SPECIES AND POPULATION DIVERSITY OF POWDERY MILDEWS ON GRAIN LEGUMES IN THE US PACIFIC NORTHWEST

By

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To the Faculty of Washington State University:

The members of the committee appointed to examine the thesis of RENUKA NILMINI ATTANAYAKE KITHUL-PELAGE find it satisfactory and recommended that it be accepted.

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Chair

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SPECIES AND POPULATION DIVERSITY OF POWDERY MILDEWS

ON GRAIN LEGUMES IN THE US PACIFIC NORTHWEST

Abstract

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Powdery mildew is a serious disease of pea *(Pisum sativum* L.) and other food legumes in most temperate production regions of the world. Powdery mildew of pea is caused by *Erysiphe pisi*, a biotrophic ascomycetous fungus belonging to the order Erysiphales. Managing powdery mildew relies on planting resistant cultivars and applying fungicides. In pea breeding programs for powdery mildew resistance, a given breeding line may respond differently to powdery mildew infection under different conditions. Some pea breeding lines showed resistance in the greenhouse, but were susceptible in the field. Such inconsistent responses might be due to different pathogen populations found in various environments.

The objectives of this research were to study the pathogen variation of pea powdery mildew found in greenhouse and field conditions using morphological and sequences of the internal transcribed spacer (ITS) region and to find alternative hosts of pea powdery mildews. Powdery mildew samples were collected from infected pea plants as well as other cultivated and wild legumes such as lentil, black medic, and sweet clover from production fields, greenhouses, and natural ecosystems in the Palouse region.

Pea powdery mildew samples from fields exhibited morphological characteristics and ITS sequences in agreement with those of *E. pisi*. Samples obtained from greenhouse-grown pea

plants contained either *E. pisi* or *E. trifolii* depending on the season of sampling and greenhouse location. *E. trifolii* was the cause of lentil powdery mildew. This is the first report of *E. trifolii* from lentil and pea. In addition, *Leveillula taurica* is reported for the first time on chickpea in Washington State.

These data suggest that more than one *Erysiphe* species infect pea. Therefore, the powdery mildew pathogens infecting pea are more diverse than previously assumed. These findings may explain the inconsistent responses of pea breeding lines in various environments. Powdery mildews from *Medicago lupulina* and *Melilotus albus* yielded ITS sequences virtually identical to each other and to *E. trifolii* from lentil and to some greenhouse-originated pea. These weedy legumes could be inoculum sources for pea powdery mildews in greenhouses and potentially serve as alternative hosts for powdery mildew of cultivated legumes.

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DEDICATION

This dissertation is dedicated to my parents, and my uncle and aunt who have supported me unconditionally and inspired me to reach my potential.

CHAPTER ONE

SPECIES AND POPULATION DIVERSITY OF POWDERY MILDEWS ON GRAIN LEGUMES IN THE US PACIFIC NORTHWEST

GENERAL INTRODUCTION

Pea

Field pea (*Pisum sativum* L.), native to Southwest Asia, is an important grain legume cultivated over 25 million acres worldwide. Pea provides a good source of protein and dietary fiber. Dry peas contain 60.0% carbohydrates, 22.9% proteins, 1.4% fat, and 1.4% crude fiber (Hulse, 1994). It is believed to be among the first crops cultivated by mankind (Yamaguchi, 1983). Pea is the fourth most important cultivated legume after soybean, ground nut and dry bean (Hulse, 1994). Major pea producing countries are France, Ukraine, Denmark, Russia and UK in Europe, China and India in Asia, Canada and USA in North America, Australia in Oceana, Ethiopia in Africa and Chile in South America (FAO, 1994). Major pea exporting countries include Australia, Canada and USA, and most of the pea exports from USA are to India, Haiti, Peru and the Philippines (Directory of US suppliers and Industry information, 1996). In the USA, dry pea production is concentrated mainly in the Palouse region and the northern Great Plains.

Major diseases of pea include Ascochyta blight, Fusarium wilt, downy mildew, Aphanomyces root rot, several virus diseases (e.g. *Pea enation mosaic virus*, *Pea top yellows* and *Pea seed-borne mosaic virus*), and powdery mildew (Muehlbauer *et al*., 1983; Duke, 1981).

Lentil

The scientific name for lentil, *Lens culinaris* Medik., came from the Latin term ‘lens’ a disk shaped object) and ‘culinaris’, culinary use and edibility, giving a meaning of a disk shaped

edible grain. It is an excellent source of protein, vitamins and other minerals such as, potassium, phosphorous, iron, zinc and selenium (Bhatty, 1988). It is high in the amino acids lysine and tryptophan, and consumption of lentil with wheat or rice gives a balance of essential amino acids for human nutrition. Lentils, belonging to the family Fabaceae, have the ability to fix nitrogen and therefore, lentil is a very good rotational crop (Bhatty, 1988). Lentil straw is also a good source of animal diet. Lentil is among the earliest crops domesticated in the Fertile Crescent of the Near East and became one of the major grain legumes in the world. It is especially important in Afghanistan, Bangladesh, India, Nepal, Iran, Syria, Turkey, Tanzania, Egypt and Iraq. The crop has been grown in Australia, Canada, and the USA during the past two decades and is an important export agricultural product.

Chickpea

Chickpea is a major food legume crop grown in tropical, subtropical and temperate regions. Major chickpea growing countries are India, Pakistan and Turkey in Asia; Ethiopia in Africa and the Pacific Northwest (PNW) and California in the United States as well as Mexico; and Australia (FAO, 1994). Historic evidence shows that chickpea was first domesticated in the Middle East and then widely cultivated in India, the Mediterranean region, Middle East, Ethiopia and much later introduced into the USA (Duke, 1981). However, Ladizinsky (1975) reported that the center of origin of chickpea was southeastern Turkey. Van der Maesen (1987) based the suggestion that the southwestern part of Turkey adjacent to Syria is the possible center of origin of chickpeas on the presence of closely related annual species. There are two types of chickpeas grown worldwide, ‘Kabuli’, with larger seeds, and ‘Desi’ type with small angular seeds. Important diseases that limit the yield of chickpea include Fusarium root rot, Ascochyta blight and Rhizoctonia dry root rot (Muehlbauer *et al.,* 1988).

Powdery mildew

Powdery mildew fungi, belonging to Phylum Ascomycota, order, Erysiphales, are common, obligate plant pathogens distributed throughout the world. They are classified in a single family, Erysiphaceae within filamentous ascomycetes. They produce one-celled ascospores borne in asci within non-ostiolate ascocarp. Number of asci and ascospores vary. Modern taxonomic studies of powdery mildews started with Léveillé's work in 1851 (Braun *et al*., 2002). He categorized genera based on the chasmothecial appendage morphology and number of asci per ascus. Ten years later, in 1861, Tulasne brothers, de Bary in 1863 and deBary and Wronin in 1870, identified the taxonomic importance of conidia and appressorial morphology (Braun *et al.,* 2002). Today, the characteristics of the anamorphic states are the basis for the modern generic taxonomy of the Erysiphales while the teleomorphic state is important for the species taxonomy.

Powdery mildew is a serious disease on a variety of plants such as cereals, vegetables, fruit trees and ornamentals. The disease is easily recognized, as the name indicates, by the powdery appearance of lesions on vegetative parts of the plant. Powdery mildews are cosmopolitan, but occur mainly in the temperate regions of the northern hemisphere (Braun, 1987, 1995). Amano (1986) recognized two groups of plant families, named group A and group B, based on the number of hosts for powdery mildews. Group A consists of plant families with few or no host species for powdery mildews and group B consists of plant families having more than 100 host species. Among the members of group B, Poaceae, Rununculaceae, Brassicaceae, Rosaceae, Fabaceae, Apiaceae, Boraginaceae, Lamiaceae, Scrophulariceae and Asteraceae include more than 200 host species from each family for powdery mildews. Most of the members of group A are distributed in the southern hemisphere or tropics whereas most members of group B are

commonly distributed in the northern hemisphere. A few families of group B such as Fabaceae and Asteraceae are distributed all over the world. According to Amano (1986), powdery mildews infect 9,838 host species in 1,617 genera, 169 families and 44 orders of angiosperms, which indicates a broad host range concept. There are no known hosts belonging to the gymnosperms or pteridophytes. Most hosts are dicots whereas only 8 host families are the monocots and most of them are Poaceae.

In North America, taxonomic research on powdery mildews lagged behind other parts of the world (Braun *et al.,* 2002), but research on powdery mildew in North America has advanced greatly in recent years (Glawe, 2006). It is important to carryout detailed morphological and phylogenetic research to provide more information about powdery mildews in North America. The research described herein focused on powdery mildews of grain legumes in the PNW and will be an important preliminary study for a future comprehensive study.

Using rDNA sequences to aid powdery mildew species determination

Advances in molecular biology and software for analyzing nucleic acid sequence data have greatly aided the determination of fungal species in the past 20 years. Molecular characters not subject to environmentally-induced variation are useful in the absence of particular spore states of fungi. Among many DNA regions used in assignment of fungal isolates to species and phylogenetic analysis, the nuclear rDNA is frequently used. Eukaryotic rDNA includes tandemly repeated clusters of 18s, 5.8s and 28s rRNA genes (Raué and Planta, 1995). In the rDNA, internal transcribed spacer regions, ITS-1 and ITS-2, are located between genes encoding the 18s, 5.8s, and 28s nuclear ribosomal RNA (nrRNA) subunits (Fig. 1.1). The universal and important function of ribosomes in processing DNA in the protein syntheses requires a high level of uniformity among the copies of rDNA (Polanco *et al*., 1998). The multiple copies of the

members of the multigene family, rDNA, appear to homogenize quickly (Ohta, 1991). Homogeneity among repeated copies within a genome and among individuals of a population is maintained through a process called concerted evolution (Li, 1997). ITS regions have been regarded as nonfunctional sequences, and are less conserved during evolution. Therefore, mutations may accumulate more rapidly in these non-coding regions. rDNAs are highly uniform within a species, but maintain varying levels of diversity between species (Polanco *et al.,* 1998).. Therefore, this region has been used in numerous systematic studies at the generic and species levels of a wide array of organisms including powdery mildew pathogens (Baldwin, 1992; Bruns *et al.,* 1991; Samuels and Seifert, 1995). ITS regions of rDNA have also been used to link anamorphic specimens of powdery mildews with their teleomorphs and to identify the anamorphic herbarium specimens (Cunnington *et al.,* 2003). Takamatsu *et al.* (1998) predicted the secondary structures of ITS-2 region of powdery mildews and suggested that the secondary structure of ITS-2 is necessary for the processing of precursor molecules. Consideration of secondary structure is important for phylogenetic analysis. In the precursor molecules of rRNA, several stem-loop structures are present and some conserved regions are found in the stem regions making it possible to design PCR primers that work on a wide range of the powdery mildews (Takamatsu *et al.,* 1998). The PCR direct sequencing method reported by White *et al.* (1990), has been applied extensively and is an excellent method applicable to most of the organisms including powdery mildews. Nucleotide sequences of these regions are useful taxonomically because they are highly conserved and are found universally in living cells. Takamatsu (1998), Cunnington (2003), Sang *et al.* (1995) and Bruns *et al.* (1991) showed that sequences of ITS regions are useful in distinguishing closely allied powdery mildew species. Based on availability of a large number of ITS sequences in GenBank, lack of Erysiphales-

specific PCR primers for other genes and difficulty of amplifying single copy genes from a minute amount of available DNA, ITS region is the most suitable nucleic acid sequence available for molecular taxonomy of powdery mildews. Another advantage of using ITS region is that several hundred copies of this region exist per individual cell and therefore, it is easer to amplify with a small amount of template (Lee and Taylor, 1990). This is a great advantage over using single-copy gene regions of the genome specifically for biotrophs, where artificial culture is not possible. Therefore, ITS sequences are important for molecular taxonomy of powdery mildew species, especially when a teleomorphic state is lacking and when studying sparse herbarium specimens. In some instances the lack of variation in the ITS regions (such as in small-spored *Alternaria* species) means that they are not always useful in differentiating species (Kusaba and Tsuge 1994). However, ITS sequence data have been shown to be powerful in distinguishing closely related species of powdery mildews on Fabaceae, such as *E. pisi* and *E. trifolii* (Cunnington *et al*., 2003).

This research focused on assessing variability of powdery mildews of grain legumes in the US Pacific Northwest. In previous pea breeding experiments, pea breeding lines reacted inconsistently to powdery mildew infections in greenhouses and fields (McPhee, Personal communication, 2007). Such inconsistent responses of pea breeding materials in different environments might be due to variability of the powdery mildew populations occurring in different environments. Therefore the objectives of this study were to: 1) determine to species and compare the powdery mildew pathogens collected on pea from greenhouses and pea production fields using morphological and molecular characters, 2) use and assess the *in-vitro* bioassay of pea powdery mildew to access possible alternative hosts by cross inoculation and, 3)

determine the powdery mildew species attacking chickpea and lentil in the Palouse region using morphological characters and ITS sequences.

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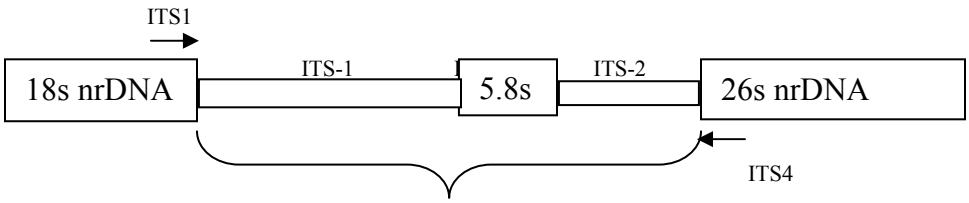

ITS region

Figure 1.1- A map of the ribosomal DNA region containing ITS-1, ITS-2 and 5.8s rDNA gene and ITS1 and ITS4 primer sites (not drawn to scale).

CHAPTER TWO

DIVERSITY OF PEA POWDERY MILDEW SPECIES IN THE GREENHOUSE AND FIELD ENVIRONMENTS OF THE PACIFIC NORTHWEST

ABSTRACT

Species diversity of pea powdery mildew pathogens in different environments was investigated. Previous experience showed that individual pea breeding lines reacted inconsistently to powdery mildew infection in different environments. Some pea lines resistant to powdery mildew in the greenhouse appeared susceptible to powdery mildew in the field. It was hypothesized that the pathogen populations found in the greenhouse are different from those found in the fields. Powdery mildew-infected pea plants were collected from several greenhouses and farms. Morphological and molecular characterization was carried out in order to determine the fungal species identity. Morphological characters of all field isolates from pea conformed to descriptions of *Erysiphe pisi*, a known causal agent of pea powdery mildew, and the ITS sequences of these isolates also 99% matched that of previously determined sequences of *E. pisi* available in GenBank. However, greenhouse isolates from pea varied depending on the sampling season of the year and greenhouse locations. Some of the greenhouse isolates were identical to those found in the fields, but some greenhouse isolates differed in morphology and in ITS sequences. The isolates of the latter group had similar ITS sequences, but differed from *E. pisi* in 14 nucleotide positions, and their morphological characters were generally similar to *E. trifolii*, suggesting that the pathogen populations in these environments are genetically different. These findings that different species were found on peas in different environments might explain inconsistent reactions to powdery mildew infection of pea breeding materials in greenhouses and field conditions.

INTRODUCTION

Powdery mildew of pea (*Pisum sativum* L.) is caused by *Erysiphe pisi* DC., (often referred to as *E. communis* or *E. polygoni*), (Barun, 1987; Falloon *et al*., 2001). It is a serious disease of pea in all over the world. *Medicago, Vicia, Lupinus, and Lens* are some other plant genera of the family Fabaceae, infected by *E. pisi* (Braun, 1987). *Erysiphe pisi* has been reported on pea from Afghanistan, Australia, Brazil, India, Iran, Iraq, Japan, Kenya, Korea, China, Spain, Pakistan, USA and several other countries all over the world (Amano, 1986). This disease adversely affects total biomass yield, number of pods per plant, number of seeds per pod, plant height and number of nodes (Gritton and Ebert, 1975). Yield loss due to the disease averages about 10% (Dixon, 1978). Under warmer and optimal conditions for the pathogen, yield losses vary from 10-65% (Tiwari *et al.,* 1997). The specialized form of *Erysiphe pisi* f. sp. *pisi* is adapted to infect pea plants. Applying fungicides and planting resistant cultivars are the main methods used to control the disease. Growing resistant cultivars is an economic and environmental friendly way to manage the disease. Previously limited number of germplasm sources available for powdery mildew resistance exhibited several undesirable traits such as, low yield, susceptibility to lodging and other problems. Improved resistant cultivars are available today. Resistance to powdery mildew is controlled by recessive genes *er-1* and *er-2*. Gene *er-1* confers full resistance whereas, gene *er-2* provides only leaf resistance (Heringa *et al.,* 1969). These genes are inherited independently from each other (Heringa *et al.,* 1969; Tiwari et *al*., 1997). Pea breeding programs have been carried out at the Grain Legume Genetics and Physiology Research Unit, USDA, ARS, at Washington State University, Pullman, to find powdery mildew resistant pea varieties with favorable traits. During the breeding program, early generations of breeding materials are tested in greenhouse conditions and later generations are

tested in field. However, breeding lines respond differently to powdery mildew infection in greenhouse and field conditions (McPhee, Personal communications, 2007). Some pea breeding lines showed resistance in the greenhouse, but were susceptible to powdery mildew in the field. Such inconsistent response of pea lines could be due to different disease pressures in the greenhouse versus field, selection of plants under a low virulence strain of *E. pisi*, appearance of new race(s) of powdery mildew with virulence to *er-1* gene, or occurrence of another powdery mildew species (Ondřej *et al*., 2005). Taylor (2008), in a study of clover, observed a breakdown of powdery mildew resistance on red clover and assumed that the climatic differences enabled existing pathogen races to become more aggressive. Another possible explanation for the inconsistent response of individual pea breeding lines could be due to differences in pathogen populations found in greenhouse versus field environments. The pathogen populations found in greenhouse environments may be avirulent on some breeding lines whereas the pathogen populations found in the field environments may be virulent on the same breeding lines. Deployment of reliable criteria for species determination of powdery mildews on legumes is essential for examining these multiple possibilities.

Therefore, the objective of this research was to clarify the taxonomy of the species of pea powdery mildews in greenhouse and field environments by using morphological and molecular characters. Hence, determination of powdery mildew pathogens of pea in different environments is a key element of this project. Although precise taxonomic clarification of powdery mildews may not be vital to the immediate control through IPM, fungicide treatments and cultural practices, it is critical in developing forecasting systems, in breeding for resistance, and in dealing with imported plants in quarantine.

Traditionally, powdery mildew genera were distinguished from one another mainly by teleomorphic features such as chasmothecial appendages, chasmothecial diameter, number of asci per chasmothecium and number of ascospores per ascus (Braun, 1987). Species with multiple asci and dichotomously branched chasmothecial appendages were grouped into *Microsphaera,* whereas otherwise similar, mycelioid appendage-bearing species were classed within *Erysiphe*. Earlier authors stressed that only a limited number of characters should be used in taxonomy of powdery mildews, specifically teleomorphic characters (Salmon, 1900). But production of a sexual state is dependant on complex environmental conditions and sexual states may not always be present in all conditions. Therefore, anamorphic features were also identified as important in powdery mildew taxonomy (Boesewinkel, 1980). Braun (1995) suggested that mycelium (epiphyllous, hypophyllous, amphigenous, effusiveness, and color), vegetative hyphae (width, color, branching), appressoria (shape, size, arrangement), conidiophores (size, shape and size of the foot cells, number of following cells), conidia (in chains or formed singly, shape, size, contents such as fibrosin bodies) and conidial germination are also taxonomically important (Braun *et al.*, 2002).

At the species level, molecular characters also are used in taxonomy. Comprehensive examination of nucleotide sequences of the rDNA ITS region of powdery mildew fungi have been carried out recently by Takamatsu (1998, 1999), Mori *et al.* (2000), Saenz and Taylor (1999) and by several other authors. In the study of Saenz and Taylor (1999) morphological features were compared with the phylogenetic analysis based on ITS nucleotide sequences; they observed that mycelioid appendages were distributed throughout the tree and occurred in every major clade. Based on previous comprehensive studies of nucleotide sequences of the rDNA ITS region of powdery mildew fungi (Takamatsu *et al.,* 1998, 1999; Saenz and Taylor, 1999; Mori *et*

al., 2000) and SEM examination data (Cook *et al.,* 1997), Braun and Takamatsu (2000) re-organized the classification of powdery mildew genera *Erysiphe, Microsphaera* and *Uncinula.* The anamorphs of these three genera are relatively uniform and produce Pseudoidium type conidial structures. According to their results, *Microsphaera* and *Erysiphe* did not group into separate monophyletic lineages; instead, species of these genera are dispersed within the same clade. In other words, the genus *Microsphaera*, as defined by morphology, could not be distinguished phylogenetically by ITS sequences from genus *Erysiphe* section *Erysiphe*. Therefore, they concluded that branched chasmothecial appendages do not have taxonomic value at the genus level, but may have value at the species level and therefore reduced *Microsphaera* and *Uncinula* to synonymy with *Erysiphe*.

Both morphological characters and ITS sequence data were used in the current study to test the hypothesis that pea powdery mildew pathogen populations found in greenhouses are different from the pathogen populations found in field conditions.

MATERIALS AND METHODS

Isolates

Powdery mildew-infected pea plants were collected from several greenhouses and commercial and experimental fields in eastern Washington and northern Idaho. Isolates were labeled using the year and location where they were collected (Table 2.1). In addition, a known powdery mildew-resistant cultivar 'Lifter' was grown in two different greenhouses among the powdery mildew-infected susceptible pea cultivars. Cultivar 'Lifter' is known to have resistance to *E. polygoni* (or *E. pisi*) (McPhee and Muehlbauer, 2002). Powdery mildew-infected wild soybean (*Glycine* spp.) isolate was obtained from USDA Soybean Germplasm Collection at University of Illinois.

Morphological characterization

Chasmothecia and conidia were removed using an insect needle from infected leaves and mounted in water. Taxonomic characters examined and recorded included diameter of chasmothecia, number of asci per chasmothecium, lengths and widths of asci, number of ascospores per ascus, lengths and widths of ascospores, lengths and widths of conidia and lengths and widths of conidiophore foot cells. Appendage morphology also observed and recorded. At least 50 measurements made for each character and results were compared with the descriptions found in Braun (1987).

ITS sequence studies

DNA isolation. Total genomic DNA was isolated from fungal materials using the FastDNA® Kit (BIO 101 Inc, Carlsbad, CA) described by Chen *et al*. (1999) with a few modifications. About 100 mg of powdery mildew conidia or mycelia were collected and homogenized with 1 ml of the buffer CLS-Y supplemented with FastDNA® Kit, for 20 seconds at an intensity setting of 4.0 in a FastPrep® homogenizer. The mixture was centrifuged at 14,000 x g for 5 minutes and 600 µl of the supernatant was transferred to a micro-centrifuge tube and DNA was bound to the supplied binding matrix, washed and eluted with 60 µl of extraction buffer.

PCR assay. Polymerase Chain Reaction (PCR) was performed using the ITS1 and ITS4 primer pair (White *et al.*, 1990). In some instances additional primers were designed to avoid the amplification of ITS regions of the host plant or any other contaminating tissues. To design specific primers, ITS sequences of *Erysiphe* species available from GenBank were aligned using ClustalW2 (http://www.ebi.ac.uk/Tools/clustalw2/index.html) algorithm and conserved sequences were used for designing an *Erysiphe*-specific primer pair, EryF

(5'TACAGAGTGCGAGGCTCAGTCG3') and EryR (5'GGTCAACCTGTGATCCATGTGACTGG3'). Total genomic DNA was used in the amplification of the ITS region. PCR reaction mixtures (20 µl total volume) consisted of 2 units of Taq polymerase (Promega), 1X PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 40 ng of the genomic DNA and 10-20 pmol of each primer. Thirty-five thermal cycles were carried out under the following parameters: initial hot start for 10 min at 95 °C prior to the amplification cycles of denaturation at 95 °C for 1 min, annealing at 52 °C for 30 sec, extension at 72 °C for 1 min, and final extension at 72 °C for 5 min. Amplified PCR products were separated on a 1% agarose (Bioline, Randolph, MA) gel in 1X tris-borate-EDTA buffer (45mM Tris, 45mM Boric acid, 1mM EDTA, pH 8.0), stained with ethidium bromide (0.6 µg of ethidium bromide/1 ml agarose) and visualized under ultraviolet light. PCR products were subjected either to direct sequencing or were cloned into a vector and sequenced. For direct sequencing, 5 µl of the PCR product was treated with 2µl of ExoSAP-IT® (USB, Cleveland, Ohio) following the manufacturer's instructions to remove excess template, primer-dimers and oligonucleotides from the PCR product. Reaction mixture was incubated at 37 °C for 15 min followed by 15 min at 80 °C to inactivate the enzyme. Purified PCR products were subjected to direct sequencing, double stranded DNA template 0.5-1.0 µg or 10-90 ng of single stranded/PCR DNA, 3.2 picomoles of primer, 4 µl of Big Dye Mix and required amount of distilled water was added to a final volume of 10 µl and sequencing reaction was carried out as follows: 25 cycles of initial denaturation at 95 °C for 10 sec, annealing at 50 °C for 15 sec and extension at 60 °C for 4 min and reaction products were sequenced at the Sequencing Core Facility of Washington State University. Nucleotide sequences were determined from both strands using an ABI PRISM 377 automatic sequencer (Applied Biosystems, USA). For cloning, PCR products were ligated to the

pCR2.1TOPO plasmid (Invitrogen Crop, Calsbad, CA) and transformed into One Shot® Topo10 chemical competent cells following manufacture's instructions. The transformation mixture (10-50 µl) was spread on LB agar plates containing 40 µg/ml X-Gal (5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside), and 50 µg/ml kanamycin for blue-white selection. At least 5 white colonies from each isolate were selected and colony PCR was performed using M13F and M13R primers. Positive colonies were grown overnight in LB broth containing 50 µg/ml kanamycin at 37 °C. Plasmids were isolated by alkaline lysis method using the Montage® Life Science Kit (Millipore Corporation, Bedford, MA 01730, USA). Plasmids containing inserts were further verified by restriction digestion with EcoRI and separated by gel electrophoresis. Sequencing reactions were carried out with M13F or M13R primers. Sequences were compared with the GenBank (http://www.ncbi.nlm.nih.gov/BLAST) database using the Basic Local Alignment and Search Tool (BLAST). Sequence alignments were carried out using ClustalW2, a general purpose multiple sequence alignment program from EMBL-EBI. Alignments were inspected visually and adjusted manually using a word processing program to omit ambiguous portions of the alignment. All the alignments are presented in Appendix 1.

RESULTS

Morphological data

Powdery mildew symptoms were observed in the greenhouse and field grown peas (Fig. 2.1A and B). Mycelia of all the isolates were mainly epiphyllous, in white or effuse patches often covering the entire upper and lower sides of leaves, stems and pods. Hyphae were branched, septate, hyaline, thin-walled; lobed appressoria were solitary or in opposite pairs (Fig. 2.2). Single conidia formed terminally on conidiophores. Conidiophore foot cells were erect, straight, cylindrical and sometimes flexuous (Fig. 2.3). Conidia were ellipsoid or cylindrical or doliform

without fibrosin bodies (Fig. 2.4) subtended by one or two shorter conidiophore cells or by cells of about the same length. These characteristics observed in all specimens indicated that the fungus belongs to the mitosporic genus *Oidium* subgenus *Pseudoidium*.

Chasmothecia were observed in some field isolates and in some greenhouse isolates. Chasmothecia were scattered or gregarious, with irregularly polygonal peridial cells. Mature dark brown chasmothecia enclosed several sessile or short-stalked asci (Fig. 2.5). Ascospores were ellipsoid to ovoid. Chasmothecial appendages of the isolates from Fairfield and Spillman Agronomy Farm were short mycelioid, simple, often interwoven with each other and with other mycelia, septate, brown colored at the base and paler towards the tip and hyaline at the upper half (Fig. 2.6). Chasmothecial appendages of the GH N 07 isolate was rather long, flexuous, dichotomously branched, sometimes arising from the upper half of the chasmothecia, horizontally spread, hyaline or faintly colored at the base, aseptate or with very few septa. Some chasmothecia were embedded in colonies forming aerial hyphae without conidia formation (Fig. 2.7). Appendage apices displayed 3-5 times loosely branched, diffuse, often with deeply cleft and straight (not curved), richly branched tips, (Fig. 2.8A & B). Taxonomically-important characteristics and dimensions of all the field and greenhouse isolates in comparison with descriptions in Braun (1987) are shown in Table 2. Extreme values are shown within parentheses. Anamorphic characters of SP 07, GE 07, GH M 07 and GH 07 N were highly similar to the descriptions of *E. pisi* and most of the teleomorphic characters and dimensions overlap among the isolates as well as with the descriptions of *E. pisi, E. diffusa, E. trifolii* and *E. baeumleri* in Braun (1987). Powdery mildew symptoms were observed on the known resistant cultivar, 'Lifter', grown in the new greenhouse (Fig. 2.9). Morphological measurements from powdery mildew on the cultivar 'Lifter', were taken. Conidia were 28-45 x 11-16 µm,

conidiophore foot cells were 30-45 x 6.5-10.5 µm, chasmothecia were 107-133 µm, asci were 63-78 x 40-52 µm, ascospores were 22-33 x 12-17 µm and displayed (2-) 3-5 (-6) ascospores per ascus. All these features were similar to those in *E. trifolii* descriptions (Braun, 1987, 1995). Powdery mildew on wild soybean formed rather short conidia (Fig. 2.10) which measured 28-38 x 12.5-16 µm.

ITS sequence data

ITS 1 and ITS 2 regions were amplified successfully with ITS1 and ITS4 primers. Amplified products were about 650 bp in length (Fig. 2.11) in all the isolates. PCR products were cloned into vector pCR2.1TOPO. When selected positive colonies were subjected to another colony PCR with M13F and M13R primers to confirm the insertion of correct size inserts, about 850 bp products were observed on 1% agarose gel (Fig 2.12). ITS sequence alignments showed that there were sequence differences among the isolates. ITS sequences of all the field isolates and GH M 07, GH 04 and GH 06-119 isolates were identical to one another and designated as group 1. GH N 07, GH 05 and GH 06 isolates were identical to each other and designated as group 2. However, there were about 14-nucleotide differences between those two groups of isolates (Appendix 1). BLAST search results showed that all the isolates of group 1 were 99% similar to an *E. pisi* (AF011306) sequence deposited by Saenz and Taylor (1999) from *Lathyrus latifolius*. BLAST results showed that the isolates of group 2 were 99% similar to an *E. trifolii* (AB163926) sequence deposited by Takamatsu *et al.* (2004). A summary of BLAST search results are shown in the Appendix 2. These data suggested that there are two possible pathogen species, *E. pisi* and *E. trifolii* causing powdery mildew on peas in different environments. ITS sequence of a wild soybean (*Glycine* sp.) powdery mildew isolate showed

100% ITS sequence similarity to an *E. diffusa* sequence (EF196675) deposited by Almeida *et al.* (2008).

DISCUSSION

Tiwari *et al*. (1997) studied pathogenic variation in *Erysiphe pisi* on pea using powdery mildew isolates from six locations (Morden and Plum Coulee, Manitoba; Melfort and Indian Head, Saskatchewan; Lacombe, Alberta and Pullman, Washington). Disease reactions of those individual isolates on 14 powdery mildew-resistant and susceptible pea lines were evaluated using detached leaf assays. Except for the isolates from Plum Coulee, Manitoba and Lacombe, Alberta, other isolates failed to produce symptoms on previously known resistant pea lines and produced symptoms on previously known susceptible pea lines. They did not find high variability in virulence among the powdery mildew populations on pea. However, the isolates of Plum Coulee, Manitoba and the isolate of Lacombe, Alberta showed slight virulence on detached leaves of previously known resistant pea cultivars indicating that, there was a slight variance among the powdery mildew isolates tested. Schroeder and Provvidenti (1965) also reported that resistance to powdery mildew conferred by the presence of *er-1* was overcome by a naturally occurring powdery mildew strain from peas and suggested that there was a low level of variability in virulence among the pathogen populations of pea powdery mildew. In these studies no attempt was made to identify the pathogen species. Ondřej *et al.* (2005), in the Czech Republic, observed successful powdery mildew colonization on homozygous pea lines (with *er-1* gene) resistant to *E. pisi.* The naturally occurring pathogen attacked at the locations Rapotín, and Temenice in field trials in the Czech Republic, during the years 2000-2003 and infected all pea lines tested which were known previously as resistant to *E. pisi*. The pathogen was identified by the authors as *E. baeumleri* based on morphology.

In this study, for the first time in the USA, we attempted to observe whether the pea powdery mildew pathogen populations found in greenhouses are different from the pathogen populations found under field growing conditions in the Palouse region, WA and we used both molecular and morphological data to determine the pathogen species. The taxonomically important morphological features were documented and were used to compare greenhouse isolates with field isolates. Most of the dimensions of morphological characters are more or less overlapping amongst *E. pisi* and *E. trifolii* sometimes even with *E. diffusa* in Braun (1987), and there was analogous overlap amongst the isolates observed in this study. Anamorphic characters of all the field isolates were similar to each other and similar to the *E. pisi* descriptions. Greenhouse isolates, GH 07-119 and GH M 07 (*E. pisi* by ITS criteria) displayed conidial lengths and widths similar to *E. pisi* descriptions, but so did GH N 07 (*E. trifolii* by ITS criteria).

In most cases, variably-shaped conidia were observed in greenhouse isolates (Fig. 2.13) making it difficult to differentiate them into groups on the basis of conidial dimensions. It has been reported that individual conidial sizes in a particular collection vary depending on humidity, host, age of the host leaves and season (Braun, 1995; Homma, 1937). Dried conidia are smaller than fresh conidia. By multiplication widths of dry conidia by the factor of 1.2 and the lengths by 1.15, one can estimate widths and lengths of fresh conidia (Braun, 1995). Following this calculation for the dried isolates, fresh conidial length and width would be 39-50 x 15-19 µm of FF 06 isolate and it would be 40-52 x 15-20 µm for the GH 04 isolate. These values are in a strong agreement with *E. pisi* descriptions of Braun's Monograph (1987). ITS sequences of these isolates also were highly similar to *E. pisi* as described earlier. For isolates GH 05 and GH 06 data on conidia were lacking.

Morphological features of teleomorphs overlap among collections but they also are similar in descriptions of *E. pisi, E. trifolii*, *E. baeumleri* and even with *E. diffusa* (Braun, 1987). However, chasmothecial appendages differ between *E. trifolii* and *E. pisi* in the published records (Braun, 1987). Similarly, there were clear differences in the chasmothecial appendage morphology between the field isolates and some greenhouse isolates. Field isolates formed short, mycelioid, simple appendages; often interwoven with each other and with mycelia. Appendages were septate, brown-colored at the base and paler towards the tip and hyaline at the upper half. These characteristics agree with the standard descriptions of *E. pisi* (Fig 2.6) (Braun, 1987). Chasmothecial appendages of GH N 07 isolate were diffuse, loose, highly branched (3-6 times) (Fig. 2.8A and B). This appendage branching pattern resembles that described for *E. diffusa* f. sp. *elongata* descriptions in Braun (1987). However, considering the length and flexuous nature of the chasmothecial appendages (which are about 6.5 times as long as the chasmothecial diameter), NGH 07 isolate is in a good agreement with *E. trifolii* (Braun, personal communication. 2008). Although the longer appendages seem to agree with *E. diffusa* f. sp *elongata,* those in *E. diffusa* f. sp *elongata* exhibit a more compact and regular branching pattern. This fungus is not conspecific with *E. diffusa* (Braun, personal communication, 2008). Interestingly there was a considerable difference in conidial size between the standard descriptions of *E. diffusa* and the NGH 07 isolate (Braun, 1987). Conidia of NGH 07 isolate were much longer than the conidia described in *E. diffusa* and longer conidial size is suggestive of *E. trifolii*. Morphological evidence suggests that, NGH 07 from pea belongs to the *E. trifolii* complex, differing in the production of highly branched chasmothecial appendages. It is known that appendage branching pattern can be highly plastic and may not be a reliable distinguishing character between *E. diffusa* and *E. trifolii* (Braun and Takamatsu, 2000).

As previously described, powdery mildew determination at the species level can be ambiguous when based solely on morphology. To avoid the confusion resulting from overlapping morphological measurements, it is important to consider the molecular data. In the absence of teleomorphic data and when only DNA samples are present as in the case of isolates GH 05 and GH 06, species determination can only be carried out with molecular data (Cunnington *et al*., 2003). According to the ITS sequence data of all the isolates, GH 05, GH 06 and GH N 07 were identical to each other and 99% similar to sequences of *E. trifolii*. All the field isolates and isolates GH 07-119 and GH 04 were identical to each other and 99% similar to sequences of *E. pisi* (Appendix. 1 and 2). Among these two groups of isolates there were about 14 nucleotide differences. This suggests variability among pathogens in two different environments.

ITS regions of twenty positive colonies from GH N 07 were sequenced and all the sequences were 99.8-100% identical to each other. When these sequences were used in a BLAST search of GenBank, the sequence accession, AB163926 of *E. trifolii* deposited by Takamatsu *et al.* (2004) was most similar. Eight colonies from GH 07-119 isolate were sequenced and seven colonies showed maximum sequence similarity (99%) to *E. pisi* sequence accession AF011306, whereas one sequence was highly similar (99%) to *E. trifolii* sequence accession AB163926. This indicates that in the greenhouse *E. pisi* and *E. trifolii* may occur together and *E. trifolii* occurs at a low frequency (observed to be about 12.5%). Eight positive colonies were sequenced from both directions from the isolate SP 07 and 7 colonies were identical to each other and they were highly similar (99.4%) to the *E. pisi* (AF011306) sequence. Sequence of the other colony was highly similar to *E. trifolii* (AB163926). This indicates that in July 2007, at Spillman Farm, the dominant species of powdery mildew pathogen was *E. pisi*, but *E. trifolii* may also present at

a low frequency (about 12.5%). Twenty positive colonies were sequenced from the FF 06 isolate and all were 99.85% similar to each other indicating that in 2006 the Fairfield population was dominated by *E. pisi* and was a uniform population. However, only 5-6 powdery mildew infected pea plants were available for study and therefore to reach a solid conclusion more diseased plants from different parts of the field would have been needed. From the Spillman farm, powdery mildew-infected pea plants were collected from 10 different sites and conidia from all plants were pooled together for DNA isolation and sequencing studies. Therefore, it can be assumed that sequence data for SP 07 is a better representation of the Spillman farm population. Based on these observations it can be concluded that the pathogen species found in field and greenhouse differ from time to time and place to place and the incidence of each pathogen species found also vary.

Ribosomal genes exist as multiple gene families because of the need for large amount of products for which they code. In addition, as ribosomal function is important in cell functioning, uniformity among all the copies is essential (Ohita, 1991). Therefore, within an individual species there is a high degree of uniformity among the rDNA copies. Observed one or two nucleotide differences among the sequences of the same isolate may be due to single base pair substitutions, insertion deletion events (Baldwin *et al.,* 1995), or may be due to inherent problems with big dye terminator reactions.

Occurrence of a pathogen other than *E. pisi* was further validated by successful infection of powdery mildew on a known resistant cultivar to *E. pisi.* The resistant cultivar studied in this project was ‘Lifter’. ‘Lifter’ is a known cultivar carrying *er-1* homozygous recessive genes. Excessive colonization was observed. Morphological measurements were in agreement with *E.*

trifolii although highly branched appendage morphology was similar to the *E. diffusa* f. sp. *elongata* or *E. baeumleri* which are poorly described and uncertain species.

Erysiphe diffusa, traditionally is known to attack soybean. Lehman (1947) described chasmothecial appendage features of *Microsphaera* sp. on soybean and Paxton and Rogers also identified *Microsphaera diffusa* Cooke & Peck. as the causal agent of powdery mildew on soybean (Paxton and Rogers, 1974). Since then, *E. diffusa* infection on soybeans has been reported from Georgia (Demski and Phillips, 1974), Iowa (Dunleavy, 1976), Delaware (Leath and Carroll, 1981) and from several other states in the USA, but no ITS sequence of *E. diffusa* from authentically-identified specimens is available in GenBank. The available sequences of *E. diffusa* (EF196675) from soybean were deposited by Almeida *et al*. (2008) from Brazil. They did not observe the teleomorphic state of soybean powdery mildew and the species determination of that specimen was based on several features such as, the ITS sequence obtained was rather different from *E. glycines,* which is another powdery mildew species reported to infect soybean, the historical occurrence of *E. diffusa* in America and relatively small sized conidia. Therefore, they concluded that the powdery mildew pathogen on soybean was *E. diffusa* (Almeida, personal communication, 2008). Morphological and molecular characteristics of a powdery mildew isolate from a wild soybean (*Glycine* spp.) isolate obtained from USDA Soybean Germplasm Collection at University of Illinois were observed in this study to determine if greenhouse-originated pea powdery mildew was similar to those of *E. diffusa* on soybean. ITS sequence of the wild soybean (*Glycine* spp.) isolate was identical to *E. diffusa* (EF196675) GenBank sequence deposited by Almeida *et al*., 2008 and had much smaller conidia than the pea powdery mildew isolates observed in this study. Therefore, it can be concluded that the observed greenhouse-originated pea powdery mildew isolate is different from *E. diffusa* although it had

highly branched appendages. Unfortunately the teleomorph was not found *Glycine* spp. and so it was not possible to compare the chasmothecial appendages with those of greenhouse-originated pea powdery mildews. Cunnington *et al*. (2003) concluded that ITS sequence data can be used successfully to distinguish very similar species such as *E. pisi* and *E. trifolii* which have similar anamorphs and overlapping host ranges. Therefore, based on available morphological data, molecular data and following the keys in the pages 287, 176-177 and 69-71 in Braun (1987), the pathogen found on peas from NGH 07 was identified as a member of *E. trifolii* complex.

It is documented that some powdery mildew pathogens have the ability to infect plants outside their normal host range (Amano, 1986). Those infections are known as accidental hosts and therefore there is a possibility that *E. trifolii*, a known pathogen of *Trifolium medium* L., accidentally infecting peas, but further validation using cross-inoculation studies with *Glycine max* and *Trifolium* sp. are needed.

These data demonstrate that more than one *Erysiphe* species infect pea under greenhouse conditions. In some fields *E. trifolii* was found together with *E. pisi*. Therefore, powdery mildew fungi infecting pea are more diverse than previously assumed. These findings may explain the inconsistent responses of individual pea breeding lines to powdery mildew infection in various environments. If breeding experiments were carried out in a greenhouse where only *E. trifolii* is present, those breeding lines may respond differently in the fields where *E. pisi* were present. Therefore, pea breeders should consider the presence of both these pathogens when developing and screening resistant breeding lines.

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Table 2.1- Collection date, location and names of pea powdery mildew isolates

Collection year	Location	Isolate name
2004 December	Greenhouse 112, WSU	GH 04
2005 November	Greenhouse 112, WSU	GH 05
2006 July	Greenhouse 112, WSU	GH 06
2006 August	Greenhouse 119, WSU	GH 06-119
2007 December	Greenhouse, Moscow	GH M 07
2007 December	Plant Growth Facility (New Wheat Greenhouse) Room 134, WSU	GH N 07
2006 August	Fairfield, WA	FF 06
2007 July	WSU Spillman agricultural Farm	SP 07
2007 July	Genesee, US 196 Chamber Road	GE 07

Table 2.2- Dimensions of anamorphic and teleomorphic features of all greenhouse and field isolates compared with three *Erysiphe* species in Braun, 1987.

Characters and Isolates	***Erysiphe* species From Braun, 1987**			**Field isolates**			**Greenhouse isolates**			
	E. diffusa	***E. pisi***	***E. trifolii***	**FF 06 (dried)**	**SP 07**	**GE 07**	**GH 04 (dried)**	**GH 07-119**	**GH N 07**	**GH M 07**
Conidia length x width	25-35 x 11-17.5	24-55 x 13.5-22	30-45 x 16-21	(24-)32-43(-51) x 12.5-17	35-55 x 16-18	(23-)32-45(-47) x 13-20	35-46 (-50) x 13-20	34-44 x 16.5-20	35-55 x 12-20	33-59 x 14-16
Conidiophore foot cell length x width	25-38 x 7.5-10	(15-)20 -50(-70) x 6-10	(15-)25-38 (-55) x 6.5-9	17- 22 x 4.5-6	31-45 x 6.5-9	24-47 x 7-14	n/d	27-44 x 6-10	30-40 (-56) x 6.5-9	35-43 x 6-8
Diameter of chasmothecia	75-135	(80-) 85-150	(80-)90-150 (-180)	102-124 (-187)	100-135	n/a	90-135 (-161)	117-145 (-158)	100-122	n/a
Length of appendage/ diameter of chasmothecia	1.5-2.5 or 2-4.5	(0.25-) 0.5-3.5 (-5)	2-6	n/d	n/d	n/a	1.5-2.5	n/d	4-6.5	n/a
Number of asci per chasmothecia	4-10	(3-)4-8 (-13)	3 -12	6-8	8-10	n/a	n/d	(5-)8-13 (-15)	4-8	n/a
Length x width of asci	40-75 x 25-45	40-85 x 25-55	45-80 x 25-50	(39-)64-77 x 32-44	61-75(-81) x (-25)30-37	n/a	65-80 x 33-49	73-84 x (26-)35-48	70-78 x 32-52	n/a
Number of ascos--pores per ascus	3-6	(2-)3-6	(2-)3-5(-6)	3-5	(3-)4-5	n/a	(2-)4-6	4-5	(2-)3-5 (-6)	n/a
Length x width of ascospores	16-24 x 9-15	(15-)18-25(-28)x 10-16.5	18-30 x 10-16	19-25 x 13-16	18-22 x 10-14	n/a	22-29 x 13-18	21-26 x 12-15	24-35 x 13.5-18	n/a

n/d = not determined, n/a = not available
Extreme values are in parenthesis

Figure 2.1- A. Powdery mildew infected pea plants in the greenhouse. **B**. Powdery mildew infected pea plants in the field (Courtesy K. McPhee)

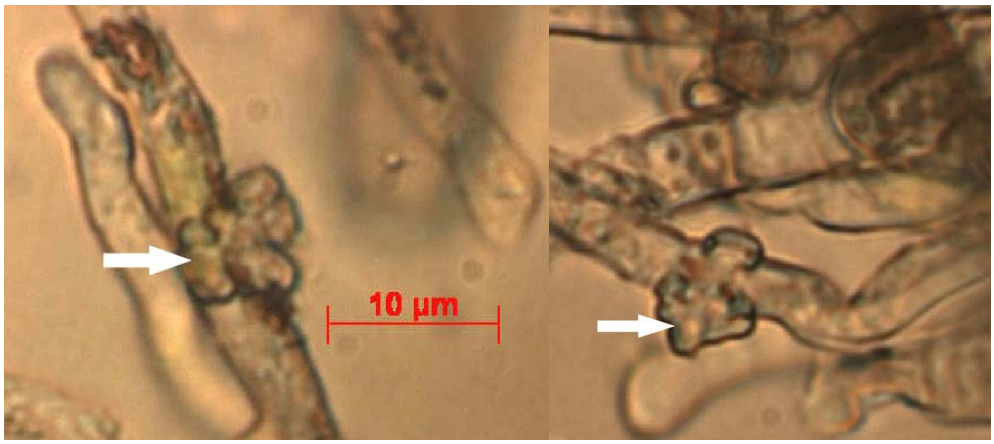

Figure 2.2- Lobed appressoria (arrows) formed by *Erysiphe trifolii* on pea. Bar = 10 µm.

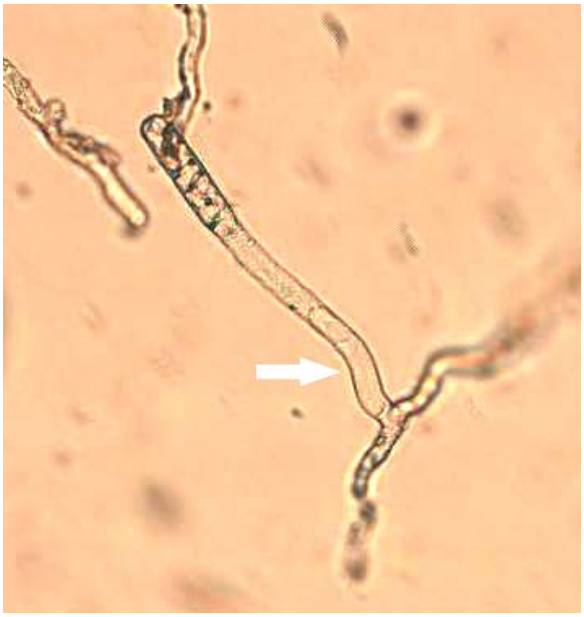

Figure 2.3- Foot cell (arrow) of flexuous conidiophore bearing single conidia

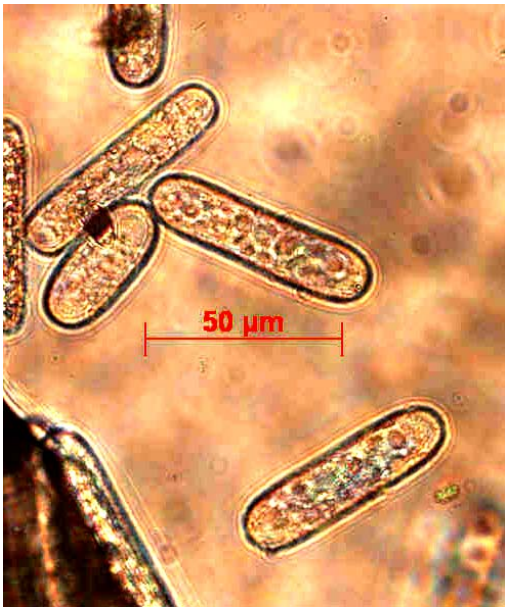

Figure 2.4- Ellipsoid and cylindric conidia of *Erisiphe pisi* on pea. Bar = 50 µm.

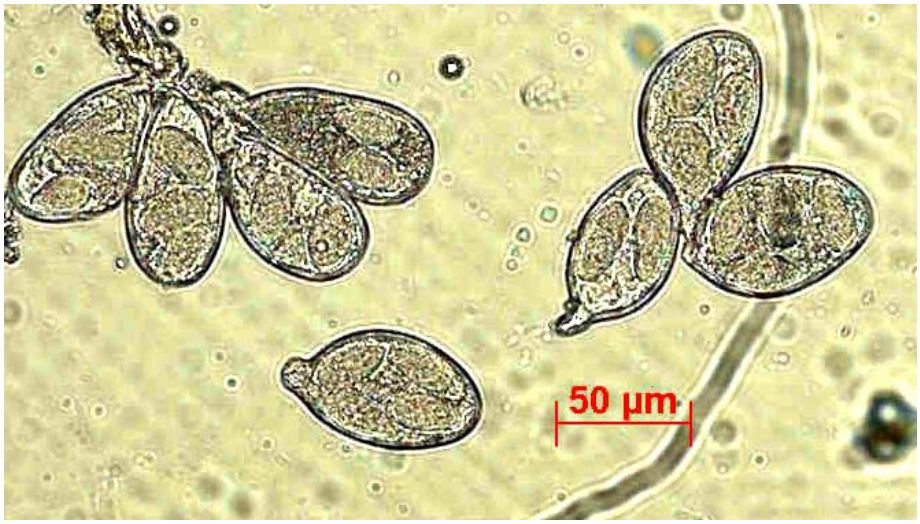

Figure 2.5- Short-stalked or sessile asci of *Erysiphe pisi* on pea. Bar = 50 µm.

Figure 2.6- Short, mycelioid chasmothecial appendages of *Erysiphe pisi* on pea. Bar = 50 µm.

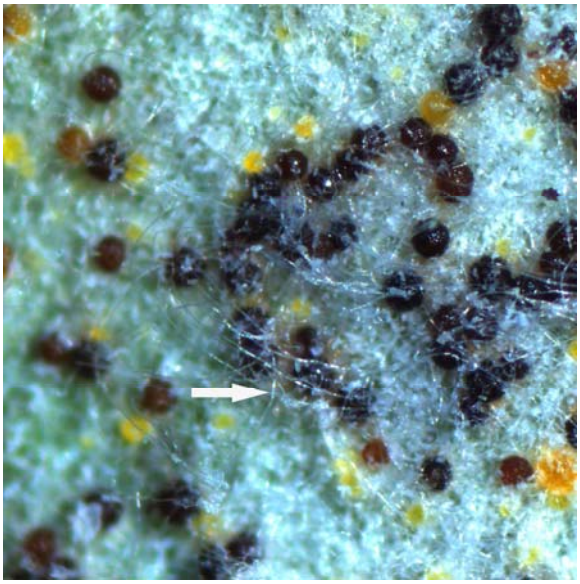

Figure 2.7- Chasmothecia of *Erysiphe trifolii* embedded in colonies forming aerial hyphae (arrow head) without conidia formation on a pea leaf.

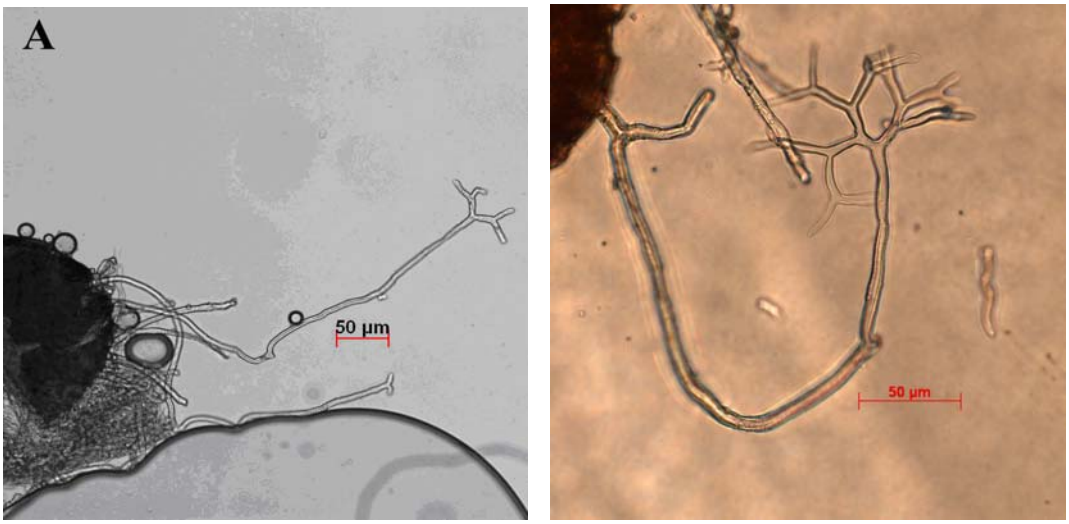

Figure 2.8- Long, diffuse, often deeply cleft and richly branched chasmothecial appendages of *E. trifolii* on pea. *Erysiphe diffusa* is reported with similar appendages. **A.** Early stage of development **B.** Later stage of development. Bar = 50 µm.

Figure 2.9- Production of chasmothecia of *Erysiphe trifolii* on pea cv. 'Lifter'.

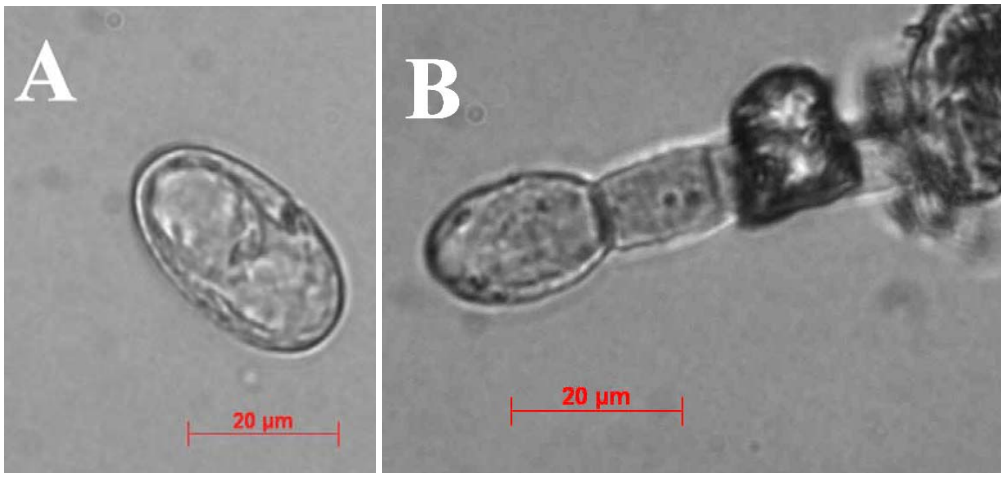

Figure 2.10- A. Conidia **B.** Conidiophore of *E. diffusa* on *Glycine* spp.

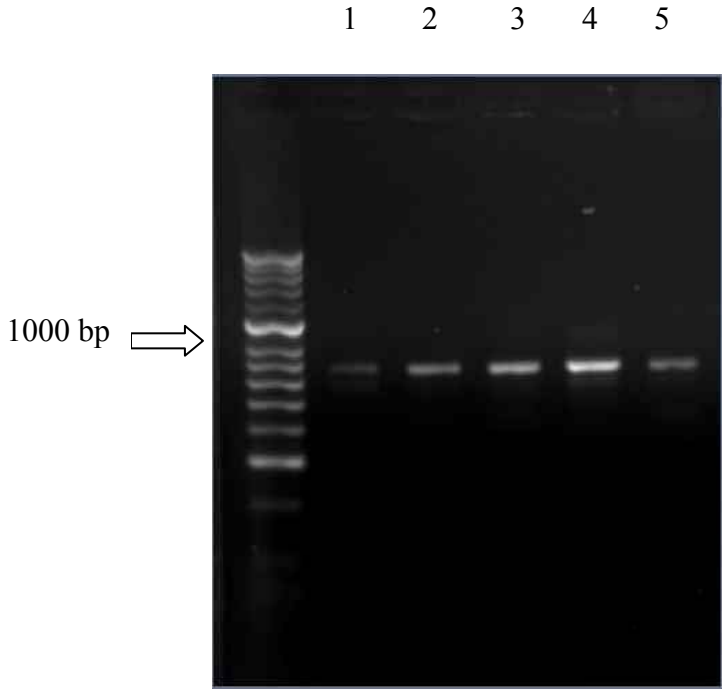

Figure 2.11- Ethidium bromide-stained 1% agarose gel showing PCR products from genomic DNA of pea powdery mildew isolates amplified with ITS1 and ITS4 primers. Lanes 1, 2, 3, 4 and 5 are GE 07, SP 07, FF 06, GH N 07 and GH 05 respectively. Standard size marker (Hyper ladder II) is on the left.

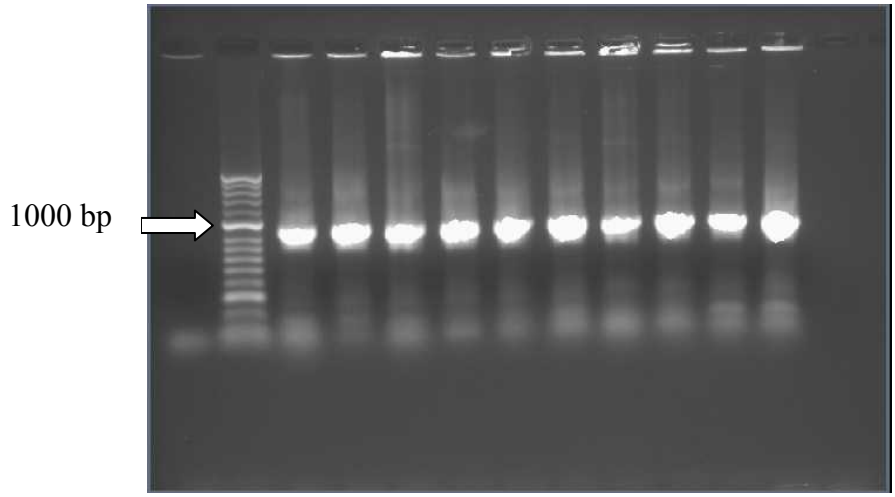

Figure 2.12- Ethidium bromide-stained 1% agarose gel showing PCR products from positive colonies of the GH N 07 isolate amplified with M13F & M13R primers. Standard size marker (Hyper ladder II) on the left.

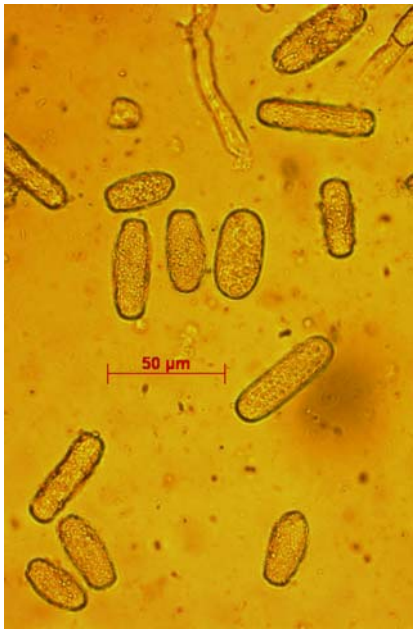

Figure 2.13- Conidia of *Erysiphe* sp. with different sizes and shapes on pea. Bar = 50 µm.

CHAPTER THREE

TAXONOMY OF POWDERY MILDEW PATHOGENS ON LENTILS

ABSTRACT

Taxonomy of the fungus causing powdery mildew on lentil under greenhouse and field production conditions in the US Pacific Northwest was investigated using morphological features and rDNA ITS sequences. Morphological characters, such as conidial sizes, chasmothecial appendage lengths and the flexuous nature of appendages were in strong agreement with descriptions of *E. trifolii*. In addition, ITS sequences were identical to one another and (99.5%) similar to an *E. trifolii* GenBank accession AB163926. Appendages of chasmothecia were highly branched at the apices resembling descriptions of *E. diffusa*, but appendage length relative to chasmothecial diameter was much longer than that of *E. diffusa,* and more similar to *E. trifolii*. Based on morphological and molecular evidence, it is concluded that the pathogen causing powdery mildew on lentil in the US Pacific Northwest is *E. trifolii sensu lato*. The morphological features and ITS sequences were nearly identical to those of the powdery mildew isolate on pea, previously determined to be *E. trifolii*. Therefore, the evidence supports the conclusion that lentil and pea plants were infected by the same powdery mildew species, *E. trifolii*. However, in one greenhouse sample, *E. pisi* was found together with *E. trifolii* on lentils and this determination was confirmed by the ITS sequence data. Based on these observations, it is concluded that the powdery mildew on lentil and pea is caused by both *E. trifolii* and *E. pisi* and that pea plants can act as an alternative host for lentil powdery mildew pathogens.

INTRODUCTION

Lentil is an important grain legume in North America and in the world. In the United States, much of the area growing lentils are in the Palouse region of eastern Washington and northern Idaho, and in the Northern Great Plains region of Montana and North Dakota. Lentil seed is a rich source of protein and other minerals and a very important source of protein, especially for people living in developing countries where lentil often is a part of staple food. This important crop faces several major biotic stresses which limit the yield and include Ascochyta blight, Botrytis stem rot, Fusarium root rot and Rhizoctonia root rot (Bayaa, 1998; Morrall *et al*., 1972). In addition, powdery mildew has been reported from various parts of the world including South Asia, the Middle East, the Mediterranean, East Africa, Eastern Europe, the former USSR, South America, and, more sporadically, from North America (Allen and Lenné, 1998). It is usually considered a minor disease, but may be severe on some cultivars and severe in some parts of the world including South Asia, particularly India (Allen and Lenné, 1998), and is a problem on breeding materials in greenhouse environments. *Erysiphe pisi* DC. and *Leveillula taurica* (Lév.) Arnaud. are the powdery mildew pathogens reported on lentils. *Erysiphe pisi* on lentil has been reported (often as *E. communis* or *E. polygoni*) from various parts of the world including Argentina, Chile, India, Italy, Jordan, Mexico, Romania, Sudan, Tanzania, and the former USSR (Amano, 1986; Farr *et al*., n.d.). Indeed, some reports refer only to *Erysiphe* sp. or *Oidium* sp., i.e., species assignment of *Erysiphe* sp. attacking lentil has often been ambiguous. Recently, *Erysiphe diffusa (as Microsphaera diffusa*) was reported as infecting lentils in Canada and the species has been determined solely on morphology (Banniza *et al*., 2004). Infections by *Eryisphe* species typically result in small white colonies on leaf surfaces.

Resulting lesions expand to cover entire leaf surfaces and pods. Infection can be especially extensive at flowering, and when severe infections, leaves become chlorotic, then curled and necrotic prior to abscission. Yield decline may result and plants sometimes die. Infection by *Leveillula taurica* results in lesions of varying size on leaves and stems, with areas of infection displaying dense, felt-like mycelium.

Life cycles of powdery mildews on lentils are just as inadequately investigated as are species assignments within *Erysiphe*. Teleomorphs can be uncommon in mild winter areas such as the coastal Pacific Northwest, where teleomorphs of *E. pisi* and *L. taurica* are infrequently encountered; in such regions asexual states may be important in over-wintering (Falloon and Viljanen-Rollinson, 2000; Glawe, 2006). In areas with colder winter temperatures the sexual state of *E. pisi* can form abundantly, but survival rates can be low (Tiwari *et al.*, 1999). If chasmothecia survive the winter, ascospores probably serve as the primary source of inoculum. Conidia act as secondary inoculum and enhance population growth and spread of the fungus. *E. pisi* can be seed borne and seeds themselves may serve as over-wintering inoculum, and seeds may also be responsible for long distance dispersal of the pathogen (Falloon and Viljanen-Rollinson, 2000). Colonies of *E. pisi* tend to be apparent on legume hosts in mid-summer, with chasmothecia first appearing in late summer. Spores are carried by wind and can germinate in the absence of free water. Moderate temperatures and shady conditions generally are favorable for powdery mildew growth. Relatively dry weather favors spread and frequent rain inhibits disease spread and development (Anon n.d.). Climatic change has been proposed as a hypothetical cause of increased aggressiveness of some powdery mildew species. Taylor (2008) proposed that powdery mildew on red clover caused by *E. polygoni* DC. may become aggressive on certain resistant cultivars when climate change occurs. To control powdery mildew on lentils, use of

cultivars resistant to powdery mildew is currently the most practical method and screening for resistance is being conducted in a number of countries (Agrawal and Prasad, 1997; Gupta and Sharma, 2006; Mishra, 1973; Pandy and Promod, 1996).

In October 2007, powdery mildew-infected lentil plants were observed in a field near Pullman, Whitman County, Washington. Typical powdery mildew lesions with dense white covered mycelia were observed on lentil plants. This research was undertaken to determine the powdery mildew species occurring on lentil to determine whether pea and lentil powdery mildew found in neighboring fields were similar to each other, and whether the same species infects both pea and lentils.

MATERIALS AND METHODS

Fungal isolates

Powdery mildew-infected lentil plants were collected from greenhouses and from a field and isolates were labeled based on the location and date of collection as cited in Table 3.1. Powdery mildew-infected wild soybean (*Glycine* spp.) samples were obtained from the USDA Soybean Germplasm Collection at the University of Illinois.

ITS sequence studies

Total DNA was extracted from conidia or mycelia pooled from infected lentil plants using the FastDNA® kit described by Chen *et al.* (1999) as described in the previous chapter. PCR amplification of the ITS region from each isolate was performed using general primers ITS1 and ITS4 (White *et al.*, 1990), or *Erysiphe* specific primers EryF (5'TACAGAGTGCGAGGCTCAGTCG3') and EryR (5'GGTCAACCTGTGATCCATGTGACTGG3') (described in the previous chapter). Single PCR reaction (20 µl total volume) consisted of 2 units of Taq polymerase (Promega), 1X PCR

buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 40 ng of the template and 10-20 pmol of each primer, subjected to the following parameters: initial hot start was 10 min at 92 °C prior to 35 cycles of denaturation at 94 °C for 1 min, annealing at 52 °C for 30 sec, extension at 72 °C for 2 min, and final extension at 72 °C for 10 min. A negative control without template DNA also was included in each set of PCR reactions. PCR products were separated and product sizes were estimated on a 1% agarose gel along with standard DNA size markers. All experimental conditions were the same as described in the previous chapter. For cloning, amplified DNA fragments were ligated to the pCR2.1TOPO plasmid (Invitrogen Crop, Calsbad, CA) and transformed into One Shot® Topo10 chemical competence cells by following the manufacture's protocol. Fifty to one hundred µl from each transformation mixture was spread and grown on LB agar plates containing 40 µg/ml of X-Gal (5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside) and 50 µg/ml of kanamycin for blue-white colony selection. At least 5 white colonies from each isolate were selected and colony PCR was performed with M13F and M13R primers at 50 °C as primer annealing temperature. Positive colonies detected by PCR were grown overnight in LB broth containing 50 µg/ml kanamycin at 37 °C. Plasmids were isolated using the Montage® life science kit (Millipore Corporation, Bedford, MA 01730, USA) following the manufacturer's instructions. Plasmids containing inserts were further verified by restriction digestion with EcoRI restriction enzyme and separated on 1% agarose gel. Sequencing reactions were carried out directly with purified PCR product or with isolated plasmids using one of the four primers: ITS1, ITS4, M13F and M13R, as described in the previous chapter and products were sent for direct sequencing at the Sequencing Core Facility of Washington State University. Nucleotide sequences were determined from both strands using an ABI PRISM 377 automatic sequencer (Applied Biosystems, USA). Sequences were used as query in BLAST searches against

GenBank (http://www.ncbi.nlm.nih.gov/BLAST) database. Sequence alignments were carried out using the ClustalW program.

Morphological characterization

Powdery mildew samples from symptomatic leaves from the field and greenhouse were examined using 100-1000x using bright field light microscopy. Teleomorphic characters such as diameter of chasmothecia, chasmothecial appendage morphology, lengths and widths of asci, number of ascospores per ascus, lengths and widths of ascospores, and anamorphic characters such as lengths and widths of conidia and conidiophore foot cells were measured. At least 50 measurements for each character were taken from each sample to determine the ranges that were compared with the descriptions of powdery mildew species recorded on lentils (Braun, 1987 and 1995).

RESULTS

Morphological data

Infected plants from the greenhouse and field showed initial symptoms of small white lesions on leaf surfaces (Fig. 3.1) and expanded to cover the entire leaf surface (Fig. 3.2A) and pods at the later stages. Infection was especially extensive at flowering and under severe infection leaves became chlorotic, then curled and necrotic prior to abscission (Fig 3.2B). Hyaline mycelium occasionally was found in patches with abundant production of single, ellipsoid-cylindric conidia (Fig 3.3). Chasmothecia were scattered to gregarious, initially light yellow to tan and turning dark and rusty brown when they approaching maturity (Fig. 3.4). Some chasmothecia were in colonies that formed aerial hyphae but no conidia. Chasmothecial appendages were branched 3-6 times at the apex; branches were rather loose, diffuse, often deeply cleft with tips that were straight, not curved (Fig. 3.5). Appendages averaged 5.5 times as

long as the chasmothecial diameters (Fig. 3.6). Mature chasmothecia contained several asci (Fig. 3.7). Appressoria were moderately lobed (Fig. 3.8). A comparison of morphological characters with standard descriptions of the *Erysiphe* species previously recorded on lentils, plus *E. trifolii*, is shown in Table 3.2. Conidia of wild soybean (Glycine spp.) powdery mildew were rather small, oval shape and measured 28-36 x 12.5-16 µm (Fig. 2.10).

ITS sequence data

PCR amplification using primers ITS1 and ITS4 was successful with all isolates. Amplified products were about 650 bp (Fig. 3.9). DNA sequences from both strands were obtained. Except for the LSP 07 isolate, full length of ITS region (646 bp) was obtained for all the isolates from lentil. Amplified sequences were identical among all lentil powdery mildew isolates. When the complete ITS sequence of lentil powdery mildew was used as query in a BLAST search, the sequence from accession AB163926 of *E. trifolii*, deposited by Takamatsu *et al*. (2004), showed the highest sequence similarity (99.4%, 4 nucleotide difference). The sequence of *E. pisi* accession AF011306 deposited by Saenz and Taylor (1999) and of *E. diffusa* accession EF196675 deposited by Alemida *et al.* (2008) showed 97% and 96% similarities respectively. Twenty positive colonies were selected and colony PCR was performed with the M13F and M13R primer pair. When the PCR product of each colony was separated on 1% agarose gel, one 850 bp band was observed (Fig.3.10). Both strands were sequenced from each clone to determine whether there are different species of powdery mildews occurring together on lentils. Out of 18 complete sequences, 17 were 99.5-100% similar to each other and 99.4% matched with *E. trifolii* GenBank sequence. Only one sequence was 99.5% similar to an *E. pisi* (AF011306) GenBank sequence (Appendix 2). ITS sequence of the powdery mildew from wild

soybean was identical to an *E. diffusa* GenBank sequence deposited by Almeida *et al*. (2008). A summery of sequence similarities are shown in the Appendix 1 and 2.

DISCUSSION

Although powdery mildew is not a major problem on lentil in US production fields it is a problem on lentil breeding materials in the greenhouse (Chen, Personal communication, 2007). According to previous records, pathogens causing powdery mildew on lentils are *Erysiphe pisi, E. diffusa* and *Leveillula taurica* (Farr *et al*., n.d.). *Leveillula taurica* is easily distinguished from the other two pathogens, based on its sub-epidermal mycelium, branched or unbranched conidiophores emerging through stomata singly or in groups and primary lanceolate conidia and secondary ellipsoid to cylindrical conidia. The isolates observed on lentil in this study definitely were not *Leveillula taurica* (Braun, 1987). *Erysiphe pisi* and *E. diffusa* are very similar in their anamorphic states, but their sexual states are more readily distinguished (Kapoor, 1967a, b). According to Braun, 1987 the major distinguishing feature between *E. pisi* and *E. diffusa* is the morphology of chasmothecial appendages. In *E. pisi* mature chasmothecia are 85-150 µm in diameter with simple appendages ranging 0.5 – 3 times as long as the chasmothecial diameter, very rarely irregularly branched, often interlaced with each other or with the mycelium. Considering the anamorphic characters, *E. pisi* has longer conidia than *E. diffusa*, generally ranging from 24-55 x 13.5-22 µm. Based on all these morphological characters and ITS sequence data it was concluded that the powdery mildew observed on lentil was not *E. pisi.* In contrast, *E. diffusa* forms chasmothecia with diameters ranging from 75 to135 µm and chasmothecial appendages are stiff, 1.5-2.5 as long as chasmothecial diameter (*E. diffusa* f. sp. *diffusa*) or 2-4.5 times as long as the chasmothecial diameter (*E. diffusa* f. sp. *elongata*), 3-6 times branched, loose, diffuse and deeply cleft (Braun, 1987). However, this fungus is

undoubtedly not conspecific with *E. diffusa* and seems to represent another species (Braun, personal communications 2008). The highly branched appendage morphology observed on lentil powdery mildew was similar to *E. diffusa* but most other morphological characters, such as appendage length, flexuous nature of the appendages, conidia length and width and conidiophore foot cell length and width, strongly agreed with descriptions of *E. trifolii* (Braun, 1987). Therefore, the species-level taxonomy of powdery mildew on lentil was not straightforward. Traditionally, *E. diffusa* is the powdery mildew species reported from soybean and, therefore, morphological and molecular characters of wild soybean powdery mildew were studied to observe whether the lentil powdery mildew is the same as or distinct from soybean powdery mildew. ITS sequence of wild soybean powdery mildew was identical to an *E. diffusa* GenBank accession. Furthermore, conidia of soybean powdery mildew were shorter than those of the lentil powdery mildew. Conidial dimensions for soybean powdery mildew were in good agreement with those described for *E. diffusa* and, therefore, it was concluded that the powdery mildew on lentil is not *E. diffusa*.

E. trifolii displays 1-2 times branched, long appendages with loose branching and widely forked tips. Appendages are 2-6 times as long as chasmothecial diameter and they are flexuous (Braun, 1987). Conidia of *E. trifolii* are longer than those of *E. diffusa* (Braun, 1987). The lentil powdery mildew isolates observed in this study also displayed long (6-7 times as long as chasmothecial diameter), flexuous appendages but with frequent branching. Braun and Takamatsu (2000) in their comprehensive report described that; appendage branching pattern has no taxonomic value at the genus level. It has been recognized that some anamorphic features are taxonomically informative, although in the very early reports they were neglected (Boesewinkel, 1980). The conidial length and width and conidiophore foot cell dimensions of lentil powdery

mildew were in an agreement with *E. trifolii* descriptions. Apart from anamorphic characters, ITS sequences also have been identified as an important tool in determining powdery mildew species, especially when teleomorphic states are absent and in separating closely related species such as *E. pisi* and *E. trifolii* (Cunnington *et al.*, 2003). The fact that the ITS sequence was highly similar to *E. trifolii* (EF196675) GenBank accession supported the morphological observations. After considering all these morphological and molecular characters, it was concluded that although highly branched appendages were present on chasmothecia of lentil powdery mildews, all the other characters were in a strong agreement with *E. trifolii*. Taxonomic studies of legume powdery mildews are incomplete in scope and, *E. trifolii* has been referred to as a complex consisting of *E. trifolii, E. baeumleri* and *E. asteragali* (Braun, 1987). The nature of this complex is yet incompletely determined (Braun, personal communication, 2008). Interestingly, in North America *E. trifolii* has been recorded from *Vicia* spp. and, therefore, there is a possibility of infection by this species on lentils. This determination was further validated by following the keys on pages 287, 176 and 69-71 in Braun's Monograph (1987). Therefore, it was determined that the lentil powdery mildew isolate is a member of *E. trifolii* complex. It is doubtful whether *E. baeumleri* is actually distinct from *E. trifolii* because, very few differences are exhibited between *E. baeumleri* and *E. trifolii.* Due to the plastic nature of appendage morphology and the fact that most classifications are based on appendage morphology, powdery mildew taxonomy is rapidly evolving. Especially, powdery mildew on legumes has to be revised (Braun, personal communications 2008).

In conclusion, powdery mildew on peas grown in the greenhouse was determined using morphological and molecular characters as *E. trifolii*. This fungus exhibited an ITS sequence identical with that of *E. trifolii* (chapter 2). Therefore, it can be concluded that both pea and lentil

can act as alternative hosts for *E. trifolii.* Consequently, removal of volunteer pea plants is a control strategy for powdery mildew on lentils. These findings may be helpful in the future if sudden outbreaks of lentil powdery mildew occur and knowing the species involved in causing disease will be very important in the future breeding programs and developing disease forecasting systems.

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Table 3.1- Collection date, location and names of lentil powdery mildew isolates.

Date of collection	Location	Name of the isolate
2007 July	Spillman	LSP 07
2007 Dec	Plant Growth Facility (New Wheat Greenhouse) Room 134, WSU	LGH N 07
2006 Sept	Grain Legume Greenhouse, WSU	LGH 06
2007 Aug	Greenhouse 119 WSU	LGH 07-119

Table 3.2- Diagnostic characters of three *Erysiphe* species: *E. pisi*, *E. trifolii* and *E. diffusa* in Braun (1987, 1995), compared with lentil powdery mildew isolate.

Characters	***Erysiphe* species from Braun, 1987 & 1995**			**Powdery mildew on lentil**
	E. pisi	***E. trifolii***	***E. diffusa***	
Conidia length x width	24-55 x 13.5-22	30-45 x 16-21	25-35 x11-17.5	25-43 x13-22
Conidiophore foot cell length x width	(15-)20-50 (-70) x 6-10	(15-)25-38(-55) x 6.5-9	25-38 x 7.5-10	27-39 x 7-9
Diameter of chasmothecia	(80-) 85-150	(80-)90-150 (-180)	75-135	(78-)83-130
Number of asci per chasmothecia	(3-)4-8(-13)	3 -12	4-10	3-6 (-8)
Length x width of ascus	40-85 x 25-55	45-80 x 25-50	40-75 x 25-45	(46-)57-75 (-91) x 40-54 (-58)
Number of ascospores per asci	(2-)3-6	(2-)3-5(-6)	3-6	1-5
Length x width of ascospores	(15-)18-25(-28) x 10-16.5	(15-)18-25(-28) x 10-16.5	16-24 x 9-15	19-30 x (9-)12-19

Extreme values are shown in parentheses.

Figure 3.1- Early sign of powdery mildew (*Erysiphe trifolii*) infection on a lentil leaf (Courtesy W. Chen).

Figure 3.2– A. Lesions of lentil powdery mildew (*Erysiphe trifolii*) expand to cover the entire leaf (Courtesy W. Chen). **B.** Leaf necrosis

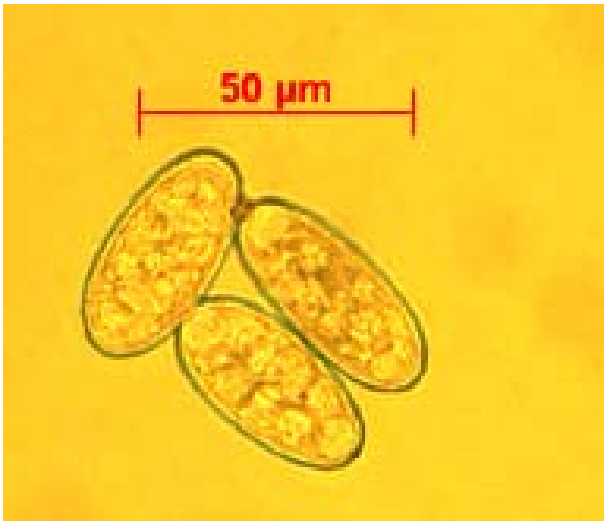

Figure 3.3- Conidia of *Erysiphe trifolii* on lentils. Bar = 50µm.

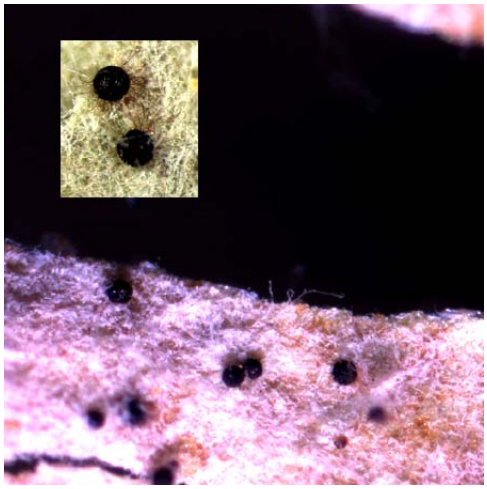

Figure 3.4- Chasmothecia of *Erysiphe trifolii* on a lentil leaf (inset courtesy D. A Glawe)

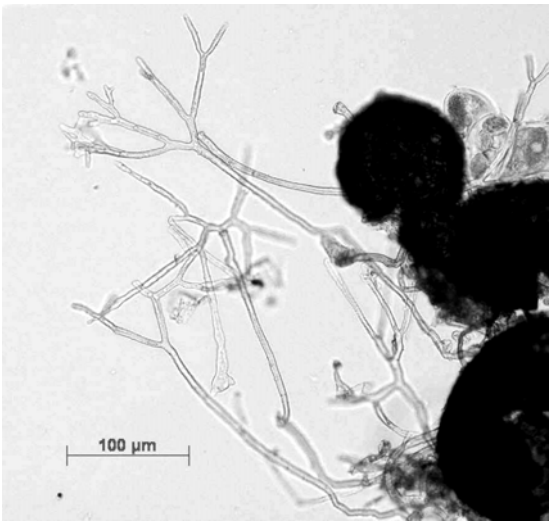

Figure 3.5- Three-six times dichotomously-branched chasmothecial appendages of *Erysiphe trifolii* produced on lentils can resemble those of *E. diffusa*. Bar = 100 µm.

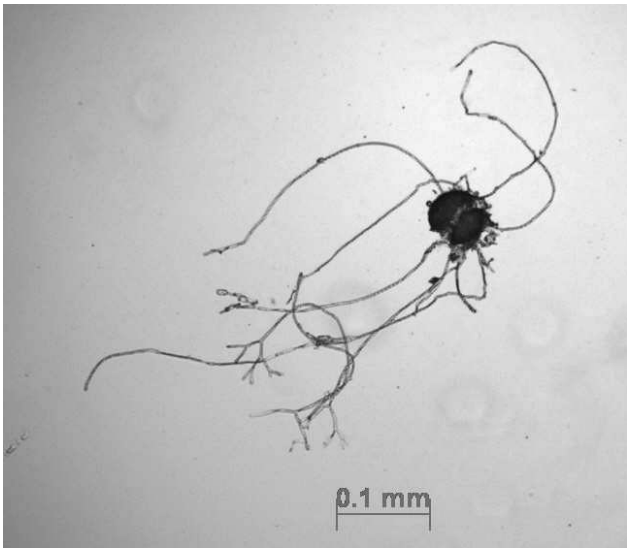

Figure 3.6- A chasmothecium of *Erysiphe trifolii* on lentils having long flexuous appendages up to seven times the diameter of the chasmothecium. Bar = 100 μm.

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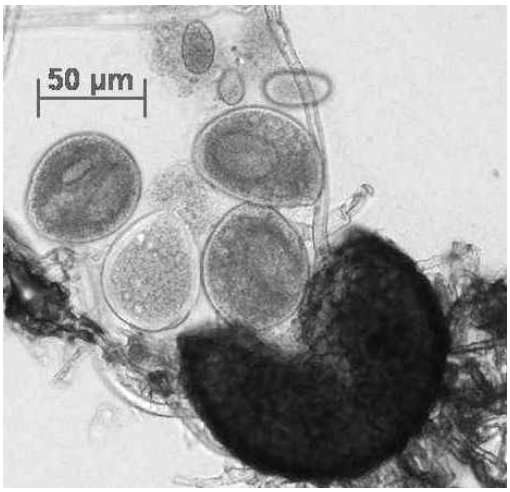

Figure 3.7- A ruptured chasmothecium showing asci and ascospores of *Erysiphe trifolii* on lentils. Bar = 50 µm.

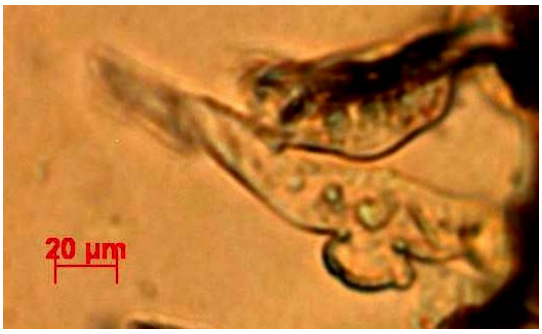

Figure 3.8- Lobed appressorium of *Erysiphe trifolii* on lentils. Bar = 20 µm.

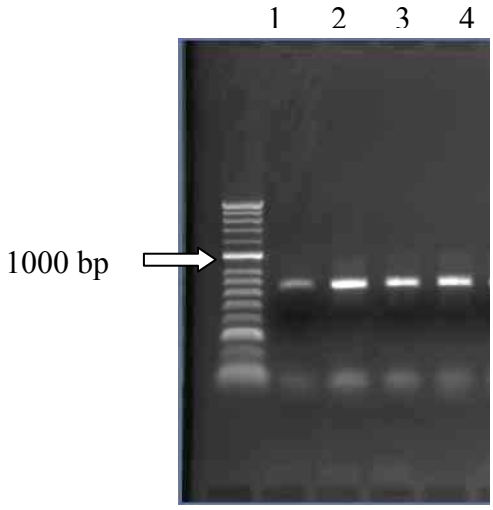

Figure 3.9- Ethidium bromide-stained 1% agarose gel showing PCR products from genomic DNA of lentil powdery mildew isolates amplified with ITS1 and ITS4 primers. Standard size marker (Hyper ladder II) on the left. Lanes 1, 2, 3 and 4 are LSP 07, LGH N 07, LGH 06 and LGH 07-119 respectively.

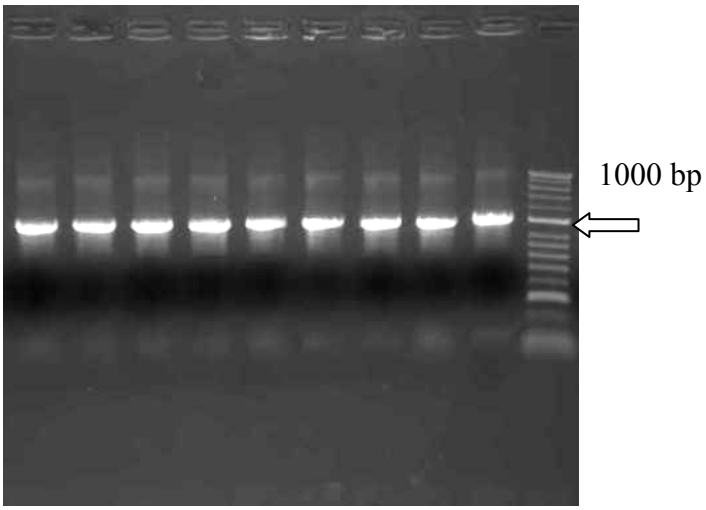

Figure 3.10- Ethidium bromide-stained 1% agarose gel showing PCR products from positive colonies of LGH N 07 isolate amplified with M13 F and M13R primers. Standard size marker (Hyper ladder II) on the right.

CHAPTER FOUR

FIRST REPORT OF POWDERY MILDEW OF CHICKPEA (C*icer arietinum* L.) CAUSED BY *Leveillula taurica* IN WASHINGTON STATE

ABSTRACT

Powdery mildew on chickpea plants was observed during October 2007 in two fields on an experimental farm near Pullman, Whitman County, Washington. Microscopic observation and molecular characterization using ITS sequences were carried out with the objective of determining the pathogen species. The pathogen was determined to be *Leveillula taurica* (Lév.) Arnaud. based on dimorphic conidia, endophytic mycelia, branched conidiophores emerging through stomata and morphological dimensions of conidia. BLAST results using the ITS sequence showed that it was identical to fifteen accessions of *L. taurica* in GenBank including a local isolate (AY912077) reported previously from the monocot *Triglochin maritima* (Juncaginaceae). This is the first report of *L. taurica* on chickpea in Washington State. The disease occurred late in the growing season. Therefore, its economic impact could not be assessed but no control measures appeared necessary. Further research on control could be useful if the disease occurs earlier in the season when potential for economic losses could be greater.

INTRODUCTION

Chickpea (*Cicer arietinum* L., Fabaceae), is an important grain legume grown in tropical, subtropical and temperate regions. Dehulled chickpea contains about 38-59% carbohydrate, 25.3-28.9% protein content, 4.8-5.5% fiber, 0.2% calcium, 0.3% phosphorous and other minerals (Hulse, 1991; Huisman and Van der Poel, 1994). In the U.S.A., major chickpea production areas include the Palouse region of north central Idaho and southeastern Washington, central California, and the northern Great Plains. Ascochyta blight, Botrytis gray mold, Fusarium wilt and dry root rot are the major diseases of chickpea (Nene, 1984). Powdery mildew of chickpea is a minor disease and causes minor economic damage (UTSPP.ICARDA n.d.) but with the potential for major impact on some susceptible cultivars. The disease occurs widely in the Middle East through South Asia, East Africa, Mexico and the United States. The most common pathogens causing powdery mildew on chickpea are *Leveillula taurica* (Lév.) Arnaud [anamorph: *Oidiopsis sicula* Scalia] and *Erysiphe communis*. *Leveillula taurica* is reported on chickpea from India, Iran, Ethiopia and Sudan (Haware, 1998; Mahmudi *et al*., 2006), Morocco, Pakistan, Turkey, the former U.S.S.R. and Zambia (Amano, 1986; Farr *et al.,* n.d.; Kannaiyan and Haciwa, 1989). One source lists Australia as well (UTSPP.ICARDA n.d.). In North America it has been reported on chickpea from California (Buddenhagen *et al.,* 1988).

Different spore states and their roles in the life cycle are not well documented for *L. taurica*, but in general it appears that the teleomorph is seldom observed on this host. It produces chasmothecia with mycelioid appendages on other hosts.

Erysiphe communis (a synonym used for *E. pisi* DC., [Braun, 1987]) is recorded from chickpea in Jordon, Lebanon and Mexico; and an unidentified *Oidium* sp. is reported from India (Farr *et al.,* n.d.). *Erysiphe communis* or *E. pisi,* is better known as a pathogen on pea than on

chickpea and has been investigated primarily on *Pisum*. On pea, chasmothecia are readily formed and over winter, but apparently with low survival (Tiwari *et al.,* 1999). It may be that both ascospores and conidia function as primary inoculum for *E. pisi*, with conidia playing the major role in subsequent disease spread (Falloon and Viljanen-Rollinson, 2000; Tiwari *et al.*, 1999). To control powdery mildew on chickpea, growing resistant cultivars and cultural practices are effective. Nearly all reports of chickpea powdery mildew are based on *L. taurica*, to which many chickpea cultivars are resistant (Nene, 1988). However, Nene (1988) reported that a chickpea cultivar, PRR-1 obtained from Mexico, was severely infected by *L. taurica* when it was cultivated in India whereas a large number of Indian landraces remained resistant. This indicates that the inoculum is already present in that geographic location in India and the native chickpea cultivars/ landraces possess resistance to the pathogen.

In October 2007, powdery mildew was found in two chickpea fields in the Spillman experimental farm near Pullman, Whitman County, Washington. Although disease signs were observed on all chickpea cultivars in the fields, high incidence (>80%) was seen only on cvs. Dwelley and Spanish White. Dwelley is widely cultivated in the Palouse region due to its resistance to pathotype I of Ascochyta blight, whereas Spanish White is more common in areas with low disease pressure of Ascochyta blight. The objective of this study was to determine the fungus causing powdery mildew on chickpea using morphological and molecular characters. Reporting new pathogen records is important for improving our understanding of pathogen behavior and for providing information on accidental and alternative hosts for pathogens. Such information is of utility for applying proper quarantine practices, developing disease forecasting systems and identifying the sources of transmission.

MATERIALS AND METHORDS

Powdery mildew-infected chickpea plants were collected from the chickpea fields in an experimental farm near Pullman, Whitman County, Washington. Morphological characters and ITS sequences were studied. Conidia and mycelia were removed using an insect needle from the infected leaves and mounted in water or cotton blue stain. Lengths and widths of conidia and of conidiophore foot cells were documented. No teleomorphic stage was observed. Observations, including aforesaid dimensions, were compared with species descriptions in Braun's Monograph of Erysiphales (Braun, 1987). For molecular characterization, total DNA was isolated from fungal material with few modifications of the FastDNA® Kit (BIO 101 Inc, Carlsbad, CA) described by Chen *et al*. (1999) and described in the previous chapters. Polymerase chain reaction (PCR amplification) was performed by using the ITS1 and ITS4 primer pair to amplify internal transcribed spacer (ITS) region (White *et al.*, 1990). To avoid amplification of ITS regions from other sources such as from the host plant, thrips, nematodes and other fungi species, *Leveillula*-specific primer pair, Lev3F (5'GACTGCCTAGCGGTCCTCTG3') and Lev3Rb (5'GAAAGCACCACCGGCACCGCCACTG3'), was designed. Total genomic DNA was used in the amplification of the ITS region. PCR reaction with genomic DNA was performed as described in the previous chapters (Chapter 2 and 3). Amplified PCR products were separated by 1% agarose (Bioline, Randolph, MA) gel electrophoresis in 1X tris-borate-EDTA buffer (45 mM Tris, 45 mM Boric acid, 1 mM EDTA at pH 8.0), stained with ethidium bromide (0.6 µg of ethidium bromide/1 ml of agarose) and visualized under ultraviolet light. PCR products were subjected to direct sequencing as described in the previous chapters. Reactions were sent for sequencing at the Sequencing Core Facility of Washington State University. Nucleotide sequences were determined from both strands using an ABI PRISM 377 automatic sequencer

(Applied Biosystems, USA). Sequences were used in BLAST searches to query the GenBank (http://www.ncbi.nlm.nih.gov/BLAST) database.

RESULTS

Typical powdery mildew symptoms and signs were observed on plants in early senescence. Affected areas were usually small, but sometimes coalesced into larger areas under severe disease conditions. Petioles and adaxial leaf surfaces exhibited dense, white powdery patches, with areas beneath the fungal growth initially turning chlorotic, then necrotic, followed by defoliation (Fig. 4.1). Morphologically distinguished dimorphic conidia were observed that fit criteria for primary and secondary conidia (Braun, 1987). Primary conidia were lanceolate, with a pointed apical tip whereas secondary conidia were ellipsoid to cylindric (Fig. 4.2). Photomicrographs of conidiophores were taken in air and short chains of primary and secondary conidia were observed (Fig. 4.3). Primary conidia were measured (47.5-) 50-66.5(-68.5) x (13.5-) 14.5-20.5 µm and secondary conidia were 46.5-62 x 14.5-18.5 µm. Branched or unbranched conidiophores (Fig. 4.4) typically developed from internal mycelium and emerged through stomata singly or in groups of one to five (Fig. 5.5) or rarely arose from external mycelia. The teleomorph was not observed.

PCR products were visualized as a single band about 375 bp in size on 1% agarose gel. Sequence comparisons using BLAST searches (http://www.ncbi.nlm.nih.gov/BLAST) determined that the sequence was identical to fifteen accessions of *L. taurica* in GenBank including a local isolate (AY912077) reported from the monocot *Triglochin maritima* (Juncaginaceae) (Glawe *et al.*, 2005). The ITS sequence of the chickpea isolate was deposited at GenBank (accession number EU437785).

DISCUSSION

According to Nene *et al.*, (1991) the most conspicuous sign of infection by *L. taurica* is diffuse, powdery sporulation on leaves and pods and stems. Other symptoms include early senescence and smaller seeds (UTSPP.ICARDA n.d.). Most genera of powdery mildew are strictly ectophytic. Three genera, *Phyllactinia, Leveillula,* and *Pleochaeta* form endophytic mycelia (Braun, 1995). The endophytic mycelia and morphology and sizes of dimorphic conidia of the chickpea powdery mildew isolate in this study matched descriptions of *L. taurica* (Lév.) Arnaud (Braun, 1987).

BLAST searches determined that the ITS sequence was identical to fifteen accessions of *L. taurica* in GenBank including a local isolate (AY912077) reported from the monocot *Triglochin maritima* (Juncaginaceae) (Glawe *et al.,* 2005).

Leveillula taurica occurs on a broad host range comprised of more than 70 plant families including monocots and dicots from all over the world. In the Fabaceae, species of *Acacia, Cicer, Lathyrus, Lens, Lupinus, Medicago, Melilotus, Phaseolus, Pisum, Vicia* and *Vigna* are few hosts (Braun, 1987). In the Pacific Northwest *Leveillula taurica* was unknown before the 1980s and the only known *Leveillula* species recorded is *Leveillula taurica* (Glawe, 2006). After the 1980s there have been several new records of *Leveillula taurica* on various hosts i.e. *Allium cepa* L. (du Toit *et al.*, 2004; Mohan and Molenaar, 2005), *Capsicum annuum* L. (Cerkauskas and Buonassis, 2003), *Cucumis sativus*. L. and *Lycopersicon esculentum* P. Mill (Forster, 1989), *Gaillardia x grandiflora* (Glawe *et al*., 2006), *Solanum tuberosum* (Glawe *et al.,* 2004) and *Triglochin maritma* L. (Glawe *et al*., 2005). Therefore, *L. taurica* is considered as an emerging plant pathogen in the PNW. These records suggest that this pathogen has become established in the Pacific Northwest and the wide host range may complicate management practices for this

pathogen on chickpea and other crops in the region. In California, *L. taurica* on chickpea was reported (Buddenhagen, 1988) in 1988 and to our knowledge this is the first record of powdery mildew caused by *L. taurica* on chickpea in Washington state. In Washington state, the disease occurred late in the growing season in 2007, and there was no economic impact on the chickpea crop. Therefore, no control measures were necessary. However, further research on control could be needed in this region if the disease occurs earlier in the season when it could be more damaging.

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Figure 4.1- Chickpea leaflets with signs of infection by *Leveillula taurica*.

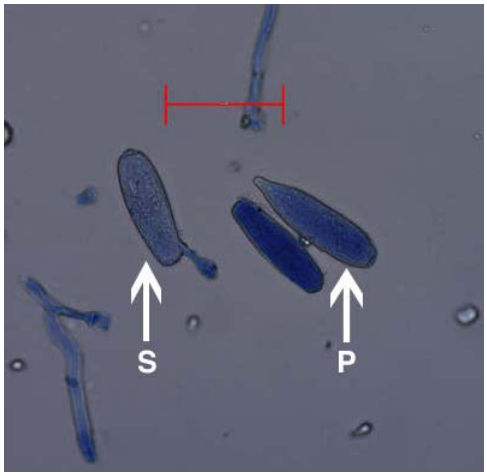

Figure 4.2- Detached primary lanceolate conidium(P), and secondary cylindrical conidia (S) of *Leveillula taurica* on chickpea. Bar = 50 µm.

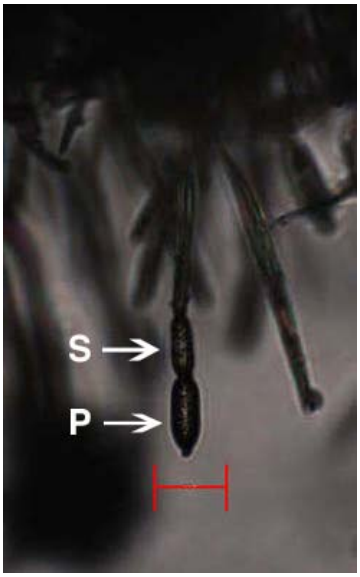

Figure 4.3- Primary (P) and secondary (S) conidia of *Leveillula taurica* on chickpea, photographed in air. Bar = 50 µm.

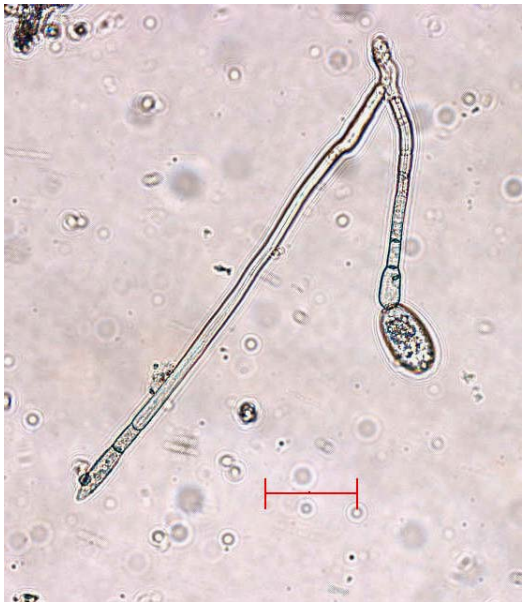

Figure 4.4- Branched conidiophore of *Leveillula taurica* on chickpea**.** Bar = 50 µm.

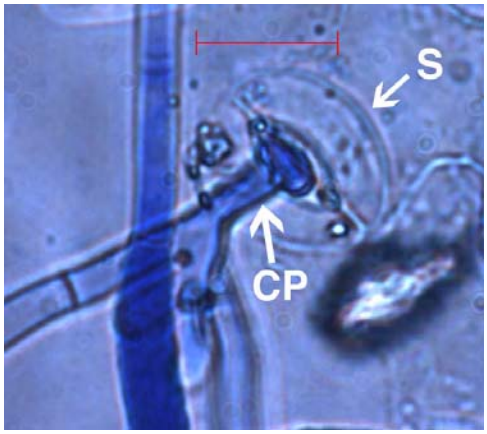

Figure 4.5- Conidiophore (CP) of *Leveillula taurica* emerging through a stoma (S). Bright field. Bar = 50 µm.

CHAPTER FIVE

EXAMINATION OF POSSIBLE ALTERNATIVE HOSTS OF PEA POWDERY MILDEW PATHOGENS AND CROSS INOCULATION STUDIES

ABSTRACT

In the Palouse region of the inland Pacific Northwest, peas are planted in the spring and harvested in the fall. But pea plants are grown in greenhouses for experimental purposes throughout the year, often during winter months. Greenhouse-grown pea plants often become infected with powdery mildew. The source of the inoculum has been unknown, because there are no pea fields available to serve as a source of inoculum in the region during this time. In August and September of 2007 powdery mildew on *Lens culinaris, Melilotus albus, Medicago lupulina*, plants were observed in some fields near by experimental pea fields, natural ecosystems and in roadsides. ITS sequence data of those powdery mildew isolates were highly similar to *Erysiphe trifolii* (AB163926) GenBank sequence. At the same time, ITS sequences of the pea powdery mildew isolates found in some greenhouses were highly similar to *E. trifolii*. Therefore, those wild legumes and lentil plants may serve as alternative hosts for *E. trifolii.* ITS region of the powdery mildew from wild *Lathyrus* sp. was identical to *E. pisi* (AF011306) GenBank accession and to the field grown powdery mildew isolate from peas. Therefore, *Lathyrus* may serve as alternative host for *E. pisi*.

A detached leaf assay was developed to use in cross-inoculation studies and results using it confirmed that detached leaves of *Lens* and *Melilotus* showed symptoms when greenhouse-originated pea powdery mildew was inoculated onto them. When *Melilotus* powdery mildew was inoculated onto pea leaves, successful colonization was observed. Hence, it was concluded that

these wild legumes can serve as alternative hosts for pea powdery mildew and removal of alternative hosts and volunteer lentil plants are possible control strategies.

INTRODUCTION

Powdery mildew fungi are biotorphs, needing a living host to complete their life cycles and they cannot grow on artificial media in the laboratory. Greenhouse-grown plants and detached-leaf assays have been used to study various aspects of the powdery mildew diseases (Doster and Schnathorst, 1985). Several studies tested the effectiveness of the detached-leaf assay to determine the resistance to powdery mildews and to determine the powdery mildew reactions of cultivars (Cohen, 1993; Warkentin, 1995; Olmstead, 2000). For resistance studies, a detached leaf assay can be very useful and reliable, because dependence on natural field infection is unreliable if the disease pressure is very low (Welty and Barker, 1993). This method has been used traditionally as a powdery mildew storage method with periodical sub-culturing onto new leaves or new cotyledons (Nicot *et al.*, 2002). Similarly a modified detached leaf bioassay is useful in the cross-inoculation studies and pathogenicity tests.

After pea harvest in the US PNW, pea powdery mildew pathogens may survive as colonies on volunteer pea plants, on alternative hosts or as resting states (chasmothecia) on plant debris until the next season. Previous experiments determined that *E. pisi* and *E. trifolii* both can infect pea plants (Attanayake *et al.*, 2008). *Erysiphe trifolii* was frequently observed in greenhouses whereas *E. pisi* was frequently observed in the fields of the Palouse region during the time period 2006-2008 (Chapter 2). The host range of *E. pisi* putatively includes broad range genera of Fabaceae including *Arachis, Astragalus, Baptisia, Dolichos, Dorycnium, Hymenocarpus, Lathyrus, Lens, Lespedeza, Lotus, Lupinus, Medicago, Melilotus, Phaseolus, Pisum, Sophora, Thermopsis, Trifolium* and *Vicia* (Braun, 1987). *E. trifolii* also occurs on

common hosts with *E. pisi* and infects species of *Melilotus, Trifolium, Acacia Arachis* and *Lathyrus* (Braun, 1987).

The objectives of this study were to test cross-infectivity of powdery mildews found on various legume host plants in the US Pacific Northwest and to discover alternative hosts for powdery mildews of field and greenhouse grown peas. Another objective was to assess the detached leaf assay for suitability in cross inoculation studies of pea powdery mildews. The hypothesis tested was that wild and cultivated legumes grown nearby the pea fields and found in the natural and artificial ecosystems in the Palouse region are alternative hosts for the powdery mildew pathogen of pea.

METHODS AND METHODOLOGY

Powdery mildew samples were collected from infected pea plants as well as other cultivated and wild legumes such as lentil (*Lens culinaris*), black medic (*Medicago lupulina*), sweet pea (*Lathyrus* sp.) and sweet clover (*Melilotus albus*) from production fields, greenhouses, road sides, natural and artificial ecosystems in the Palouse region. Morphological observations were made as described in the previous chapters and taxonomically important dimensions were recorded (Table 5.1). Total DNA was isolated from fungal materials with few modifications of the FastDNA® Kit (BIO 101 Inc, Carlsbad, CA) described by Chen *et al*. (1999). As a preliminary screening method, polymerase chain reaction was performed to amplify the ITS region of powdery mildews of wild plant species with previously designed *Erysiphe*-specific primers, EryF (5'TACAGAGTGCGAGGCTCAGTCG3') and EryR (5'GGTCAACCTGTGATCCATGTGACTGG3'). If PCR amplification with specific primers were positive, the entire ITS sequence was determined through amplification with the ITS1 and ITS4 primer pair (White *et al*., 1990). PCR products were purified and directly sequenced at the

Sequencing Core Facility of Washington State University. Nucleotide sequences were determined from both strands using an ABI PRISM 377 automatic sequencer (Applied Biosystems, USA). Sequences were used as query in BLAST searches against GenBank (http://www.ncbi.nlm.nih.gov/BLAST) database. Morphological data of powdery mildews on naturally-infected wild and cultivated legumes were assessed to determine lengths and widths of conidia and of conidiophore foot cells and teleomorphic characters if teleomorphic state was produced. Those features were compared with Braun (1987, 1995).

Development and test the suitability of *in-vitro* bioassay

To access the suitability of *in-vitro* bioassay, the method described by Tiwari *et al.* (1997) was followed with some modifications. Highly susceptible pea cultivars, 'Dark Skin Perfection' and 'Melrose' were used for this test and grown in a separate greenhouse to obtain powdery mildew free leaves. When plants were about 20 days old, second or third leaves below the apex were harvested from pea plants and surface sterilized with 70% ethanol for 30 seconds followed by three serial washings with sterilized distilled water (Spurr, 1979). Leaves were air-dried in a laminar flow cabinet after surface-sterilization. Petioles were trimmed with a sterilized blade. Moist chambers were made with sterile wet filter papers kept inside Petri dishes (Warkentin *et al*., 1995). Leaf petioles were immersed in sterile 1% sucrose solution inside a 200-µl micropipette tip with its narrow tip end sealed with a flame. To prevent free moisture formation on the leaf surface a piece of sterile 4 x 4 cm metal mesh was kept between the filter paper and the leaf. This helps prolong the greenness of the leaf tissue. Leaves were oriented abaxial surface up. Fresh pea powdery mildew conidia were inoculated with a fine paint brush (Lim, 1973) on the abaxial surface of the leaves. At each operation the paint brush was rinsed with 90% ethanol and dried before the next inoculation. Non-inoculated leaves were kept as

controls under the same condition. The incubation conditions were maintained with a 12 hr photoperiod under high intensity fluorescent light. Disease development was observed at two day intervals under a dissecting microscope.

Cross inoculation tests with wild and cultivated legumes

As a preliminary study, seeds of *Melilotus albus* (PI 90186), *Melilotus officinalis* (PI539020), *Medicago polymorpha* (PI 186329), *Medicago lupulina* (PI 189128), *Medicago scutellata* (PI 161415), *Lathyrus latifolius* (PI 358888), *Trifolium pratense* (PI 631906), *Vicia cracca* (PI 371785), and *Vicia amoena* (PI 428330) were obtained from the North Central Plant Introduction Station at Iowa. *Vicia faba* seeds were obtained from Mountain Valley Inc. (Salt Lake City, UT) and grown in greenhouse 119 and in Plant Growth Facility Room 134 (New Wheat green house), WSU, among the powdery mildew infected pea plants. Disease development was observed and recorded in Table 5.2. Previously described *in-vitro* bioassay was used to test the pathogenicity of powdery mildew from wild and cultivated legumes on pea plants for more precise environmental control. Cross inoculation studies were carried out only with susceptible pea cultivars 'Dark Skin Perfection' and 'Melrose', a susceptible lentil cultivar 'Crimson', and *Melilotus albus.* All were grown in a powdery mildew-free greenhouse to obtain the powdery mildew free leaves for the detached leaf assay. Powdery mildew infected wild *Melilotus albus* was brought from its original location to another greenhouse and replanted to maintain powdery mildew inoculum. Pea powdery mildew conidia originated from greenhouse 119 were used in this study. Previously it was determined that *E. pisi* is the frequently occurring species (~ 82%) in this greenhouse and *E. trifolii* was also present at a lower frequency (~ 12%) (Chapter 2). Pea powdery mildew conidia were inoculated on detached leaves of *Melilotus albus* and *Lens culinaris*. Conidia from the replanted powdery mildew-infected *Melilotus albus* plants

originated from the Snake River Bank, Boyer Park, WA and conidia from greenhouse-grown lentil plants were used for cross inoculation with pea leaves. Aseptic techniques were used during the inoculation procedure to minimize contamination and non-inoculated controls were used to monitor potential contaminations. Experimental units were incubated at room temperature and illuminated with white florescence light and incubated at 12hr photoperiod (Warkentin *et al.,* 1995). Three replicates per unit were used and three non-inoculated controls were also given the same treatments as others in each experiment.

RESULTS

Among the tested powdery mildew isolates, *Melilotus, Medicago, Lathyrus* and *Lens culinaris* gave positive PCR results with the EryF and EryR primer pair. ITS sequences were obtained from both strands with ITS1 and ITS4 primers. The ITS sequence from powdery mildew on *Medicago lupulina* was 99% similar to that of *E. trifolii* (AB015913) GenBank sequence submitted by Takamatsu *et al*. (1999) from the host *Trifolium vulgaris.* ITS sequences from powdery mildews on *Melilotus albus* and *Lens culinaris* were identical to each other and 99% similar to the *E. trifolii* (AB163926) sequence submitted by Takamatsu *et al*. (2004). ITS sequences of powdery mildew on *Lathyrus* sp. was 99% similar to *E. pisi* (AF011306) GenBank accession submitted by Saenz and Taylar (1999) from the host *Lathyrus latifolius*. A summary of BLAST results are shown in the Appendix 2. Morphological characters were also observed in these isolates. Morphological measurements were taken from the powdery mildew growing on plants in non-cultivated areas ("wild" plants) are shown in the Table 5.1. Anamorphic characters of the powdery mildews found on *Melilotus, Medicago*, and *Lens* were closer to *E. trifolii* than to *E. pisi*. Teleomorphic characters were not observed in the isolates except for *Lathyrus* and *Lens*.

In the preliminary study done to observe the infectivity of powdery mildew on selected wild species under greenhouse conditions, successful powdery mildew infection was observed after 14 days of planting on the plant species shown in Table 5.2.

In the test assessing the detached leaf assay as a suitable method for cross inoculation studies, pea powdery mildew conidia were inoculated onto a susceptible pea cultivar ('Dark Skin Perfection'). One week after the inoculation severe colonization on pea laves was observed (Fig.5.1). After about 3 weeks chasmothecia production also was observed (Fig. 5.2). Therefore, this procedure served to be a suitable method for cross-inoculation studies and for other studies on peas. When pea powdery mildew conidia were inoculated onto detached leaves of *Melilotus albus* and *Lens culinaris*, successful colonization was observed after about one week (Fig. 5.3A and B). Chains of conidia were observed on lentil leaves in the absence of air turbulence in the moist chamber (Fig. 5.4). Conidia production and infection on detached pea leaves was observed when pea leaves were inoculated with powdery mildew from wild *Melilotus albus* and powdery mildew from *Lens culinaris*. Non-inoculated controls remained healthy.

DISCUSSION

Pea breeding materials grown in the greenhouse during winter months frequently are infected with powdery mildews. No pea fields are available during this particular time of the year in the Palouse region and, therefore, the source of greenhouse inoculum is a mystery. Pea powdery mildew pathogens may survive on alternative hosts in the absence of pea plants. In the preliminary study of detecting the possible alternative hosts for powdery mildews for *E. pisi* or *E. trifolii*, plants of *Lathyrus latifolius, Melilotus albus, Melilotus officinalis, Medicago polymorpha, Medicago lupulina, Medicago scutellata, Vicia faba, Vicia amoena, Vicia cracca* and *Lens culinaris* were found as potential alternative hosts. Interestingly in any of the

greenhouse studies, *Trifolium pratense* remained resistant and there may be several reasons. The accession of *T. pratense* used in the experiment may be resistant to the powdery mildew species found in greenhouse 119, it may be resistant in the juvenile stage but may become susceptible at later growth stages or there may be some other forma species specifically adapted to infect *Trifolium pratense*. Further studies are necessary to verify this hypothesis. Greenhouse inoculation tests are not specific and the infection may depend on the various environmental factors (Nicot *et al*., 2002; Glawe, 2008). It is impossible to do cross inoculation studies more precisely in greenhouse conditions. Detached leaf assay is important as a more specific method.

Warkentin *et al*. (1995) studied the effectiveness of a detached leaf assay to determine the reaction of pea plants to powdery mildew and concluded that results were highly correlated with the reactions of the whole plant tests. Chasmothecia production on detached leaves also was observed in our study and the method appears useful for cross inoculations. Cross inoculation studies have not always supported this broad host range concept, as when isolates from pea were inoculated on chickpea, lentil, field bean and faba bean without production of disease (Tiwari *et al*., 1999). But in this study detached pea leaves were colonized successfully when powdery mildews from *Lens culinaris* or *Melilotus albus* were used as inoculum. Likewise, positive infection results were obtained when detached leaves of *Melilotus* and *Lens* were inoculated with powdery mildew originated on pea plants. Results of cross inoculation studies confirmed that *Melilotus albus* and *Lens culinaris* can act as alternative hosts for pea powdery mildew pathogens.

ITS sequences were highly similar among these differently-sourced powdery mildews i.e. from pea, *Lens, Medicago* and *Melilotus* (Appendix 1 & 2). This further validated the results of cross inoculation experiments.

Therefore, it can be concluded that both sweet clover and lentil can act as alternative hosts for pea powdery mildews. Removal of volunteer pea plants from lentil fields and volunteer lentil plants from pea fields and powdery mildew-susceptible varieties of *Melilotus* and *Medicago* plants from both pea and lentil fields and surrounding areas may be a control strategy for powdery mildew on peas and lentils. These wild legumes can be alternative hosts for powdery mildew on greenhouse grown peas and lentils. The ITS sequence of powdery mildew from *Lathyrus* plants was highly similar to an *E. pisi* GenBank sequence and identical to the powdery mildew isolates originated from field grown pea plants. Therefore, *Lathyrus* may serve as an alternative host for *E. pisi*. *Lathyrus* species also were reported in the host range for *E. trifolii* (Braun, 1987). Furthermore, based on the preliminary study conducted in the greenhouse, it was concluded that *Vicia* sp. also can serve as an alternative host for pea powdery mildew pathogens. These results suggest that the removal of volunteer lentil plants and other wild legumes from the pea fields may be useful as a potential cultural practice to control powdery mildew on peas.

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Table 5.1- Morphological dimensions of wild legumes and cultivated lentils (*Lens culinaris*) found in the Palouse region, 2007 compared with the *Erysiphe pisi* and *E. trifolii* in Braun, 1987.

Characters and Isolates	**Braun (1987)**		**Isolates**		
	E. trifolii	***E. pisi***	***Lathyrus* spp.**	***Melilotus albus***	***Lens culinaris***
Conidia length x width	30-45 (-50) x 16-21	24-55 x 13.5-22	32-40 x11-15	30-40 x 12- 17	25-43 x 13-22
Conidiophore foot cell length x width	(15-) 25-38 (-55) x 6.5-9	(15-)20-50 (-70) x 6-10	n/d	29-37 x 6-8	27-39 x 7-9
Diameter of chasmothecia	(80-) 90-150 (-180)	(80-) 85-150	100-130	n/a	(78-)83-130
Number of asci per chasmothecia	3-12	(3-)4-8(-13)		n/a	3-6 (-8)
Length x width of asci	45-80 x 25-50	40-85 x 25-55	51-78 x 31-43	n/a	(46-)57-75 (-91) x 40-54 (-58)
Number of ascospores per ascus	(2-)3-5(-6)	(2-)3-6		n/a	1-5
Length x width of ascospores	18-30 x 10-16	(15-)18-25(-28) x 10-16.5	20-24 x 10.8-12	n/a	19-30 x (9-)12-19

n/a = not available
n/d = not determined
Extreme values are shown within parenthesis

Table 5.2- Powdery mildew reaction on greenhouse grown wild plant species

Plant species	**Greenhouse 119 (*E. pisi* and *E. trifolii* present)**	Plant Growth Facility (New Wheat Greenhouse) Room 134, WSU **(*E. trifolii* present)**
Melilotus albus	infected	non-infected
Melilotus officinalis	infected	non-infected
Lathyrus latifolius	infected	infected
Medicago polymorpha	infected	infected
Medicago lupulina	infected	infected
Medicago scutellata	infected	not germinated
Trifolium pratense	non-infected	very few lesions
Vicia faba	infected	infected
Vicia amoena	infected	infected
Vicia cracca	infected	infected
Lens culinaris	infected	infected

Figure 5.1- Colony of *E. pisi* on a detached pea leaf (10 days after inoculation)

Figure 5.2- Chasmothecia production of *Erysiphe pisi* on a detached pea leaf (3 weeks after the inoculation)

Figure 5.3- Development of powdery mildew colonies on detached pea leaves when inoculum was from *Melilotus* **(A)** or *Lens culinaris* powdery mildew **(B).**

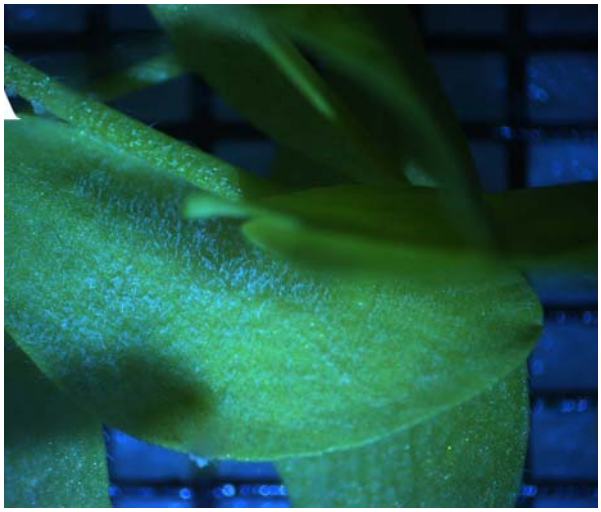

Figure 5.4- Development of powdery mildew on detached lentil leaves when the inoculum was from pea powdery mildew.

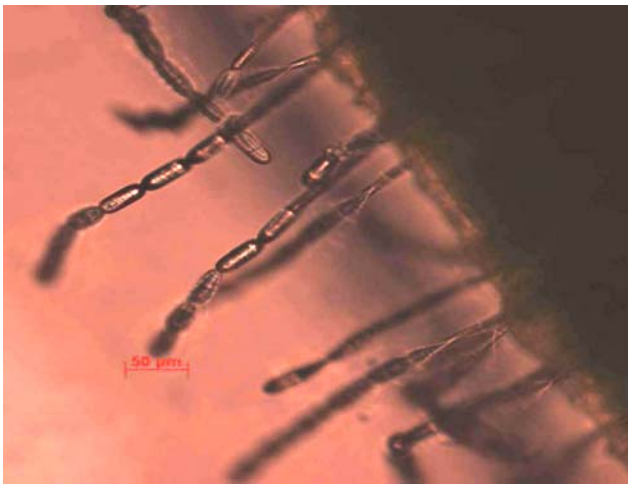

Figure 5.6- In the absences of air turbulence conidia produced and remained in chains on detached lentil leaves in the moist chamber. Bar = 50 µm.

CHAPTER SIX

GENERAL CONCLUSIONS

This research provided a detailed investigation on species identity and cross infectivity of powdery mildews on legumes in the PNW. Previously it was assumed that *Erysiphe pisi* is the pathogen causing powdery mildew on pea. The findings of this research show that inaddition to *E. pisi, E. trifolii* is also a pathogen on peas. In the absence of the teleomorph, *E. pisi, E. trifolii* and *E. diffusa* can be distinguished using the ITS sequences and anamorphic characters. Both *E. trifolii* and *E. pisi* were found to infect pea in greenhouse environments, and *E. trifolii* was found at low frequency infecting peas in the field. The powdery mildew species found depended on the sampling location and time of sampling. It can be concluded that due to the variation of pathogens found in different environments, pea breeding lines may respond differently.

Erysiphe diffusa, E. pisi and *Leveillula taurica* have been reported as pathogens causing powdery mildew on lentils. But from this research it was found that *E. trifolii* is also a potential pathogen on lentils. *Erysiphe diffusa* was reported from Canada, determined on the basis of teleomorphic characters. Powdery mildew-infected lentil plants observed in the Canadian study would were *E. trifolii* with frequently branched chasmothecial appendages. Results of the current study revealed that powdery mildews on peas and lentils are taxonomically more diverse and complex than previously assumed. Powdery mildews on *Melilotus albus, Medicago lupulina*, and *Lens culinaris* were highly similar to *E. trifolii*. Cross inoculation results confirmed that lentils and sweet clover can serve as alternative hosts for pea powdery mildew pathogens. Chasmothecial appendages of the observed *E. trifolii* isolates on lentils and peas were highly branched. The branching pattern was similar to the *E. diffusa* descriptions. But ITS sequences and anamorphic characters of these isolates were highly similar to *E. trifolii* rather than to *E.*

diffusa. Although the branching pattern was similar to *E. diffusa*, the length of chasmothecial appendages and flexuous nature of appendages were also in good agreement with the descriptions of *E. trifolii* (Braun, 1987). Moreover, the observed wild soybean (*Glycine* spp.) powdery mildew isolate showed ITS sequence identity to an *E. diffusa* GenBank sequence (EF 196675). Conidia of wild soybean powdery mildew were smaller than lentil and pea powdery mildew isolates. Traditionally *E. diffusa* has been regarded as a pathogen causing powdery mildew on soybean, and based on all these data, it can be concluded that the lentil and pea powdery mildew isolates were different from *E. diffusa* although branching pattern of chasmothecial appendage apices resembled *E. diffusa*. Branching pattern of chasmothecial appendages is highly plastic and it seems to have less taxonomic importance as previously described in some reports. Therefore ITS sequences and anamorphic characters seem to be more informative for taxonomic studies. *Erysiphe trifolii* may produce highly branched appendages on lentils and peas in the PNW. *E. trifolii* is a poorly described species which may be easily confused with *E. baeumleri* which has highly branched appendages, but with overlapping characters among them. Furthermore, it is doubtful whether *E. trifolii* and *E. baeumleri* are actually two different species or both belong to the same species. Taxonomic revision of powdery mildew on legumes should be a priority.

Taxonomic studies on legume powdery mildews in the USA have lagged behind other countries. Hence a phylogenetic analysis was done using all the isolates found in this study except *Leveillula taurica*. Additional ITS sequences of legume powdery mildew species from GenBank were obtained and the species name, database ID number, country of origin and author reference of the GenBank sequences are shown in the Table 6.1. The DNA sequences initially were aligned using the ClastalW integrated within BioEdit v 7.0.5.3 (Hall, T. A. 1999), a general

purpose multiple sequence alignment program. The alignments were inspected visually and adjusted manually using BioEdit to omit ambiguous portions of the alignment and improve the alignment. The data were analyzed using the maximum likelihood methods with PAUP 4.0β10 (Swofford, 2002). The best model used for maximum likelihood analysis was determined by Modeltest 3.8 (Posada and Crandall, 1998). Maximum likelihood phylogenies were also estimated in a Bayesian framework with Markov Chain Monte Carlo (MCMC) sampling in MrBayse version 3.1 (Ronquist and Huelsenbeck, 2003). The maximum likelihood tree with bootstrap values obtained with 100 replications is shown in the Fig. 6.1.

Erysiphe glycines was used as the out group because it was reported to infect *Glycine max,* another legume, and is reported from the USA. In the ML tree, all field isolates, GHM 07, GH 07-119, GH 04 and wild *Lathyrus* isolate clustered together with an Australian isolate of *E. pisi* and a USA isolate from pea. This cluster is very well supported with maximum bootstrap values and designated as the pisi clade. This group is more closely related to *E. diffusa* than to any other species included in this analysis. All the other isolates, GH 05, GH 06, GH N 07, *Lens*, *Melilotus albus* and *Medicago lupulina* are identical to each other, formed a separate cluster, and are designated as the trifolii clade. Separation of the cluster of Japanese isolates of *E. trifolii* from rest of the isolates was not well supported with bootstrap values.

According to Amano (1986), plant family Fabaceae belongs to group B which comprises more than 200 host species for powdery mildews and is cosmopolitan. A majority of powdery mildew species are commonly found in the northern hemisphere, but in North America powdery mildew studies have, until recently, lagged behind studies in Europe, Japan and Australia. A comprehensive study of legume powdery mildews may help in applying and improving disease control strategies, breeding programs, developing forecasting systems and plant quarantine

programs. This study is important as a preliminary investigation of powdery mildew on Fabaceae in the US PNW. Furthermore, powdery mildew on chickpea was found in Washington State for the first time and species was determined to be *Leveillula taurica*. This research will lay a foundation for such a comprehensive study.

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Table 6.1- Species name, database ID number, country of origin and author reference of the sequences used from the GenBank for the phylogenetic analysis.

Fungus name	**Data base ID number**	**Country of origin**	**Author reference**
E. pisi	AF011306	California, USA	Saenz and Taylor, 1999
E. pisi	AF073348	Australia	Cunnington *et al.,* 2003
E. trifolii	AB015913	Japan	Takamatsu *et al.,* 1999
E. trifolii	AB163926	Japan	Takamatsu *et al.,* 2004
E. diffusa	EF196675	Brazil	Almeida *et al*., 2008
E. glycines	AB015927	Japan	Takamatsu *et al.,* 1999
E. baeumleri	AB015933	Japan	Takamatsu *et al*., 1999

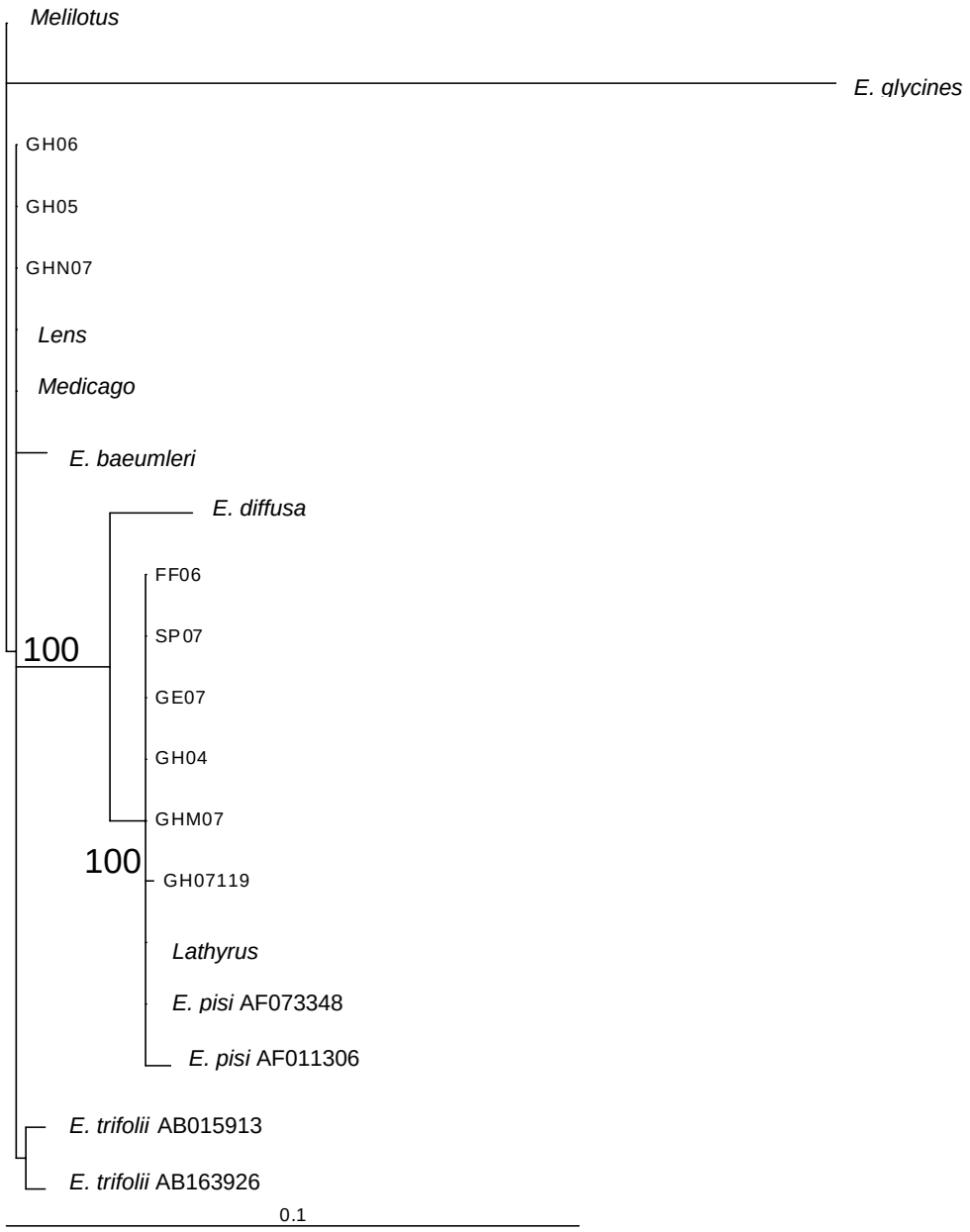

Figure 6.1 Maximum Likelihood tree based on the sequences of ITS-1 and ITS-2 region of two *E. trifolii*, two *E. pisi* and one *E. baeumleri* and the out group *E. glycines* from GenBank and the isolates used in this study. The bar indicates a distance of 0.1 base changes per hundred nucleotide positions. The numbers above branches represent the percent of 100 bootstrap replications which supported the branch.

APPENDIX 1

CLUSTAL 2.0.8 multiple sequence alignment of all the isolates used in this study and few GenBank sequences with their accession numbers.

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E.trifoliiAB015913      ----------------------AGTTCAGCGGGTATTCCTACCTGATTCGAGGTCAACC 37
E.trifoliiAB163926      TCCTCCGCTTATTGATATGCTTAAGTTCAGCGGGTATTCCTACCTGATTCGAGGTCAACC 60
GH05                    TCCTCCGCTTATTGATATGCTTAAGTTCAGCGGGTATTCCTACCTGATTCGAGGTCAACC 60
Medicago                ------------------------------------------------GGTCAACC 8
GHN07                   TCCTCCGCTTATTGATATGCTTAAGTTCAGCGGGTATTCCTACCTGATTCGAGGTCAACC 60
GH06                    TCCTCCGCTTATTGATATGCTTAAGTTCAGCGGGTATTCCTACCTGATTCGAGGTCAACC 60
Lens                    TCCTCCGCTTATTGATATGCTTAAGTTCAGCGGGTATTCCTACCTGATTCGAGGTCAACC 60
Melilotus               TCCTCCGCTTATTGATATGCTTAAGTTCAGCGGGTATTCCTACCTGATTCGAGGTCAACC 60
E.pisiAF011306          TTTTCCGCTTATTGATATGCTTAAGTTCAGCGGGTATTCCTACCTGATTCGAGGTCAACC 60
E.pisiAF073348          ------------------------------------CCTACCTGATTCGAGGTCAACC 22
GHM07                   TCCTCCGCTTATTGATATGCTTAAGTTCAGCGGGTATTCCTACCTGATTCGAGGTCAACC 60
SP07                    TCCTCCGCTTATTGATATGCTTAAGTTCAGCGGGTATTCCTACCTGATTCGAGGTCAACC 60
GH07-119                TCCTCCGCTTATTGATATGCTTAAGTTCAGCGGGTATTCCTACCTGATTCGAGGTCAACC 60
FF06                    TCCTCCGCTTATTGATATGCTTAAGTTCAGCGGGTATTCCTACCTGATTCGAGGTCAACC 60
GH04                    TCCTCCGCTTATTGATATGCTTAAGTTCAGCGGGTATTCCTACCTGATTCGAGGTCAACC 60
GE07                    TCCTCCGCTTATTGATATGCTTAAGTTCAGCGGGTATTCCTACCTGATTCGAGGTCAACC 60
Lathyrus                TCCTCCGCTTATTGATATGCTTAAGTTCAGCGGGTATTCCTACCTGATTCGAGGTCAACC 60
E.diffusaEF196675       TCCTCCGCTTATTGATATGCTTAAGTTCAGCGGGTATTCCTACCTGATTCGAGGTCAACC 60
E.baeumleriAB015933     ---------------------------------------TACCTGATTCGAGGTCAACC 20
E.glycinesAB015927      ----------------------GTTCAGCGGGTATTCCTACCTGATTCGAGGTCAACC 36
                                                                        ********

E.trifoliiAB015913      TGTGA--TCCATGTGACTGGA----GCAAAAGAGGGGTGTTCTGGCAAGCCACCGTCGTC 91
E.trifoliiAB163926      TGTGA--TCCATGTGACTGGA----GCAAAAGAGGGGTGTTCTGGCAAGCCACCGTCGTC 114
GH05                    TGTGA--TCCATGTGACTGGA----GCAAAAGAGGGGTGTTCTGGCAAGCCACCGTCGTC 114
Medicago                TGTGA--TCCATGTGACTGGA----GCAAAAGAGGGGTGTTCTGGCAAGCCACCGTCGTC 62
GHN07                   TGTGA--TCCATGTGACTGGA----GCAAAAGAGGGGTGTTCTGGCAAGCCACCGTCGTC 114
GH06                    TGTGA--TCCATGTGACTGGA----GCAAAAGAGGGGTGTTCTGGCAAGCCACCGTCGTC 114
Lens                    TGTGA--TCCATGTGACTGGA----GCAAAAGAGGGGTGTTCTGGCAAGCCACCGTCGTC 114
Melilotus               TGTGA--TCCATGTGACTGGA----GCAAAAGAGGGGTGTTCTGGCAAGCCACCGTCGTC 114
E.pisiAF011306          TGTGA--TCCATGTGACTGGA----GCAGATGAGGGTTGTTCTGGCAAGCCACCGTCGTC 114
E.pisiAF073348          TGTGA--TCCATGTGACTGGA----GCAGATGAGGGTTGTTCTGGCAAGCCACCGTCGTC 76
GHM07                   TGTGA--TCCATGTGACTGGA----GCAGATGAGGGTTGTTCTGGCAAGCCACCGTCGTC 114
SP07                    TGTGA--TCCATGTGACTGGA----GCAGATGAGGGTTGTTCTGGCAAGCCACCGTCGTC 114
GH07-119                TGTGA--TCCATGTGACTGGA----GCAGATGAGGGTTGTTCTGGCAAGCCACCGTCGTC 114
FF06                    TGTGA--TCCATGTGACTGGA----GCAGATGAGGGTTGTTCTGGCAAGCCACCGTCGTC 114
GH04                    TGTGA--TCCATGTGACTGGA----GCAGATGAGGGTTGTTCTGGCAAGCCACCGTCGTC 114
GE07                    TGTGA--TCCATGTGACTGGA----GCAGATGAGGGTTGTTCTGGCAAGCCACCGTCGTC 114
Lathyrus                TGTGA--TCCATGTGACTGGA----GCAGATGAGGGTTGTTCTGGCAAGCCACCGTCGTC 114
E.diffusaEF196675       TGTGA--TCCATGTGACTGGA----GCAAAAGAGGGTTGTTCTGGCAAGCCACCGTCGTC 114
E.baeumleriAB015933     TGTGA--TCCATGTGACTGGA----GCAAAAGACGGGTGTTCTGGCAAGCCACCGTCGTC 74
E.glycinesAB015927      TGTAAAATCCGTATGACCGGACTTGGTTAAAAGGGGGTTTTTTGGCTGATCATCGTCGTC 96
                        *** *  *** * **** ***    *   *    ** * ** ****    ** *******

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E.trifoliiAB163926      ACTCTGTCGCGAGAAGCAAGTTACTACGCGTAGAGCCCACGCCGGGACCGCCACTGTCTT 174
GH05                    ACTCTGTCGCGAGAAGCAAGTTACTACGCGTAGAGCCCACGCCGGGACCGCCACTGTCTT 174
Medicago                ACTCTGTCGCGAGAAGCAAGTTACTACGCGTAGAGCCCACGCCGGGACCGCCACTGTCTT 122
GHN07                   ACTCTGTCGCGAGAAGCAAGTTACTACGCGTAGAGCCCACGCCGGGACCGCCACTGTCTT 174
GH06                    ACTCTGTCGCGAGAAGCAAGTTACTACGCGTAGAGCCCACGCCGGGACCGCCACTGTCTT 174
Lens                    ACTCTGTCGCGAGAAGCAAGTTACTACGCGTAGAGCCCACGCCGGGACCGCCACTGTCTT 174
Melilotus               ACTCTGTCGCGAGAAGCAAGTTACTACGCGTAGAGCCCACGCCGGGACCGCCACTGTCTT 174
E.pisiAF011306          ACTCTGTCGCGAGAAGCAAGTTACTACGCGTAGAGCCCACGCCGGGACCGCCACTGTCTT 174
E.pisiAF073348          ACTCTGTCGCGAGAAGCAAGTTACTACGCGTAGAGCCCACGCCGGGACCGCCACTGTCTT 136
GHM07                   ACTCTGTCGCGAGAAGCAAGTTACTACGCGTAGAGCCCACGCCGGGACCGCCACTGTCTT 174
SP07                    ACTCTGTCGCGAGAAGCAAGTTACTACGCGTAGAGCCCACGCCGGGACCGCCACTGTCTT 174
GH07-119                ACTCTGTCGCGAGAAGCAAGTTACTACGCGTAGAGCCCACGCCGGGACCGCCACTGTCTT 174
FF06                    ACTCTGTCGCGAGAAGCAAGTTACTACGCGTAGAGCCCACGCCGGGACCGCCACTGTCTT 174
GH04                    ACTCTGTCGCGAGAAGCAAGTTACTACGCGTAGAGCCCACGCCGGGACCGCCACTGTCTT 174
GE07                    ACTCTGTCGCGAGAAGCAAGTTACTACGCGTAGAGCCCACGCCGGGACCGCCACTGTCTT 174
Lathyrus                ACTCTGTCGCGAGAAGCAAGTTACTACGCGTAGAGCCCACGCCGGGACCGCCACTGTCTT 174
E.diffusaEF196675       ACTCTGTCGCGAGAAGCAAGTTACTACGCGTAGAGCCCACGTCGGAACCGCCACTGTCTT 174
E.baeumleriAB015933     ACTCTGTCGCGAGAAGCAAGTTACTACGCGTAGAGCCCACGCCGGGACCGCCACTGTCTT 134
E.glycinesAB015927      ACTCTGTCGCGAGAATCAAGTTACTACGCGTAGAGCCCACGCCGGGACCGCCACTGTCTT 156
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E.trifoliiAB015913      TAAGGGCCGCCGCAT--CGCGACGTGCCCCAACACCGCAGCCACACAAAGGCAGCTGGAG 209
E.trifoliiAB163926      TAAGGGCCGCCGCAT--CGCGACGTGCCCCAACACCGCAGCCACACAAAGGCAGCTGGAG 232
GH05                    TAAGGGCCGCCGCAT--CGCGACGTGCCCCAACACCGCAGCCACACAAAGGCAGCTGGAG 232
Medicago                TAAGGGCCGCCGCAT--CGCGACGTGCCCCAACACCGCAGCCACACAAAGGCAGCTGGAG 180
GHN07                   TAAGGGCCGCCGCAT--CGCGACGTGCCCCAACACCGCAGCCACACAAAGGCAGCTGGAG 232
GH06                    TAAGGGCCGCCGCAT--CGCGACGTGCCCCAACACCGCAGCCACACAAAGGCAGCTGGAG 232
Lens                    TAAGGGCCGCCGCAT--CGCGACGTGCCCCAACACCGCAGCCACACAAAGGCAGCTGGAG 232
Melilotus               TAAGGGCCGCCGCGT--CGCGACGTGCCCCAACACCGCAGCCACACAAAGGCAGCTGGAG 232
E.pisiAF011306.         TAAGGGCCGCCGCTT--CGCGACGAGCCCCAACACCGCAGCCACACAAAGGCAGCTGGAG 232
E.pisiAF073348          TAAGGGCCGCCGCTT--CGCGACGAGCCCCAACACCGCAGCCACACAAAGGCAGCTGGAG 194
GHM07                   TAAGGGCCGCCGCTT--CGCGACGAGCCCCAACACCGCAGCCACACAAAGGCAGCTGGAG 232
SP07                    TAAGGGCCGCCGCTT--CGCGACGAGCCCCAACACCGCAGCCACACAAAGGCAGCTGGAG 232
GH07-119                TAAGGGCCGCCGCTT--CGCGACGAGCCCCAACACCGCAGCCACACAAAGGCAGCTGGAG 232
FF06                    TAAGGGCCGCCGCTT--CGCGACGAGCCCCAACACCGCAGCCACACAAAGGCAGCTGGAG 232
GH04                    TAAGGGCCGCCGCTT--CGCGACGAGCCCCAACACCGCAGCCACACAAAGGCAGCTGGAG 232
GE07                    TAAGGGCCGCCGCTT--CGCGACGAGCCCCAACACCGCAGCCACACAAAGGCAGCTGGAG 232
Lathyrus                TAAGGGCCGCCGCTT--CGCGACGAGCCCCAACACCGCAGCCACACAAAGGCAGCTGGAG 232
E.diffusaEF196675.      TAAGGGCCGCCGCAT--CGCGACGAGCCCCAACACCGCAGCCACACAATGGCAGCTGGAG 232
E.baeumleriAB015933     TAAGGGCCGCCGCAT--CGCGACGTGCCCCAACACCGCAGCCACACAAAGGCAGCTGGAG 192
E.glycinesAB015927      TAAGAGCCACCGCGAGACGCGGCGAGCCCCAACACCACAGCCACACAACGGTAGCTCGAG 216
                        **** *** ****    **** ** *********** *********** ** **** ***

E.trifoliiAB015913      GGGGTGTTATGACGCTCGAACAGGCATGCCCCTCGGAATACCAAGGGGCGCAATGTGCGT 269
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GH05                    GGGGTGTTATGACGCTCGAACAGGCATGCCCCTCGGAATACCAAGGGGCGCAATGTGCGT 292
Medicago                GGGGTGTTATGACGCTCGAACAGGCATGCCCCTCGGAATACCAAGGGGCGCAATGTGCGT 240
GHN07                   GGGGTGTTATGACGCTCGAACAGGCATGCCCCTCGGAATACCAAGGGGCGCAATGTGCGT 292
GH06                    GGGGTGTTATGACGCTCGAACAGGCATGCCCCTCGGAATACCAAGGGGCGCAATGTGCGT 292
Lens                    GGGGTGTTATGACGCTCGAACAGGCATGCCCCTCGGAATACCAAGGGGCGCAATGTGCGT 292
Melilotus               GGGGTGTTATGACGCTCGAACAGGCATGCCCCTCGGAATACCAAGGGGCGCAATGTGCGT 292
E.pisiAF011306.         GGGGTGTTATGACGCTCGAACAGGCATGCCCCTCGGAATACCAAGGGGCGCAATGTGCGT 292
E.pisiAF073348          GGGGTGTTATGACGCTCGAACAGGCATGCCCCTCGGAATACCAAGGGGCGCAATGTGCGT 254
GHM07                   GGGGTGTTATGACGCTCGAACAGGCATGCCCCTCGGAATACCAAGGGGCGCAATGTGCGT 292
SP07                    GGGGTGTTATGACGCTCGAACAGGCATGCCCCTCGGAATACCAAGGGGCGCAATGTGCGT 292
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Lathyrus                GGGGTGTTATGACGCTCGAACAGGCATGCCCCTCGGAATACCAAGGGGCGCAATGTGCGT 292
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E.glycinesAB015927      GGGGTGTTATGACGCTCGAACAGGCATGCCCCTCGGAATACCAAGGGGCGCAATGTGCGT 276
                        ******* ************************************************

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GH05                    TCAAAGATTCGATGATTCACTAAATTCTGCAATTCACATTACTTATCGCATTTCGCTGCG 352
Medicago                TCAAAGATTCGATGATTCACTAAATTCTGCAATTCACATTACTTATCGCATTTCGCTGCG 300
GHN07                   TCAAAGATTCGATGATTCACTAAATTCTGCAATTCACATTACTTATCGCATTTCGCTGCG 352
GH06                    TCAAAGATTCGATGATTCACTAAATTCTGCAATTCACATTACTTATCGCATTTCGCTGCG 352
Lens                    TCAAAGATTCGATGATTCACTAAATTCTGCAATTCACATTACTTATCGCATTTCGCTGCG 352
Melilotus               TCAAAGATTCGATGATTCACTAAATTCTGCAATTCACATTACTTATCGCATTTCGCTGCG 352
E.pisiAF011306.         TCAAAGATTCGATGATTCACTAAATTCTGCAATTCACATTACTTATCGCATTTCGCTGCG 352
E.pisiAF073348          TCAAAGATTCGATGATTCACTAAATTCTGCAATTCACATTACTTATCGCATTTCGCTGCG 314
GHM07                   TCAAAGATTCGATGATTCACTAAATTCTGCAATTCACATTACTTATCGCATTTCGCTGCG 352
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GH07-119                TCAAAGATTCGATGATTCACTAAATTCTGCAATTCACATTACTTATCGCATTTCGCTGCG 352
FF06                    TCAAAGATTCGATGATTCACTAAATTCTGCAATTCACATTACTTATCGCATTTCGCTGCG 352
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GE07                    TCAAAGATTCGATGATTCACTAAATTCTGCAATTCACATTACTTATCGCATTTCGCTGCG 351
Lathyrus                TCAAAGATTCGATGATTCACTAAATTCTGCAATTCACATTACTTATCGCATTTCGCTGCG 352
E.diffusaEF196675.      TCAAAGATTCGATGATTCACTAAATTCTGCAATTCACATTACTTATCGCATTTCGCTGCG 352
E.baeumleriAB015933     TCAAAGATTCGATGATTCACTAAATTCTGCAATTCACATTACTTATCGCATTTCGCTGCG 312
E.glycinesAB015927      TCAAAGATTCGATGATTCACTAAATTCTGCAATTCACATTACTTATCGCATTTCGCTGCG 336
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E.trifoliiAB015913      TTCTTCATCGATGCCAGAGCCAAGAGATCCGTTGTTGAAAGTTTTATCAATTTTCATAAT 389
E.trifoliiAB163926      TTCTTCATCGATGCCAGAGCCAAGAGATCCGTTGTTGAAAGTTTTATCAATTTTCATAAT 412
GH05                    TTCTTCATCGATGCCAGAGCCAAGAGATCCGTTGTTGAAAGTTTTATCAATTTTCATAAT 412
Medicago                TTCTTCATCGATGCCAGAGCCAAGAGATCCGTTGTTGAAAGTTTTATCAATTTTCATAAT 360
GHN07                   TTCTTCATCGATGCCAGAGCCAAGAGATCCGTTGTTGAAAGTTTTATCAATTTTCATAAT 412
GH06                    TTCTTCATCGATGCCAGAGCCAAGAGATCCGTTGTTGAAAGTTTTATCAATTTTCATAAT 412
Lens                    TTCTTCATCGATGCCAGAGCCAAGAGATCCGTTGTTGAAAGTTTTATCAATTTTCATAAT 412
Melilotus               TTCTTCATCGATGCCAGAGCCAAGAGATCCGTTGTTGAAAGTTTTATCAATTTTCATAAT 412
E.pisiAF011306.         TTCTTCATCGATGCCAGAGCCAAGAGATCCGTTGTTGAAAGTTTTATCAATTTTCATAAT 412
E.pisiAF073348          TTCTTCATCGATGCCAGAGCCAAGAGATCCGTTGTTGAAAGTTTTATCAATTTTCATAAT 374
GHM07                   TTCTTCATCGATGCCAGAGCCAAGAGATCCGTTGTTGAAAGTTTTATCAATTTTCATAAT 412
SP07                    TTCTTCATCGATGCCAGAGCCAAGAGATCCGTTGTTGAAAGTTTTATCAATTTTCATAAT 412
GH07-119                TTCTTCATCGATGCCAGAGCCAAGAGATCCGTTGTTGAAAGTTTTATCAATTTTCATAAT 412
FF06                    TTCTTCATCGATGCCAGAGCCAAGAGATCCGTTGTTGAAAGTTTTATCAATTTTCATAAT 412
GH04                    TTCTTCATCGATGCCAGAGCCAAGAGATCCGTTGTTGAAAGTTTTATCAATTTTCATAAT 412
GE07                    TTCTTCATCGATGCCAGAGCCAAGAGATCCGTTGTTGAAAGTTTTATCAATTTTCATAAT 411
Lathyrus                TTCTTCATCGATGCCAGAGCCAAGAGATCCGTTGTTGAAAGTTTTATCAATTTTCATAAT 412
E.diffusaEF196675.      TTCTTCATCGATGCCAGAGCCAAGAGATCCGTTGTTGAAAGTTTTATCAATTTTCATAAT 412
E.baeumleriAB015933     TTCTTCATCGATGCCAGAGCCAAGAGATCCGTTGTTGAAAGTTTTATCAATTTTCATAAT 372
E.glycinesAB015927      TTCTTCATCGATGCCAGAGCCAAGAGATCCGTTGTTGAAAGTTTTATCAATTAT-ATACT 395
                        ************************************************* * *** *

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E.trifoliiAB163926      AAAGCTGAGACGAC----ACAAACAACATGAGTTTTGGTTGGGTCTTTGGCGGGCGCGCT 468
GH05                    AAAGCTGAGACGAC----ACAAACAACATGAGTTTTGGTTGGGTCTTTGGCGGGCGCGCT 468
Medicago                AAAGCTGAGACGAC----ACAAACAACATGAGTTTTGGTTGGGTCTTTGGCGGGCGCGCT 416
GHN07                   AAAGCTGAGACGAC----ACAAACAACATGAGTTTTGGTTGGGTCTTTGGCGGGCGCGCT 468
GH06                    AAAGCTGAGACGAC----ACAAACAACAT-AGTTTTGGTTGGGTCTTTGGCGGGCGCGCT 467
Lens                    AAAGCTGAGACGAC----ACAAACAACATGAGTTTTGGTTGGGTCTTTGGCGGGCGCGCT 468
Melilotus               AAAGCTGAGACGAC----ACAAACAACATGAGTTTTGGTTGGGTCTTTGGCGGGCGCGCT 468
E.pisiAF011306.         AAAGCTGAGACGAC----ACAAACAACATGAGTTTTGGTTGGGTCTTTGGCGGGCGCGCT 468
E.pisiAF073348          AAAGCTGAGACGAC----ACAAACAACATGAGTTTTGGTTGGGTCTTTGGCGGGCGCGCT 430
GHM07                   AAAGCTGAGACGAC----ACAAACAACATGAGTTTTGGTTGGGTCTTTGGCGGGCGCGCT 468
SP07                    AAAGCTGAGACGAC----ACAAACAACATGAGTTTTGGTTGGGTCTTTGGCGGGCGCGCT 468
GH07-119                AAAGCTGAGACGAC----ACAAACAACATGAGTTTTGGTTGGGTCTTTGGCGGGCGCGCT 468
FF06                    AAAGCTGAGACGAC----ACAAACAACATGAGTTTTGGTTGGGTCTTTGGCGGGCGCGCT 468
GH04                    AAAGCTGAGACGAC----ACAAACAACATGAGTTTTGGTTGGGTCTTTGGCGGGCGCGCT 468
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Lathyrus                AAAGCTGAGACGAC----ACAAACAACATGAGTTTTGGTTGGGTCTTTGGCGGGCGCGCT 468
E.diffusaEF196675.      AAAGCTGAGACGAT----ACAAACAACATGAGTTTTGGTTGGGTCTTTGGCGGGCGCGCT 468
E.baeumleriAB015933     AAAGCTGAGACGAC----ACAGACAACATGAGTTTTGGTTGGGTCTTTGGCGGGCGCGCT 428
E.glycinesAB015927      TTTACTTAGACTACCATGACAAATATAAGAGTTTTGGGTTGGGTCTTTGGCGGACACGCT 455
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E.trifoliiAB015913      CCAGTTCTAAACCGGTGGG-CGGCCGACGCACGTCCTTGCGAACAGCGACGACGCGGCCC 504
E.trifoliiAB163926      CCAGTTCTAAACCGGTGGG-CGGCCGACGCACGTCCTTGCGAACAGCGACGACGCGGCCC 527
GH05                    CCAGTTCAAAACCGGTGGG-CGGCCGACGCAGGTCCTTGCGAACAGCGACGACGCGGCCC 527
Medicago                CCAGTTCAAAACCGGTGGG-CGGCCGACGCAGGTCCTTGCGAACAGCGACGACGCGGCCC 475
GHN07                   CCAGTTCAAAACCGGTGGG-CGGCCGACGCAGGTCCTTGCGAACAGCGACGACGCGGCCC 527
GH06                    CCAGTTCAAAACCGGTGGG-CGGCCGACGCAGGTCCTTGCGAACAGCGACGACGCGGCCC 526
Lens                    CCAGTTCAAAACCGGTGGG-CGGCCGACGCAGGTCCTTGCGAACAGCGACGACGCGGCCC 527
Melilotus               CCAGTTCAAAACCGGTGGG-CGGCCGACGCAGGTCCTTGCGAACAGCGACGACGCGGCCC 527
E.pisiAF011306.         CCAGTTGAAAACCGGTGGG-CGGCCGACGCATGTCCATGCGGACAGCGACAGCGCGGCCC 527
E.pisiAF073348          CCAGTTGAAAACCGGTGGG-CGGCCGACGCATGTCCATGCGGACAGCGACAGCGCGGCCC 489
GHM07                   CCAGTTGAAAACCGGTGGG-CGGCCGACGCATGTCCATGCGGACAGCGACAGCGCGGCCC 527
SP07                    CCAGTTGAAAACCGGTGGG-CGGCCGACGCATGTCCATGCGGACAGCGACAGCGCGGCCC 527
GH07-119                CCAGTTGAAAACCGGTGGG-CGGCCGACGCATGTCCATGCGGACAGCGACAGCGCGGCCC 527
FF06                    CCAGTTGAAAACCGGTGGG-CGGCCGACGCATGTCCATGCGGACAGCGACAGCGCGGCCC 527
GH04                    CCAGTTGAAAACCGGTGGG-CGGCCGACGCATGTCCATGCGGACAGCGACAGCGCGGCCC 527
GE07                    CCAGTTGAAAACCGGTGGG-CGGCCGACGCATGTCCATGCGGACAGCGACAGCGCGGCCC 526
Lathyrus                CCAGTTGAAAACCGGTGGG-CGGCCGACGCATGTCCATGCGGACAGCGACAGCGCGGCCC 527
E.diffusaEF196675.      CCAGTGGAACACCGGGGGGGCGGCCGACGCATGTCCATGCGGACTGCAACAGCGCGGCCC 528
E.baeumleriAB015933     CCAGTTCAAGACCGGTGGG-CGGCCGACGCAGGTCCTTGCGAACAGCGACGACGCGGCCC 487
E.glycinesAB015927      CCAGCCAAA-GCCGGTGGG-CAGCTCGCACGCGTCACTGTGAACGACGTGGACG-GTCCC 512
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E.trifoliiAB163926      GGCCCGCCAAAGCAACAAGATACAAA-TCGACACGGGTGGGAGGGTCGGCCCAGCACGCG 586
GH05                    GGCCCGCCAAAGCAACAAGATACAAA-TCGACACGGGTGGGAGGGTCGGCCCAGCACGCG 586
Medicago                GGCCCGCCAAAGCAACAAGATACAAA-TCGACACGGGTGGGAGGGTCGGCCCAGCACGCG 534
GHN07                   GGCCCGCCAAAGCAACAAGATACAAA-TCGACACGGGTGGGAGGGTCGGCCCAGCACGCG 586
GH06                    GGCCCGCCAAAGCAACAAGATACAAA-TCGACACGGGTGGGAGGGTCGGCCCAGCACG-G 584
Lens                    GGCCCGCCAAAGCAACAAGATACAAA-TCGACACGGGTGGGAGGGTCGGCCCAGCACGCG 586
Melilotus               GGCCCGCCAAAGCAACAAGATACAAA-TCGACACGGGTGGGAGGGTCGGCCCAGCACGCG 586
E.pisiAF011306.         GGCCCGCCAAAGCAACAAGATACAAA-TCGACACGGGTGGGAGGGTCGGCCCAGCACGCA 586
E.pisiAF073348          GGCCCGCCAAAGCAACAAGATACAAA-TCGACACGGGTGGGAGGGTCGGCCCAGCACGCA 548
GHM07                   GGCCCGCCAAAGCAACAAGATACAAA-TCGACACGGGTGGGAGGGTCGGCCCAGCACGCA 586
SP07                    GGCCCGCCAAAGCAACAAGATACAAA-TCGACACGGGTGGGAGGGTCGGCCCAGCACGCA 586
GH07-119                GGCCCGCCAAAGCAACAAGATACAAA-TCGACACGGGTGGGAGGGTCGGCCCAGCACGCA 586
FF06                    GGCCCGCCAAAGCAACAAGATACAAA-TCGACACGGGTGGGAGGGTCGGCCCAGCACGCA 586
GH04                    GGCCCGCCAAAGCAACAAGATACAAA-TCGACACGGGTGGGAGGGTCGGCCCAGCACGCA 586
GE07                    GGCCCGCCAAAGCAACAAGATACAAA-TCGACACGGGTGGGAGGGTCGGCCCAGCACGCA 585
Lathyrus                GGCCCGCCAAAGCAACAAGATACAAA-TCGACACGGGTGGGAGGGTCGGCCCAGCACGCA 586
E.diffusaEF196675.      GGCCCGCCAAAGCAACAAGATACAAA-TCGACACGGGTGGGAGGGTCGGCCCAGCACGCA 587
E.baeumleriAB015933     GGCCCGCCAAAGCAACAAGATACAAA-TCGACACGGGTGGGAGGGTCGGCCCAGCACGCG 546
E.glycinesAB015927      GGCCCGCCAAAGCAACAATCTATAAAATCGACACGGGTGGGAGGGTCGGCCCAGCACGTG 572
                        ******************  ** *** ****************************

E.trifoliiAB015913      GCCGACGCCG-CGACTGAGCCTCGCACTCTGTAAT------------------------- 597
E.trifoliiAB163926      GCCGACGCCG-CGACTGAGCCTCGCACTCTGTAATGATCCTTCCGCAGGTTCACCTACGG 645
GH05                    GCAGACGCCG-CGACTGAGCCTCGCACTCTGTAATGATCCTTCCGCAGGTTCACCTACGG 645
Medicago                GCAGACGCCG-CGACTGAGCCTCGCACTCTGTAATGATCCTTCCGCA------------ 580
GHN07                   GCAGACGCCG-CGACTGAGCCTCGCACTCTGTAATGATCCTTCCGCAGGTTCACCTACGG 645
GH06                    GCAGACGCCG-CGACTGAGCCTCGCACTCTGTAATGATCCTTCCGCAGGTTCACCTACGG 643
Lens                    GCAGACGCCG-CGACTGAGCCTCGCACTCTGTAATGATCCTTCCGCAGGTTCACCTACGG 645
Melilotus               GCAGACGCCG-CGACTGAGCCTCGCACTCTGTAATGATCCTTCCGCAGGTTCACCTACGG 645
E.pisiAF011306.         GCAGATGCCA-CGACTGAGCCTCGCACTCTGTATG-ATCCTTCCGCAGGTTCACCTACGG 644
E.pisiAF073348          GCAGATGCCA-CGACTGAGCCTCGCACTCTGTAAT------------------------- 582
GHM07                   GCAGATGCCA-CGACTGAGCCTCGCACTCTGTAATGATCCTTCCGCAGGTTCACCTACGG 645
SP07                    GCAGA-GCCA-CGACTGA---------------------------------------- 602
GH07-119                GCAGATGCCA-CGACTGAGCCCCGCACTCTGTAATGATCCTTCCGCAGGTTCACCTACGG 645
FF06                    GCAGATGCCA-CGACTGAGCCTCGCACTCTGTAATGATCCTTCCGCAGGTTCACCTACGG 645
GH04                    GCAGATGCCA-CGACTGAGCCTCGCACTCTGTAATGATCCTTCCGCAGGTTCACCTACGG 645
GE07                    GCAGATGCCA-CGACTGAGCCTCGCACTCTGTAATGATCCTTCCGCAGGTTCACCTACGG 644
Lathyrus                GCAGATGCCA-CGACTGAGCCTCGCACTCTGTAATGATCCTTCCGCAGGTTCACCTACGG 645
E.diffusaEF196675.      GCAGACGCCA-CGACTGAGCCTCGCACTCTGTAATGATCCTTCCGCAGGTTCACCTACGG 646
E.baeumleriAB015933     GCAGACGCCG-CGACTGAGCCTCGCACTCTGTAAT------------------------- 580
E.glycinesAB015927      GCAGATGCCAGCGACTGAGCCTCACGCTCTGTAATG------------------------ 608
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APPENDIX 2. Pair-wise comparisons of ITS sequences among samples and GenBank accessions used in study

Powdery mildew isolates & GenBank accessions	1	2	3	4	5	6	7	8	9	10	11	12
1. FF 06[a]	100											
2. GH N 07[b]	97 632/646	100										
3. LSP 07[c]	97 632/646	100	100									
4. *Melilotus albus*	97 631/646	99 644/646	99% 644/646	100								
5. *Medicago lupulina*.	97 566/580	100 580/580	100 580580	99 578/580	100							
6. *Lathyrus* spp.	100 646/646	97 632/646	97 632/646	97 631/646	97 566/580	100						
7. *Glycine* spp.	97 633/647	96 625/647	96 627/647	96 626/647	96 561/581	97 633/647	100					
8. *E. pisi* AF011306	99 642/643	97 629/644	97 629/644	97 628/644	97 566/581	99 642/643	97 629/644	100				
9. *E. trifolii* AB163926	97 630/646	99 642/645	99 642/645	99 640/645	99 577/580	97 630/646	96 626/648	97 631/650	100			
10. *E. trifolii* AB015913	97 581/597	99 594/597	99 594/597	99 592/597	99 565/568	97 581/597	96 576/598	97 580/596	100 597/597	100		
11. *E. diffusa* EF196675	97 633/647	96 628/648	96 628/648	96 626/648	96 562/582	97 633/647	100 647/647	97 651/666	96 631/655	96 576/598	100	
12. *E. baeumleri* AB015933	97 563/580	99 577/580	99 577/580	98 566/572	99 565/568	99 575/580	96 559/581	97 563/580	100 597/597	98 575/581	96 561/583	100

[a] Five additional isolates (GE 07, SP 07, GH M 07, GH 07-119 & GH 04) have identical sequences.

[b] Two additional isolates (GH 05 & GH 06) have identical sequences.

[c] Three additional lentil isolates (LGH 07, LGH 07-119, LGH N 07) have identical sequences.

In each comparison, percentage similarity and number of nucleotides similar over total number of nucleotides is shown.