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Mycobiota of *Lupinus albus* seeds from a public germplasm collection

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Abstract: Seedborne mycobiota of *Lupinus albus* was assessed using blotter paper and agar media with Rose Bengal or semi-selective media for *Pythium* or *Fusarium*. Samples of 200 seeds were taken from each of 16 inventories, comprising 14 accessions originating from Germany, France, Ukraine, Syria, Hungary or Spain, and increased 1-2 times through propagation by the USDA-ARS Western Regional Plant Introduction Station. A total of 307 fungus isolates were recovered and preserved with over 96% belonging to 10 genera: *Penicillium* (47%), *Cladosporium* (32%, including *Cladosporium*-like *Chalastospora*), *Alternaria* (4%), *Aspergillus* (4%), *Fusarium* (3%), *Rhizopus* (3%), *Ulocladium* (3%), *Chaetomium* (0.5%) and *Arthrinium* (< 0.5%). By sub-sampling, fungi were further identified by morpho-cultural characters and/or DNA sequences as *Alternaria infectoria*, *A. alternata*, and *A. tenuissima* species-groups, *Arthrinium arundinus*, *Aspergillus niger*, *Chalastospora gossypii*, *Cladosporium herbarum* and *C. cladosporioides* species complexes, *Chaetomium* sp., *Fusarium*

acuminatum, *Rhizopus stolonifer*, and *Ulocladium atrum* group. Penicillia included *P. hordei*, but most penicillia could not be assigned species names either by morphological traits or β -tubulin DNA sequence data.

Key words: germplasm collection, lupin, lupine, *Lupinus*, mycobiota, mycoflora, plant pathogen, seed borne, seed health

Introduction:

Lupine germplasm in the National Plant

Germplasm System Lupines have long been important as food and forage, and for the ornamental plant trade (Gladstones 1974; Gladstones et al. 1998). The USDA-ARS National Plant Germplasm System's regional unit, Western Regional Plant Introduction Station (WRPIS) in Pullman, Washington, collects, maintains, regenerates and distributes seed of multiple species of lupine, including *L. albus* L. Seed from WRPIS is distributed nationally and internationally, hence the importance of assessing seed health for representative germplasm.

Lupine seeds deposited into the WRPIS seed bank are produced under standard procedures. Seeds are scarified and treated with Captan fungicide solution, placed on Captan-soaked germination paper and placed in a water-curtain germinator at 20°C for 4 days. Germinated seeds are planted in transplant flats, grown in the greenhouse for 14-21 days (20-25 °C), grown in the shadehouse for 14-21 days (ambient outside air temperature), then planted in the field in mid-April to early May. At flowering, the plants are covered with mesh screen to keep out pollinating insects (to avoid cross contamination with other accessions). Flowering starts in early June; harvest is usually in August. The growing crop is inspected for symptoms of disease, and seeds are visually inspected for disease, weed seeds, and arthropods during harvest, cleaning, packaging, and during germination tests. Lupine accessions also have been tested for potyvirus (Robertson and Coyne 2009).

In spite of these measures, the potential presence in lupine seed of plant pathogenic fungi (Elmer 2002; Elmer et al. 2001; Godrane et al. 1994), of a fungus capable of causing lupinosis in livestock, *Diaporthe toxica* P.M. Will., Highet, W. Gams & Sivasith (Hill 1998), or other legume-associated mycotoxigenic fungi (Visconti et al. 1989), justifies assessment of the mycobiota of lupine seed accessions stored and distributed by WRPIS. Although there are over 100 records of fungi on *Lupinus* species in Washington State, few are from seed and no records are from *Lupinus albus* (Farr and Rossman n.d.). Accordingly, we resolved to document the most prevalent fungi associated with *Lupinus albus* seed in selected WRPIS accessions increased and maintained in eastern Washington State, and to draw appropriate inferences regarding seed health of WRPIS accessions. A secondary objective was to compare our results with similar studies on lupine and other legumes.

Previous studies of the mycobiota of lupine

Published studies of the mycobiota of lupine have focused on *Lupinus termis* Forsk. [= *L. albus* subsp. *termis* (Forsk.) Ponert.], a form with white-pink flowers that is grown in Egypt, Libya, Sudan, Ethiopia, Israel, Palestine and Syria. The taxon is putatively distinct from other subspecies of *L. albus*, including subsp. *graecus* (Boiss. & Spun.) Franko & Silva, and subsp. *albus* L., including var. *albus* (Kurlovitch and Stankevitch 2002). However, Farr and Rossman (n.d.) list *L. termis* as synonymous with *L. albus* var. *albus*, (although records for *L. termis* are displayed separately in that database) as does the National Plant Germplasm System site, GRIN (www.ars-grin.gov/npgs/).

Abdel-Hafez (1984), in Saudi Arabia, documented 15 genera and 36 species of fungi from seed of *L. termis*. Prominent, in descending order, were species in *Aspergillus* (especially *A. flavus* Link and *A. niger* Tiegh.), *Rhizopus stolonifer* (Ehrenb.) Vuill., and miscellaneous *Penicillium* spp., but also species in *Mucor*, *Fusarium*, *Chaetomium*, *Alternaria*, *Cladosporium* and others. Embaby and Abdel-Galil (2006), in Egypt, also looked at the mycobiota of *L. termis* seed; fungi included *Aspergillus niger* (by far the most numerous fungus), *A. flavus*, *Fusarium* spp. (mostly *F. oxysporum* Schltdl.), *Alternaria* spp., and *Trichoderma* spp. In a similar study from Sudan, El-Nagerabi and Elshafie (2000) reported 57 species in 14 genera of fungi from *L. termis* seeds. In descending abundance were *Rhizopus stolonifer*, *Aspergillus* (especially *A. flavus* and *A. niger*), *Fusarium* spp., *Alternaria* (most prominent were *A. alternata* (Fr.) Keissl. and *A. tenuissima* (Kunze) Wiltshire), *Chaetomium* (*C. spirale* Zopf dominant), *Curvularia* spp., *Cladosporium* (*C. oxysporum* Berk. & M.A. Curtis dominant), *Drechslera* (*D. spicifera* (Bainier) Arx dominant), plus lesser numbers of *Penicillium*, *Phoma*, *Mucor*, *Ulocladium* and other taxa.

Materials and Methods:

Plant germplasm Fourteen accessions of *Lupinus albus*, originally from different countries around the world and increased in different years in Washington State, were obtained from WRPIIS and comprised 16 collections ("inventories") (Table 1). From each of the inventories, samples of 200 seeds were randomly selected. Seeds were then examined under a dissecting microscope at 50X to classify each seed as symptomatic (discolored or blemished) or asymptomatic. For each inventory, symptomatic (< 8% of total) and asymptomatic seed were each divided into four groups for transfer to three media and blotter paper.

Plating of seeds on agar and blotter paper

Seeds were surface disinfested in 0.5% NaOCl for one min per group followed by a sterile distilled H₂O rinse, then placed on blotter paper and agar media at no more than 5 seeds per plate for recovery of fungi. The agar media were Pythium-selective medium (Mircetich and Kraft 1973), Fusarium-selective medium (MGA 2.5) (Castellá et al. 1997), and 1/2V8 Rose Bengal medium (Dugan and Lupien 2002, with slight modification). Autoclaved blotter papers were placed in plastic deep dishes (100 x 25 mm) with 7 ml sterile water; after 2 weeks, an additional 7 ml sterile water was added to prevent drying.

Recovery and preservation of isolates

Fungi were subcultured by transfer to 1/2V8 agar, with streptomycin + tetracycline for suppression of bacteria (Dugan and Lupien 2002). Cultures were stored in glycerol at -80°C or in liquid nitrogen (Smith and Onions 1994).

Identification to genus and species

Isolates were assigned to genus by colony appearance at 10-50X (Olympus SZ-PT stereomicroscope) and higher magnifications (Olympus BH-2 compound microscope). Further identification to species was preceded by sub-sampling on the basis of colony appearance, by examination of cultural and morphological characters, and on the basis of DNA sequences as described below.

Morphological identification

Alternaria: Morphological and cultural characters of single-colony isolates (each putatively derived from single conidium on a streak plate) were documented for isolates inoculated onto Potato Carrot, Hay Infusion, and V8 juice agars by procedures, keys and descriptions in Simmons (1992) and literature therein. *Alternaria alternata* and *A. tenuissima* species-groups were further distinguished from the *Alternaria infectoria* E.G. Simmons species-group by differences in color and texture on Dichloran Rose Bengal Yeast Extract Sucrose

Table 1. WRPIS seed accessions of *L. albus* used in this study.

Accession number	Species	Year of increase at WRPIS (inventory)	Country of Origin
PI 287241	<i>L.albus</i>	2000	Germany
PI 287241	<i>L.albus</i>	2007	Germany
PI 457933	<i>L.albus</i>	2007	France
PI 457935	<i>L.albus</i>	2007	France
PI 476372	<i>L.albus</i>	2001	Ukraine
PI 481558	<i>L.albus</i>	2007	Syria
PI 483072	<i>L.albus</i>	2007	Syria
PI 502652	<i>L.albus</i>	2007	Hungary
PI 505845	<i>L.albus</i>	2007	Ukraine
PI 516625	<i>L.albus</i>	2001	Germany
PI 516625	<i>L.albus</i>	Year not available	Germany
PI 533701	<i>L.albus</i>	1995	Spain
PI 533701	<i>L.albus</i>	2007	Spain
PI 533702	<i>L.albus</i>	1995	Spain
PI 533703	<i>L.albus</i>	2007	Spain
PI 533706	<i>L.albus</i>	1995	Spain

agar (DRYES, Frisvad 1983) per procedures in Andersen and Thrane (1996).

Arthriniium: A single isolate was identified by keys and descriptions in Ellis (1971).

Aspergillus: Single-colony isolates were inoculated onto Czapek Yeast Extract (CYA), Malt Extract (MEA), Czapek Yeast Extract with 20% sucrose, and Czapek agars, incubated, and characters recorded per procedures, keys, and descriptions in Klich (2002). Twenty-five ml of media were used in 100 mm Petri dishes to allow adequate space for the *Aspergillus* heads to develop (Okuda et al. 2000). Conidia were added to 2-3 ml of semi-liquid 0.2 % water agar in small

glass, screw-capped vials for inoculating plates without establishing extraneous colonies (Pitt and Hocking 1997). For observation of microscopic characters, fungal tissues from colonies on MEA were mounted in 60% lactic acid and a drop of 70% alcohol was added to remove air bubbles and excess conidia (Varga et al. 2011).

Cladosporium: Single-colony isolates were inoculated onto 2% Potato Dextrose Agar (PDA), and Synthetic Nutrient-Poor agar, then incubated and examined per procedures, keys and descriptions in Ho et al. (1999), Bensch et al. (2010), and Schubert et al. (2007).

***Fusarium*:** Morphological identification was conducted using full strength PDA for colony characters, and Carnation Leaf Agar was used for microscopic characters per procedures, keys and descriptions in Nelson et al. (1983), and Leslie and Summerell (2006). The interactive program FUSKEY (Seifert 2000) was also used for identification to species.

***Penicillium*:** Single-colony isolates were inoculated to CYA, Neutral Creatine Sucrose Agar, MEA, and 25% Glycerol Nitrate Agar, then incubated and examined per procedures, keys and descriptions in Pitt (1979, 2000) and Frisvad and Samson (2004), with conidia inoculated to media in semi-liquid water agar as described above.

***Rhizopus*:** Single-colony isolates were inoculated to MEA for analysis of morphology and temperature-dependent growth per procedures, keys and descriptions in Liou et al. (2007), Schipper and Stalpers (1984) and Schipper (1984).

***Ulocladium*:** Single-colony isolates were grown on 1/2 V8 agar and compared to illustrations and descriptions in Simmons (1998).

Identification by DNA sequence analyses

Single-colony isolates were grown in 1/2 strength Potato Dextrose Broth (Difco) for 5-9 days at 20-25°C on a shaker at 125 rpm. Mycelium and/or conidia were harvested by centrifugation and washed three times with sterile water. The washed pellet was frozen at -80 °C and then lyophilized.

DNA extraction Approximately 20 mg of lyophilized tissue was frozen in liquid nitrogen and disrupted using 3 mm glass beads in a FastPrep® homogenizer (MP Biochemicals, Solon, OH) for 20 seconds at speed 6. DNA was extracted using the standard Qiagen DNeasy®

Plant mini kit protocol per manufacturer instructions (Qiagen, Valencia, CA). DNA was eluted in 100 µl sterile water.

β-tubulin for *Penicillium* and *Aspergillus*

Amplification of a portion of the β-tubulin gene was achieved using the forward primer Bt2a (5′-GGT AAC CAA ATC GGT GCT GCT TTC-3′) and the reverse primer Bt2b (5′-ACC CTC AGT GTA GTG ACC CTT GGC-3′) (Glass and Donaldson 1995). Additional primers, Bt2c in the reverse direction and Bt2d in the forward direction (design by Shari Lupien, synthesis by MEG Operon), were used for sequencing of the Bt2a/Bt2b amplicons. PCR reactions were conducted in 50 µL reaction mixtures containing 10 µL of GoTaq 5x buffer, 1µL of 10 mM dNTP mix, 5 µL (50 pmol) of each primer, 0.5 µL of Go Taq® polymerase (Promega, Madison, WI), and 1-5 µL template. Amplifications were performed in a Veriti® 96-Well Thermal Cycler with an initial denaturation at 94 °C for 90 seconds followed by 5 cycles of denaturation at 94 °C for 90 seconds, step down annealing 1° cycle (68 °C- 63 °C), and extension at 72 °C for 2 min followed by 26 cycles of 94 °C for 1 min, 64 °C for 90 seconds, and 72 °C for 2 min, plus a final elongation step of 10 min at 72 °C.

Internal Transcribed Spacer Region (ITS) for *Chaetomium*

PCR products were sequenced in both directions. Amplification of the ITS region was achieved with primers, ITS4 as reverse primer (5′-TCCTCCGCTTATTGATATGC-3′) and ITS5 as forward primer (5′-GCAAGTAAAAGTCGTAACAAGG-3′). In addition, the internal reverse primer ITS2 was used for obtaining sequence in both directions (White et al. 1990). PCR reactions were conducted in 50 µL reaction mixtures containing 10 µL of GoTaq 5x buffer, 1µL of 10 mM dNTP mix, 5 µL (50 pmol) of each primer, 0.5 µL of Go Taq polymerase, and 1-5 µL template. Amplifications were performed in a Veriti® 96-Well Thermal Cycler. PCR conditions consisted of

an initial denaturation at 95 °C for 1 min followed by 35 cycles of 95°C for 30 sec, 52°C for 1 min, and 72°C for 2 min plus a final extension at 72°C for 10 min.

Translation elongation factor for

Fusarium Translation elongation factor 1 α gene, (TEF-1 α), was amplified with primers, EF-1 as forward primer (5'-AT GGG TAA GGAR (A or G) GAC AAGAC-3'), and EF-2 as reverse (5'-GGAR (G or A) GTACCAGTS(C or G) AT CAT GTT-3') (O'Donnell et al. 2012). The internal primers EF11 as forward and EF22 as reverse were used with the PCR primers to obtain sequences in both directions (O'Donnell and Cigelnik 1997). PCR reactions were conducted in 50 μ L reaction mixtures containing 10 μ L of GoTaq 5x Buffer, 1 μ L of 10 mM dNTP mix, 5 μ L (50 pmol) of each primer, 0.5 μ L of Go Taq polymerase, and 1-5 μ L template. Amplifications were performed in a Veriti® 96-cell thermal cycler, programmed with an initial denaturation at 95°C for 2 min followed by 35 cycles of 95°C for 30 seconds, 57°C for 30 sec, 72°C for 1 min plus an additional extension of 10 min at 72°C (Dita et al. 2010).

PCR amplicons, primer synthesis, and analyses of fungal sequences using BLAST search

PCR amplicons were prepared for sequencing using QIAquick PCR purification Kit protocol (Qiagen, Valencia, CA) per manufacturer's instructions. Sequencing reactions and primer synthesis were performed by Eurofins MWG/Operon (Huntsville, AL). Sequence data was assembled and aligned using Sequencher™ 5.0 software (Gene Codes Corp. Ann Arbor, MI). Sequence analysis and assembled data were analyzed by conducting BLASTsearches (Zhang et al. 2000).

Results: Consensus results for identifications based on both morphology and DNA sequence criteria are shown in Table 2.

Morphological identification The percentage of each genus or group from the total number of fungi was *Penicillium* (47%), *Cladosporium* (32%, including the *Cladosporium*-like fungus *Chalastospora gossypii* (Jacz.) U. Braun & Crous), *Aspergillus* (4%), *Alternaria* (4%), *Rhizopus* (3%), *Fusarium* (3%), *Ulocladium* (3%), *Chaetomium* sp. (0.5%), *Arthrinium* (<0.5%) and 3.5% nonviable or unidentified. Percentage of viable and identified fungi out of each medium: 16% from the *Pythium* medium, 36% from MGA 2.5, 30% from the Rose Bengal medium, and 16% from the blotter paper. Percentage of fungi recovered from symptomatic seed was ~7% of the total, proportional to percentage of symptomatic seed (< 8%); of seeds from which fungi were recovered, 3.2% of seeds had two fungi and 96.7% of seeds had only one fungus.

***Alternaria*:** Two representative isolates from PI 287241-2000 (one from symptomatic, the other from asymptomatic seed), and two isolates from PI 476372-2001 (both from symptomatic seed) were assigned to *A. alternata*; all were rusty olive and velutinous on DRYES, and with chain branching typical of the *A. alternata* species-group on the diagnostic media. A determination of *A. tenuissima* was made for two isolates from PI 287241-2000 (both from asymptomatic seed) that were rusty-olive and velutinous on DRYES, and exhibited long unbranched chains typical of the *A. tenuissima* species-group on the diagnostic media. One isolate (from asymptomatic seed) from PI 457935-2007, was unambiguously in the *A. infectoria* species-group by DRYES reaction (white, floccose). An isolate from PI 533702-1995 (from asymptomatic seed) gave a white-floccose reaction on DRYES indicating *A. infectoria*, but exhibited long, unbranched chains characteristic of *A. tenuissima* on V8 medium.

***Arthrinium*:** The single isolate keyed to *Arthrinium arundinis* (Corda) Dyko & B. Sutton, a common, cosmopolitan fungus.

Table 2. Fungi isolated from seed of white lupine accessions¹

Genus or group and abundance ²	Within genus/group subgeneric taxa in descending order of abundance ³
<i>Alternaria</i> (4%)	<i>A. alternata</i> <i>A. tenuissima</i> <i>A. infectoria</i>
<i>Aspergillus</i> (4%)	<i>A. niger</i>
<i>Chaetomium</i> (0.5%)	<i>Chaetomium</i> sp.
Cladosporium-like (32%)	<i>Cladosporium cladosporioides</i> <i>Chalastospora gossypii</i> <i>Cladosporium herbarum</i>
<i>Fusarium</i> (3%)	<i>Fusarium acuminatum</i> <i>Fusarium</i> spp.
<i>Penicillium</i> (47%)	Cf. unnamed <i>Penicillium</i> sp. (JN794530) <i>Penicillium</i> spp. <i>P. hordei</i>
<i>Rhizopus</i> (3%)	<i>R. stolonifer</i>
<i>Ulocladium</i> (3%)	<i>U. atrum</i>
<i>Arthrrium</i> (< 0.5%)	<i>A. arundinis</i>
Non-viable/unidentified (3.5%)	

¹307 total isolates from 14 accessions regenerated in 1-2 years each (Table 1).

²Cladosporium-like includes *Cladosporium sensu stricto* plus *Chalastospora gossypii* (= *Cladosporium malorum*).

³Determined on the basis of subsampling. In the instances of *Alternata* and *Ulocladium*, taxa are species groups *sensu* Simmons (1992, 1998); in the instance of *Cladosporium sensu stricto*, taxa are species complexes *sensu* Bensch et al. (2010) and Schubert et al. (2007).

Aspergillus: All representative *Aspergillus* isolates were *Aspergillus niger*: Two from PI 476372-2001, five from PI 481558-2007, one from PI 502652-2007, and four from PI 516625-1995, all from non-symptomatic seed.
Cladosporium: Determinations of *Cladosporium cladosporioides* (Fresen.) G.A. de Vries were recorded for single representative isolates from PI 457933-2007, PI 457935-2007 (both from asymptomatic seed), two isolates from PI 476372-2001 (one from symptomatic, the other from asymptomatic seed), and single isolates from PI 533701-1995, PI 533702-1995, PI

533703-2007, and PI 533706-1995 (all from asymptomatic seed). *Cladosporium herbarum* (Pers.) Link was the determination for single representative isolates from PI 287241-2000 and PI 287241-2007, two isolates from PI 457935-2007, and single isolates from PI 457935-2007, PI 533702-1995, and PI 533706-1995 (all from asymptomatic seed). The Cladosporium-like fungus, *Chalastospora gossypii*, long known as *Cladosporium malorum* Ruehle, was recorded from PI 457933-2007, PI 457935-2007 (two isolates), PI 481558-2007, PI 533703-2007 (three isolates), and PI 533706-1995 (all from

asymptomatic seed). Determinations of *Chalastospora gossypii* can be regarded as to species *sensu stricto*, but for *C. cladosporioides* and *C. herbarum* determinations on the basis of morphology are conservatively best regarded as for species complexes (Bensch et al. 2010; Schubert et al. 2007).

Fusarium: Results from FUSKEY resulted in determinations of *Fusarium compactum* (Wollenw.) Raillo / *F. equiseti* (Corda) Sacc. (the two highest matches) for representative isolates from PI 287241-2000 (one isolate) and PI533703-2007 (five isolates), or *F. compactum* / *F. acuminatum* Ellis & Everh. (the two highest matches) for PI533703-2007 (three isolates). The isolate from PI 287241-2000 was from asymptomatic seed. Of the isolates from PI533703-2007, half were from symptomatic, the other half from asymptomatic seed.

Penicillium: Isolates could not be identified on the basis of morphology as they did not key in Pitt (2000) or Pitt (1979). Within most individual isolates, biverticillate penicilli predominated, but terverticillate penicilli were quite readily located. With an exception (see section on identification by DNA sequence) species identification was not determined.

Rhizopus: Determinations of *Rhizopus stolonifer* were made for four representative isolates, from PI 516625-1995, one isolate from PI 533701-1995, five isolates from PI 533701-2007 (all from asymptomatic seed, except for the one from PI 533701-1995).

Ulocladium: All *Ulocladium* isolates displayed the cruciate conidia characteristic of the *U. atrum* Preuss group (Simmons 1998). Representative isolates from PI 287241-2000, PI 457935-2007 and PI 533701-1995 (two isolates each), were all from asymptomatic seed.

Identification by DNA sequence analyses

Aspergillus: Representative isolate PI 476372-2001# 41 had a Bt-2 sequence of approximately 500bp. This β -tubulin sequence (GenBank KC175288) produced nucleotide-blast hits of 100% identity with the type strain of *A. niger*, CBS 554.65 (Genbank GU296700) and with 99% query coverage (Varga et al. 2011). The substrate for the type was given as 'tannin-gallic acid fermentation' with an origin from Connecticut, USA (Varga et al. 2011). Matches of 100% were also obtained to *A. niger* strains IHEM 6350 (Genbank HQ632687) and CICC 2487 (Genbank HQ202722).

Chaetomium: Isolates PI 476372-2001#34 and PI 476372-2001#35 had ITS sequences (GenBank KC175289 and KC175290, respectively) of approximately 600 nucleotides. These two strains had 100% identity to each other and to *Chaetomium subaffine* Sergeeva C24 (Genbank Hm365247) with 100% identity and 91% query coverage. The substratum of C24 was a petiole of *Cucumis sativus* L., and the origin was Hashtgerd, Iran (Asgari and Zare 2011). The identification of C24 was on the basis of not only ITS-LSU, but also β -tubulin, so our isolates are certainly *Chaetomium*, but species is best regarded as not determined.

Fusarium: Isolates PI533703-2007#100 and PI 287241-2000#171 had TEF- α sequences (GenBank KC175292 and KC175291, respectively) of approximately 700 nucleotides. These sequences were identical to each other by 99% and 99% query coverage. These strains had 100% identity and 84% query coverage to *F. acuminatum* CBS 131258 (Genbank JQ429337), with a substratum of *Lolium* sp., and origin in Iran (Davari et al. 2012).

Penicillium: Isolate PI 533702-1995 #40 produced a β -tubulin (Bt2a.2b) sequence of approximately 500 nucleotides. The sequence (GenBank KC175293) had 99% identity to *Penicillium hordei* Stolk strain CBS 704.68 (Genbank AY674346), whose substrate was 'grain' with an origin in the Netherlands, and CBS 788.70 (Genbank AY674345) whose substrate and origin are not cataloged, and 98% identity to the type of *P. hordei*, CBS 701.68 (Genbank AY674347), whose substrate was grain of *Hordeum vulgare* L., with origin in Denmark (Samson et al. 2004b). *Penicillium* isolate PI 457933-2007#41 (GenBank KC175294) had 99% similarity to Genbank JN794530, corresponding to unnamed isolate mms351 (no published reference). BLAST results returned e-values of 0.0 for all the above comparisons.

Discussion: Overall results are positive regarding health of WRPIS accessions of *Lupinus albus* seed because none of the major pathogens of lupine seed were detected by conventional methods. However, fungi potentially able to cause damping off or minor losses were recorded, as were fungi belonging to taxa known to produce mycotoxins.

Alternaria species differ in mycotoxin production. The *A. infectoria* species-group is less toxigenic than *A. alternata* and *A. tenuissima* groups (Andersen and Thrane 1996). We detected all three groups. Some, but not all, isolates in *A. niger* produce mycotoxins (Samson et al. 2004a). All our representative *Aspergillus* isolates were in the *A. niger* group by morphological criteria and, based on 100% sequence similarity of some isolates with the type of *A. niger*, are seemingly that species. *Fusarium acuminatum* also is known to produce mycotoxins (Visconti et al. 1989), and the fungus is previously documented from our region and is pathogenic on lentil (Kaiser 1992). The presence of this fungus in lupine seed may represent a problem should it become present in greater abundance.

Penicillium species can cause significant economic damage to stored food, including grain, and several species produce mycotoxins (Pitt and Hocking 1997). Most of the isolates in this study had largely biverticillate penicilli, but with terverticillate penicilli readily located, rendering assignment to subgenus ambiguous by morphology. Pitt (2000) noted that only 70 to 80% of isolates, even from common sources, are readily identifiable by morphology. Based on our representative isolate, the closest available match in GenBank was to an unnamed isolate; close matches of β -tubulin DNA sequences to sequences bearing names were not obtained. Our results on *Penicillium* are more intriguing than conclusive, but there is a possibility of an undescribed species.

Also isolated repeatedly was the Cladosporium-like fungus, *Chalastospora gossypii* (= *Alternaria malorum* = *Cladosporium malorum*), actually more related to *Alternaria* than to *Cladosporium* (Braun et al. 2003). This fungus, sometimes considered rare (Crous et al. 2009), is world-wide and not rare in Washington State. It is a minor pathogen of several plants, and once was recorded as the dominant fungus (contaminant) on Washington wheat grain (Goetz and Dugan 2006). *Cladosporium sensu stricto* is now more readily recognized via modern criteria (Crous et al. 2007), but identification to species is more problematic. The genus *Cladosporium* includes more than 770 names (Dugan et al. 2004). Isolates in this study clearly belonged to the *C. herbarum* or *C. cladosporioides* species complexes, each of which consists of numerous species, with especially the latter complex having distinct, yet unnamed clades (Bensch et al. 2010).

A few isolates originally displayed dark setae and somewhat elongate hyaline conidia in culture, and so assignment to *Colletotrichum*, well documented as a serious seedborne pathogen in lupine (Elmer et al. 2001), was considered. The setae eventually proved to be those on aborted

perithecia. Eventually mature perithecia were obtained in small numbers, but most failed to mature. Some species in *Chaetomium* have Acremonium-like anamorphs with hyaline conidia. The identification as *Chaetomium* by ITS sequence is considered correct.

Other common seedborne fungi isolated by us included *Rhizopus* and *Ulocladium*. Of these, precise identification of the latter is the more challenging. Many morpho-characters overlap between species of *Ulocladium*, causing difficulty in identifying isolates to species (De Hoog and Horré 2002). Recent molecular analyzes have improved greatly our understanding of the phylogenetic relationships between species of *Ulocladium* (Wang et al. 2009) but even multi-gene topologies do not yet separate all morphologically distinct species, including separating *U. atrum* from some other species (Runa et al. 2009). Isolates in this study are designated as *U. atrum* group based on the character of frequent, cruciately septate conidia (Simmons 1998).

Comparison with other mycobiotic studies of seeds In comparison with our study, Abdel-Hafez (1984) recovered more species of *Aspergillus* from lupine seed, but as in this study, *A. niger* was abundant. It is hard to compare results for *Penicillium*, given that we successfully identified only one species, even with molecular methods, but *Penicillium* was also abundant in the study of Abdel-Hafez (1984). *Alternaria* and *Cladosporium* were less abundant in Abdel-Hafez (1984), and *Rhizopus* was lacking, although *Mucor* was recovered. Although there was a similar spectrum of genera in our study and the study of Emaby and Abdul-Galil (2006), *Trichoderma* was not isolated from the WRPIS material and *Cladosporium* was apparently absent in their study. The results of the study by El-Nagerabi and Elshafie (2000) were also similar to our own, including *Chaetomium*, but with the addition of *Curvularia*, *Drechslera*, *Phoma* and *Sclerotium*. Possible causes for these

differences are conditions under which seed was grown, host (*L. termis* versus *L. albus*, although the close relationship or synonymy between the two renders this explanation less likely), experimental protocols, geography, and handling. At WRPIS, seed is inspected prior to storage, and obviously defective seed is discarded. There are no indications that seed in the other studies was as carefully screened prior to recovering fungi as is seed at WRPIS.

Studies of seed mycobiota of legumes (other than lupine) enable further comparisons. Dawar et al. (2007) summarized results of several earlier studies on chickpea, wherein the genera *Alternaria*, *Aspergillus*, *Botrytis*, *Cladosporium*, *Curvularia*, *Fusarium*, *Penicillium*, *Rhizopus* and a few others were represented. In Dawar et al. (2007), *Alternaria* (including *A. alternata*), *Aspergillus* (including *A. niger*, and equally common, *A. flavus*), *Botrytis cinerea* Pers., *Fusarium* (with *F. equiseti* best represented), *Macrophomina phaseolina* (Tassi) Goid, *Myrothecium roridum* Tode, and *Rhizoctonia solani* J.G. Kühn were dominant; the aforementioned *Aspergillus* species were the most commonly recovered fungi, both on blotter paper and agar (Dawar et al. 2007). Notable is the presence of *B. cinerea*, *M. phaseolina* and *R. solani* (all pathogens) in their results. The seeds of Dawar et al. (2007), collected in a dozen geographic regions of Pakistan, were probably from farms, i.e., unlike seed in this study they were not from a germplasm collection in which seed is screened at several stages, possibly explaining the more frequent presence of pathogens in their results. In the study by Hussain et al. (2007) on mycoflora of lentil seed, *Alternaria alternata* and *Aspergillus niger* were commonly recovered both on blotter paper and agar media, as was *Fusarium moniliforme* J. Sheld – a old name encompassing more than one species (Leslie and Summerell 2006). Also present in the study of Hussain et al. (2007) were other species of *Aspergillus*, *Chaetomium* spp., *Nigrospora* spp. and *Penicillium citrinum* Thom.

Rhizopus was absent, but *Mucor hiemalis* Wehmer was recovered. The results of our lupine study also partly correspond to those of Kritzing et al. (2003) on cowpea in Africa, wherein *Aspergillus* (*A. flavus* in this instance), *Chaetomium* sp., *Cladosporium* sp., *Fusarium* spp., and *Penicillium* (*P. chrysogenum* Thom in this instance) were commonly recovered, but with *Curvularia*, *Lasiodiplodia* and *Mucor* also commonly present; interestingly, *Alternaria* was not prominent in the results of Kritzing et al. (2003) and none of the *Fusarium* spp. were *F. acuminatum*. However, they cited other studies on cowpea which recovered *Alternaria*, *A. niger*, and *Rhizopus*, taxa germane to our results. Begun et al. (2004), examining three varieties, of commercial pea seed in Pakistan, found the most common genera to be *Alternaria*, *Aspergillus*, *Fusarium*, *Rhizopus*, *Curvularia*, and *Drescheria*; although dominance varied with variety, *Alternaria* was always the most commonly recovered genus.

The study of Kaiser (1992) on commercial lentil seed from the U.S. Pacific Northwest might be the best comparison with our study because of geographic proximity. Kaiser (1992) noted the saprophytes *Alternaria* spp., *Aspergillus* spp., *Chaetomium* spp., *Fusarium* spp., *Helminthosporium* sp., *Nigrospora* sp., *Penicillium* sp., *Stemphylium* spp., *Trichoderma* sp. and Mucorales. However, he also recovered the pathogens *Botrytis cinerea*, *Fusarium acuminatum*, *Phoma medicaginis* var. *pinodella* (L.K. Jones) Boerema, *Sclerotinia sclerotiorum* (Lib.) de Bary, *Rhizoctonia solani*, *Ascochyta fabae* f.sp. *lentis* Gossen, Sheard, C.J. Beauch. & Morrall, and *Macrophomina phaseolina*. Because of the difference in host (lupine versus lentil) comparisons should be made with caution, but the carefully screened lupine seed from the WRPIS collection did not appear to contain these latter pathogenic fungi, with the exception of *F. acuminatum*.

This study on lupine seed did not recover any pathogenic oomycetes, although a medium selective for *Pythium* was used. The medium was originally used because *Pythium* had been diagnosed in roots of some greenhouse-grown accessions of WRPIS legumes, but in light of present findings sources of contamination other than seed seem most probable. Recovery of the *Fusarium* isolates was not confined to the *Fusarium*-selective medium, MGA 2.5. Hence the use of media semi-selective for a given genus seems to have been of limited utility in this study, except that the negative results for *Pythium* are of interest. Very small numbers (~ 3.5% collectively) of isolates, some originally displaying a yeast-like habit, remain unidentified and/or became non-viable (data not shown).

Considering records for both *L. albus* and *L. termis* collectively, and considering data from Abdel-Hafez (1984), El-Nagerabi and ElShafie (2000), Embaby and Abdel-Galil (2006), and Farr and Rossman (n.d.) collectively, our results represent new records for *Alternaria infectoria*, *A. tenuissima*, *Arthrinium arundinis*, *Chalastospora gossypii*, *Cladosporium cladosporioides*, and *Fusarium acuminatum*. The 115 records in Farr and Rossman (n.d.) for Washington State and encompassing all species of *Lupinus* presently lack documentation for *Alternaria*, *Arthrinium*, *Aspergillus*, *Chalastospora*, *Cladosporium* (except for *C. herbarum*), *Fusarium*, *Penicillium* and *Ulocladium*.

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