutrition Research* 59(3), pp. 193–201.

[34] White, P., French, B., Mclarty, A. and Grains research and development corporation (2008) *Producing lupins*. Available at: https://researchlibrary.agric.wa.gov.au/bulletins.

[35] Wirtz, L., MassolaJúnior, N.S., Castro, R.R.L. de, Ruge-Wehling, B., Schaffrath, U. and Loehrer, M. (2021) '*Colletotrichum*spp. from Soybean Cause Disease on Lupin and Can Induce Plant Growth-Promoting Effects'. *Microorganisms* 9(6), p. 1130. Available at: https://www.mdpi.com/2076-2607/9/6/1130.

[36] Wojakowska, A., Muth, D., Narozna, D., Mądrzak, C., Stobiecki, M. and Kachlicki, P. (2013) 'Changes of phenolic secondary metabolite profiles in the reaction of narrow leaf lupin (Lupinusangustifolius) plants to infections with Colletotrichumlupini fungus or treatment with its toxin'. *Metabolomics* 9(3), pp. 575–589. doi: 10.1007/s11306-012-0475-8.

[37] Yagovenko, G.L., Lukashevich, M.I., Ageeva, P.A., Novik, N. v. and Misnikova, N. v. (2022) 'Evaluation of the modern lupine varieties developed in the All-Russian Lupin Scientific Research Institute'. in*IOP Conference Series: Earth and Environmental Science*. IOP Publishing Ltd. doi: 10.1088/1755-1315/1010/1/012096.

