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INCIDENCE OF SEED-BORNE FUNGI ON *LUPINUS MUTABILIS* DEPENDING ON A PLANT MORPHOTYPE, SOWING DATE AND PLANT DENSITY*

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Abstract

Seeds of the Andean lupine are characterised by high nutritional value, and the plant could become an important crop in the production of food and forage. This legume continues to attract growing interest around the world. A field experiment was carried out in in Lower Silesia, Poland, in 2011-2012. Two Andean lupine morphotypes (indeterminate and determinate) were analysed. Andean lupine was grown in treatments characterised by different sowing dates and plant density per m^2. Seed yield, macronutrient content, protein content and health were evaluated at harvest. Seed yield was determined by the interaction of all experimental factors. The indeterminate form produced a significantly higher yield than the determinate form, regardless of the sowing date. The factors had little influence on the mineral content of seeds and total protein content. Andean lupine seeds were colonised mostly by saprotrophic fungi of the genera *Alternaria*, *Cladosporium, Epicoccum* and *Rhizopus* and pathogenic fungi of the genera *Botrytis, Colletotrichum* and *Fusarium*. Delayed sowing contributed to seed colonisation by fungi of the genus *Colletotrichum*. The determinate form was more susceptible to infection than the indeterminate form. Molecular analysis showed that the *Colletotrichum* isolates found in the study belong to the *Colletotrichum acutatum* species complex. The pathogen causing lupine anthracnose, isolated from the seeds of Andean lupine in the present study, was identified as *Colletotrichum lupini* (within *C. acutatum* complex) in a molecular analysis, and its DNA sequence was compared with those of the isolates deposited in the GenBank.

Keywords: Andean lupine, seed yield, macronutrients, seed health, identification of *Colletotrichum* spp.

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INTRODUCTION

The Andean lupine continues to attract growing interest on account of its good taste and high nutritional value (high content of protein, vitamins and iron) (Kohajdova et al. 2011, Falconi 2012). The species is also widely researched in Europe (Caligari et al. 2000, Stawiński et al. 2003, Sawicka -Sienkiewicz et al. 2006, Galek et al. 2007, Reinhard et al. 2006, Guemes-Vera et al. 2008). Research into Andean lupine's adaptability to the European climate was initiated in the 1970s. The first attempts to cultivate the species were made in the UK (Reading University), Germany (University of Giessen), France (INRA, Lusignan) and Poland (Botanical Garden of the Polish Academy of Sciences, Wrocław University of Environmental and Life Sciences). In 1993-1997, an international project funded by the EU (AIR3-CT93-0865 "Adaptation of *L. mutabilis* to European soil and climate conditions") supported comprehensive evaluation of Andean lupine's suitability for cultivation in Europe (Caligari et al. 2000). The agronomic potential of Andean lupine is limited in Europe due to its low and unstable seed yield, and the absence of early and uniformly maturing genotypes (Hardy et al. 1997, Caligari et al. 2000). Anthracnose is a biotic factor that limits the cultivation potential of Andean lupine and other lupine species around the world (Talhinhas at al. 2002, Falconi et al. 2013).

Anthracnose, a pathogenic infection caused by *Colletotrichum. acutatum* Simmonds, is one of the most destructive diseases affecting *L. mutabilis* (Talhinhas et al. 2002, Falconi et al. 2013). According to the research, anthracnose affecting various lupine species is caused by *Colletotrichum lupini* (Bondar) Nirenberg, Feiler & Hagedorn, a distinct group within the *C. acutatum* species complex (Nirenberg et al. 2002, Sreenivasaprasad, Talhinhas 2005, Damm et al. 2012).

The objective of this study was to determine the yield and health of Andean lupine seeds and the severity of infections caused by fungi of the genus *Colletotrichum*, subject to a morphotype, sowing date and seeding density of Andean lupine seeds.

MATERIAL AND METHODS

The experiment had a split-plot design with four replications, and it was conducted on a field in Wrocław-Pawłowice, at the altitude of 122 m above sea level, in the catchment area of the Dobra River, the right tributary of the Widawa River (17°02′E, 51°31′N) in 2011-2012. The experimental factors were: 1) Two Andean lupine morphotypes. The indeterminate form of Andean lupine was developed by NMU- and NaN3-induced mutagenesis. In comparison with parental genotypes, it is characterised by smaller height, fewer

side branches and lower node of the first side branch (Stawiński 2001, Stawiński et al. 2003). The morphotype with a determinate growth habit was the KW-1 monostem determinate mutant, developed by Römer through mutagenesis. It does not produce lateral branches, it matures early and is characterised by tall stems that are vulnerable to lodging (Sawicka 1993). In Poland, early maturing mutants with a smaller number of side branches were developed by Sawicka (1993). Parental forms characterised by the above traits were identified and crossed with the KW-1 mutant to produce (by selection) a (determinate) form for research purposes (Sawicka-Sienkiewicz, Kadłubiec 2001, Sawicka-Sienkiewicz et al. 2006, Galek et al. 2007). The determinate form is distinguished by a medium-tall stem without lateral branches, which is resistant to lodging. Generative growth begins early; 2) Sowing date: early (31.03.2011; 28.03.2012), delayed by 14 days (14.04.2011; 12.04.2012); 3) Seeding density: the number of seeds with high germination capacity sown per m^2: 60, 90, 120. Before the experiment, soil samples were taken to determine the soil's pH and the abundance of nutrients (P, K, Mg) The soil had moderately high phosphorus and potassium levels, and high magnesium levels (Table 1).

Table 1

Selected physicochemical properties of soil used in the experiment
(average of years 2011-2012)

Years	pH (l mol KCl)	Available forms of macronutrients (mg kg^{-1})		
		P	K	Mg
2011	5.83	81.11	217.00	70.62
2012	6.18	73.89	195.03	84.02

Seed yield, macronutrient content, protein content and health were determined at harvest: 18.08.2011; 30.08.2012 for early sowing and 24.08.2011; 30.08.2012 for delayed sowing. Protein content in seeds (grain) was calculated with the coefficient 6.25 (ISO 5983-1:2005). Winter wheat was the forecrop for Andean lupine in both years of the study. Due to the high risk of anthracnose, a fungal disease caused by *Colletotrichum* sp., lupine plants were protected with Amistar 250SC and Gwarant 500SC fungicides, which were applied alternately 6 times in 2011 and 5 times in 2012. The content of K, Ca, Na was determined by flame photometry using a Flapho 4 camera, whereas the concentrations of Mg and P were determined by the calorimetric method using a Spekol 10 camera.

Andean lupine seeds were subjected to mycological analyses. For quantification of fungal pathogens from lupine seeds, a total number of 200 seeds from each sample was used: 100 seeds non-sterilised and 100 seeds disinfected (in all variants of the study, in the two years of the investigation). For isolation of fungal cultures from the functional part of seed, seeds were surface-sterilised by use 1% $Na(OCl)_2$ for 3 min and 70% ethyl alcohol for 3 min.

Seeds were plated in 9 cm diameter sterile Petri dishes with PDA medium. After 7-10 days, fungal cultures were transferred to sterile Petri plates filled with PDA and after another 14 days they were identified based on morphological and microscopic characteristics (Elis 1971, Leslie, Summerell 2006, Damm et al. 2012). The number and percentage of the incidence of each fungal species recovered were calculated.

The species identified in *Colletotrichum* spp. isolates from Andean lupine seeds (determinate and indeterminate forms) were validated by isolating DNA from fungal cultures by the column-based method (Kulik et al. 2007). A PCR assay was performed with the use of the following primers: ITS4 5´-TCCTCCGCTTATTGATATGC-3´ (White et al. 1990) in conjunction with CaInt2 5'-GGGGAAGCCTCTCGCGG-3', a primer pair specific for *C. acutatum*, and in conjunction with CgInt 5'-GGCCTCCCGCCTCCGGGCGG-3', a primer pair specific for C. *gloeosporioides* (Brown et al. 1996).

The rDNA sequence analysis of *Colletotrichum* spp. was performed on 19 selected isolates with ITS1 5'-TCCGTAGGTGAACCTGCGG-3' and ITS4 5'-TCCTCCGCTTATTGATATGC-3' primers (White et al. 1990). rDNA was sequenced in both directions in the ABI PRISM 310 sequencer with the use of a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). The sequences were analysed in the BLASTn application (http://www.ncbi.nlm.nih.gov/BLAST/).

The results of yield and chemical analyses were verified statistically. Statistical calculations were performed using the Statistica software package (data analysis software system), version 10, StatSoft, Inc. (2011). www.statsoft.com, by means of the analysis of variance, with the Duncan's test. The significance of differences between means was determined at a significance level of $p = 0.05$.

RESULTS AND DISCUSSION

The seed yield of Andean lupine was determined by the interaction of experimental factors. The indeterminate form produced a significantly higher yield (2.22 and 2.02 t ha^{-1}, respectively) than the determinate form (1.70 and 1.81 t $h,^{-1}$ respectively) in both years of the study (Table 2), regardless of the sowing date.

Early sowing increased the seed yield of both analysed lupine morphotypes (Table 2). Seeding density had a changeable effect on seed yield during the study. In 2011, the highest yield was noted in treatments with 120 seeds per m^2, whereas in 2012, the highest yield was observed in treatments with 60 seeds per m^2 (Table 2).

In an international study carried out as part of an EU project, seed yield was significantly below the reference values for other legumes grown in Eu-

Table 2

Yield of Andean lupine in the years of investigation (t ha-1)

Morphotype	Sowing date	Number of seeds per 1m^2	Yield		
			2011	2012	*X*
Indeterminate form	I	60	2.361	2.503	2.430
		90	2.533	2.592	2.563
		120	2.701	2.290	2.511
	II	60	1.839	1.468	1.658
		90	1.852	1.719	1.781
		120	2.051	1.872	1.959
Determinate form	I	60	1.640	2.741	2.193
		90	1.813	1.960	1.892
		120	1.938	2.022	1.982
	II	60	1.482	1.619	1.554
		90	1.578	1.311	1.449
		120	1.721	1.212	1.461
LSD (α = 0.05)			n.s.*	0.170	0.101
Average of factors					
Indeterminate form			2.220	2.071	2.152
Determinate form			1.701	1.810	1.752
LSD (α = 0.05)			0.043	0.091	0.040
Sowing data	I		2.172	2.349	2.261
	II		1.754	1.532	1.643
LSD (α = 0.05)			0.061	0.093	0.052
Number of seeds per 1 m^2	60		1.831	2.084	1.962
	90		1.953	1.889	1.922
	120		2.111	1.851	1.983
LSD (α = 0.05)			0.052	0.093	n.s.
Year	2011				1.962
	2012				1.944
LSD (α = 0.05)					n.s.

* n.s. – non significant difference

rope. It should be noted, however, that the discussed experiment was conducted in two very dry years (Caligari et al. 2000). Seed yield from 5 European locations ranged from 0.4 to 2.1 t h^{-1}. The above results indicate that *L. mutabilis* is characterised by highly unstable yields that are significantly influenced by environmental conditions (Caligari et al. 2000).

In the present study, lupine seed yields were relatively high in comparison with other experiments carried out in Europe. Weather conditions in the region of Lower Silesia (Poland) supported generative growth in both years of the study (Table 3). In earlier studies conducted as part of the EU-funded project, Andean lupine genotypes grown in Poland were characterised by low seed yield at 0.4 t ha^{-1} in 1994 and 0.7 t h^{-1} in 1995 (Caligari et al. 2000).

Table 3

Weather conditions in 2011-2012, data from Meteorogical Station in Swojec (district of Wrocław)

Month	Mean monthly temperature (°C)			Precipitation total (mm)		
	2011	2012	mean from 1981-2010	2011	2012	mean from 1981-2010
March	4.4	6.1	3.8	45.2	13.7	31.7
April	11.9	9.8	8.3	27	27.6	30.5
May	14.8	15.8	14.1	49.4	63.7	51.3
June	19.1	17.3	16.9	95.7	94.7	59.5
July	18.2	20	18.7	170.9	108	78.9
August	19.3	19.3	17.9	78.9	73.2	61.7

In a study of Andean lupine in the province of Cotopaxi in Ecuador, seed yield was low at 124 kg ha^{-1} (Falconi 2012). The results demonstrate that Andean lupine varieties were not adapted to the climatic conditions of Cotopaxi. High rainfall was indicated as the main cause of low yield. In the cited experiment, the growth and yield of Andean lupine was also influenced by anthracnose, one of the principal biotic factors limiting the production of L. *mutabilis* (Falconi 2012, Falconi et. al. 2013).

The experimental factors had little influence on the content of macronutrients, which was modified by weather conditions in both years (Table 4). Protein amount varied from 35% for the determinate form to 38 % for the indeterminate form (Table 5). In their studies Caligari et al. (2000) and Falconi (2012) showed a higher percentage of protein (mean values 40-45 %).

Our phytopathological analyses showed a higher number of fungal isolates obtained from both lupine forms in 2011(total 2784 – 57% isolates per year) – Table 6. The above could be attributed to higher precipitation in July of 2011 than in July of 2012, significantly exceeding the multiannual average (Table 3). The number of seeds sown per m^2 had no significant influence on the severity of fungal seeds colonisation (60 seed per m^2 – 1641; 90 seed per m^2 – 1629; 120 seed per m^2 – 1590; per both year of study).

In both years of the study, saprotrophic fungi of the genera *Alternaria*, *Cladosporium* and *Rhizopus* were the predominant pathogens colonising both forms of Andean lupine seeds (Table 6). Fungi of the genera *Penicillium, Aspergillus, Fusarium, Ulocladium, Chaetomium* and *Arthrinium* were also isolated from white lupine seeds by Alomran et al. (2013). Nedzinskiene and

Table 4

Content (g kg^{-1} d.m.) of macronutrients in Andean lupine seeds (average of years 2011-2012)

Morphotype	Sowing date	Number of seeds per 1 m^2	Macronutrients				
			N	P	K	Ca	Mg
Indeterminate form	I	60	61.00	5.401	7.409	1.944	3.005
		90	58.43	5.540	7.671	1.911	2.822
		120	60.31	5.613	7.918	1.910	2.713
	II	60	59.82	5.889	8.206	2.012	2.901
		90	58.76	5.902	8.123	1.934	2.924
		120	59.51	5.731	8.001	2.002	2.845
Determinate form	I	60	57.79	5.512	7.223	2.112	3.000
		90	55.81	5.721	7.412	2.089	2.813
		120	56.38	5.721	7.190	1.991	2.890
	II	60	57.85	5.779	7.641	2.113	2.671
		90	58.90	5.923	6.888	2.000	2.813
		120	58.03	5.602	7.521	2.201	2.596
LSD (α = 0.05)			n.s.*	n.s.	n.s.	n.s.	n.s.
Average of years							
Years	2011		58.803	5.811	7.014	2.423	2.221
	2012		58.511	5.500	8.215	1.614	3.534
LSD (α = 0.05)			n.s.	n.s.	0.611	0.102	0.323

* n.s. – non significant difference

Asakaviciute (2011) isolated fungi of the genera *Alternaria, Fusarium* and *Penicillium* from the seeds of blue lupine and yellow lupine. Pathogenic fungi made up 11.7% of isolates from Andean lupine seeds in the two years of our experiment. The major pathogenic genus from lupine seeds was *Botrytis cinerea*, which constituted 5% of the fungal cultures isolated from lupine seeds. In both years, *Colletotrichum* spp. was more frequently identified in the seeds of the Andean lupine determinate form (93 isolates) and harvested from treatments with the delayed sowing date (75 isolates). In the treatments with the early sowing date, a small number of *Colletotrichum* infected lupine seeds was observed (18 isolates). The above can probably be attributed to the weather conditions, namely flowering was delayed by several days and it occurred in a period of higher humidity, which stimulated the development of the pathogen (Tables 3 and 6). *Colletotrichum* is widespread around the world, but its prevalence is the highest in tropical and subtropical regions, characterised by frequent rainfall, high humidity and high temperatures, which contribute to the proliferation of the pathogen (Thomas, Sweetingham 2004).

Table 5

Content (g kg^{-1} d.m.) of N and of total protein (%) in Andean lupine seeds (average of years 2011-2012)

Morphotype	Sowing data	Number of seeds per 1 m^2	N (g kg^{-1} d.m)	Total protein (%)
Indeterminate form	I	60	61.00	38.13
		90	58.43	36.52
		120	60.31	37.69
	II	60	59.82	37.39
		90	58.79	36.74
		120	59.51	37.19
Determinate form	I	60	57.79	36.12
		90	55.81	34.88
		120	56.38	35.24
	II	60	57.83	36.14
		90	58.90	36.81
		120	58.03	36.27
LSD (α = 0.05)			n.s.*	n.s.
Average of years				
Years	2011		58.80	37.28
	2012		58.51	35.91
LSD (α = 0.05)			n.s.	n.s.

* n.s. – non significant difference

PCR analyses of *Colletotrichum* spp. strains performed with the use of primers specific for *Colletotrichum acutatum* and *Colletotrichum gloesporioides* revealed the presence of *C. acutatum,* as indicated by the PCR product of 490 bp (data not shown). Our results corroborate the findings of Kulik et al. (2005), who isolated *C. acutatum* from the seeds of yellow lupine and Andean lupine. Anthracnose, a pathogenic infection caused by the *C. acutatum* species complex, is one of the most destructive diseases of all species of lupins (Talhinhas et al. 2002, Damm et al. 2012, Falconi et al. 2013).

Numerous studies have demonstrated morphological and phylogenetic variations in *C. acutatum* (Nirenberg et al. 2002, Talhinhas et al. 2005, Shivas, Tan 2009, Damm et al. 2012). In the work of Sreenivasaprasad et al. (1996), the level of intraspecific divergence in the ITS1 region was determined at 5.8% in *C. acutatum*, and it was higher than in other fungal species, where it was noted in the range of 2-4%. Based on the above results, the authors suggested that *C. acutatum* should be divided into two species.

The isolates in this study were amplified by PCR with ITS1/ITS4 primers. The PCR products were sequenced and were characterised by an

Table 6

Number of fungal isolates determined by traditional methods in *Lupinus mutabilis* seeds harvested in 2011 and 2012

Fungal genus/species	Indeterminate form												Determinate form											
	early sowing date						delayed sowing date						early sowing date						delayed sowing date					
	2011			2012			2011			2012			2011			2012			2011			2012		
	60	90	120	60	90	120	60	90	120	60	90	120	60	90	120	60	90	120	60	90	120	60	90	120
Acremonium strictium W.Gams														1					1					
Alternaria alternata (Fr.) Keissler	154	246	234	114	274	183	257	211	269	143	80	131	131	154	94	94	94	91	69	111	109	77	89	86
Ascochyta lupinicola Petra.															6					6				
Aspergillus spp.															3									
Arthrinium sphaerospermum Fuckel																	1					1		
Botrytis cinerea Pers. Ex Pers.	109	34		17	5			11	17		5	17			11				23		11			
Cladosporium cladosporioides (Fries.) de Vries	17			46	46	5			17		17		3		9	17	3	6		6	3	0	20	
Colletotrichum spp.	17									11					6	3	6	3	9	9	14	20		23
Epicoccum nigrum Link Schol- Schwarz				23		17		11	23				6	14		6		3	3	9	6	6	3	
Fusarium avenaceum (Corda ex Fr.) Sacc.							11		11					3										
Fusarium culmorum (W.G. Smith) Sacc.							17																	
Fusarium poae (Peck) Wollenw.				6	6	4					11	11					11						3	
Fusarium sambucinum Fuckel.								5																
Fusarium sporotrichioides Sherb.		17					4			4			3			3								
Fusarium tricinctum (Corda)				6																		3		
Fusarium spp.		11				2	2		5		4		3						6					
Mortierella alpina Peyrond			1																	1				
Mucor spp.				6					11				3	3	9									
Mycospherella pinodes (Berk and Blox).																					3			
Nigrospora spherica (Sacc.)											5													
Penicillium spp.	5	7	40				46							3	3			1		20	20			
Phoma sp.																			1					
Rhizopus spp.					17	34				80	29	23				3	3		9	9				
Trichoderma koningi Oudemans	4		23				17			23				1										
Non sporulating fungi		1																						

1-CGGCAAGAGTCCCTCCGGATCCCAGTGCGAGACGTTAGTTACTACGCAAAGGAGGCTCCGGGAGGGTCCG

71-CCACTACCTTCAAGGGCCCACGTGTGCCGTGGGGCCCCAAAACCAAGCGGTGCTTGAGGGTTGAAATGAC

141-GCTCGAACAGGCATGCTCGCCAGAATGCTGGCGAGCGCAATGTGCGTTCAAAGATTCGATGATTCACTGA

211-ATTCTGCAATTCACATTACTTATCGCATTTCGCTGCGTTCTTCATCGATGCCAGAACCAAGAGATCCGTT

281-GTTAAAAGTTTTAATTATTTGCTTGTGCCACTCAGAAGAGACGTCGTGTAAATAGAGTTTGGTTTCCTCC

351-GGCGGGCGCCCCGTCCCCGTGGTGGGGGCCGGCGCCGGGAGGGGAGGCCCGCGAGAGGCTTCCCCTGCCC

421-GCCGAA

Fig. 1. Partial sequence of the 5´-end of ITS1 - 5.8S – ITS4 rDNA of 19 *C. lupini* isolates from Andean lupine seeds (100% homology)

identical DNA sequence (100% homology) with a length of 426 nucleotides in the analysed fragment of the rDNA region (Figure 1).

The sequence was compared to *Colletotrichum* spp. sequences in the GenBank with the use of the NCBI BLAST program (http://blast.ncbi.nlm.nih.gov/). The analysis revealed 100% homology with 30 isolates deposited in the NCBI database: *C. lupini* (19 isolates), *C. acutata* (10 isolates) and *Colletotrichum* spp. (1 isolate). The above results corroborate the findings of Rosskopf et al. (2014), who demonstrated that *C. lupini* was the causal agent of anthracnose in *L. hartwegii* and *L. mutabilis* in Florida. The above species was identified by comparison to GenBank sequences (with the use of ITS4 and ITS5 primers), and it demonstrated 99% homology with *C. lupini* var. *setosum,* strain BBA 71310, isolated from *L. luteus* in Poland, accession number AJ301968. The isolate analysed in our study was compared to GenBank sequences of the ITS region in the BLAST program, and it demonstrated 100% homology with the Florida isolate, accession number KF207599.

Damm et al. (2012) demonstrated that *C. lupini* was a phylogenetically distinct species of the *C. acutatum* complex. *C. lupini* emerged as a separate species based on an analysis of nearly all genes (ITS, CHS-1, GAPDH, TUB2 and HIS3) except ACT, and the TUB2 gene was the greatest source of diversity. In the cited study, phylogenetic analysis supported the detection of 5 main clades and 29 subclades in *C. acutatum sensu lato*, which were considered representative of the differences within the genus *Colletotrichum* (Damm et al. 2012).

The pathogen causing lupine anthracnose isolated from the seeds of Andean lupine in the present study was identified as *Colletotrichum lupini* in a molecular analysis, and its DNA sequence was compared with those of the isolates deposited in the GenBank.

The results of our study demonstrate that the soil and climate of the region Lower Silesia (Poland) in 2011-2012 were more supportive for the growth, development and yield of the indeterminate than the determinate form of Andean lupine.

The research showed that the main pathogenic species inhabiting the seeds of *Lupinus mutabilis* are *B. cinerea, C. Lupini* and species of the genus *Fusarium*. The presence of the pathogens identified in the seeds of plants indicates an infection during the growing season. Moreover, affected seeds can be a source of inoculum in the next growing season.

More precise identification of fungal species of the genus *Colletotrichum*, the major cause of anthracnose in lupine, in particular Andean lupine, requires further work. The results of numerous studies indicate that multiple genes should be used in phylogenetic analyses of fungal species (Shivas, Tan 2009, Damm et al. 2012).

The courrent data provide preliminary information on yield and health of Andean lupine seeds grown in a field experiment. However, further research is needed to achieve some improvement of the existing forms of Andean lupine and their adaptation to soil and weather conditions in Poland.

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