A NEW DISEASE OF GRASSPEA (*LATHYRUS SATIVUS*) CAUSED BY *ASCOCHYTA LENTIS* var. *LATHYRI*

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SUMMARY

Ascochyta lentis var. *lathyri* is described for the first time infecting grasspea (*Lathyrus sativus* L.) in Italy. This fungus causes necrotic lesions on leaves and stems of grasspea plants. Fungal isolates obtained from these lesions were able to infect grasspea under controlled environmental conditions and induced symptoms similar to those observed in the field. Moreover, these isolates were not pathogenic to seedlings of nine other leguminous species including lentil. Sequence analysis of the nuclear ribosomal internal transcribed spacer, as well as fast-evolving protein-coding loci chitin synthase, translation elongation factor 1-alpha, glyceraldehyde-3-phosphate dehydrogenase, revealed 99.6% to 100% similarity to sequences from *Ascochyta lentis* Vassiljevsky. Morphological analyses using elliptic fourier analysis and MANOVA demonstrated that conidial dimensions of the grasspea fungus were significantly different from those of *A. lentis*. Crosses between the grasspea fungus and *A. lentis* were successful and produced progeny with normal cultural morphology and growth rates. Hybrid status of the progeny was confirmed by segregation of mating type and microsatellite markers. These results indicate that the fungus infecting grasspea is a pathogenic and morphological variant of *Ascochyta lentis* and should be named *Ascochyta lentis* var. *lathyri.*

*Keywords***:** legumes, fungi, taxonomy, EFA analysis, host specificity.

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INTRODUCTION

There is a great potential for the cultivation of grasspea (*Lathyrus sativus* L.) worldwide due to its agronomic potential as source of protein in drought prone marginal areas (Vaz Patto *et al*., 2006) and as a source of disease resistance for several important legume crops (Almeida *et al*., 2015). Grasspea is considered as an important source of resistance to ascochyta blight of pea caused by *Peyronellaea pinodes* (Berk. & A. Bloxam) Aveskamp, Gruyter & Verkley (synonym: *Mycosphaerella pinodes* (Berk. et Bloxam) Vestergren (Gurung *et al*., 2002). In Italy, grasspea is a minor legume crop of local and historical interest and is often restricted to private gardens. Nevertheless, in recent years this plant has gained renewed interest for low input and organic agricultural systems of central and southern Italy for the preservation of crop biodiversity (Crinò *et al*., 2004) and as an historically important food source in Italy and the Mediterranean. Powdery mildew caused by *Erysiphe pisi* DC, rust caused by *Uromyces pisi* (Pers. Schrot), downy mildew caused by *Peronospora lathipalustris* Gaumann, and the parasitic weed *Orobanche crenata* are considered the major diseases infecting grasspea (Campbell *et al*., 1994; Vaz Patto and Rubiales, 2014). *Ascochyta lathyri* Trail was described as causing leaf spot of *Lathyrus sylvestris* in 1887 and subsequently re-examined by Mel'nik and Braun (1999). Recently, the holotype of this species (*Ascochyta lathyri* CBS 423.67) has been re-classified as *Boeremia exigua* var. *pseudolilacis* using a polyphasic approach with multi-locus sequence data (Chen *et al*., 2015). Thirteen grasspea ecotypes (Pietranera, Altamura, Calitri, S. Gerardo, Colliano, S. Rufo, Grottaminarda, Carife, Monti Frentani, Palazzo S. Gervasio, Castelcivita, Bari, Marchigiano) collected from Central and Southern Italy, were grown in experimental trials performed in Battipaglia (Salerno, Italy, 40°34'59.8"N 14°58'53.1"E), and Corleto Perticara (Potenza, Italy, 40°22'01.89"N 16°04'33.02"E) for agronomic adaptation (Lioi *et al*., 2011). The first site was located at the CREA-ORT experimental

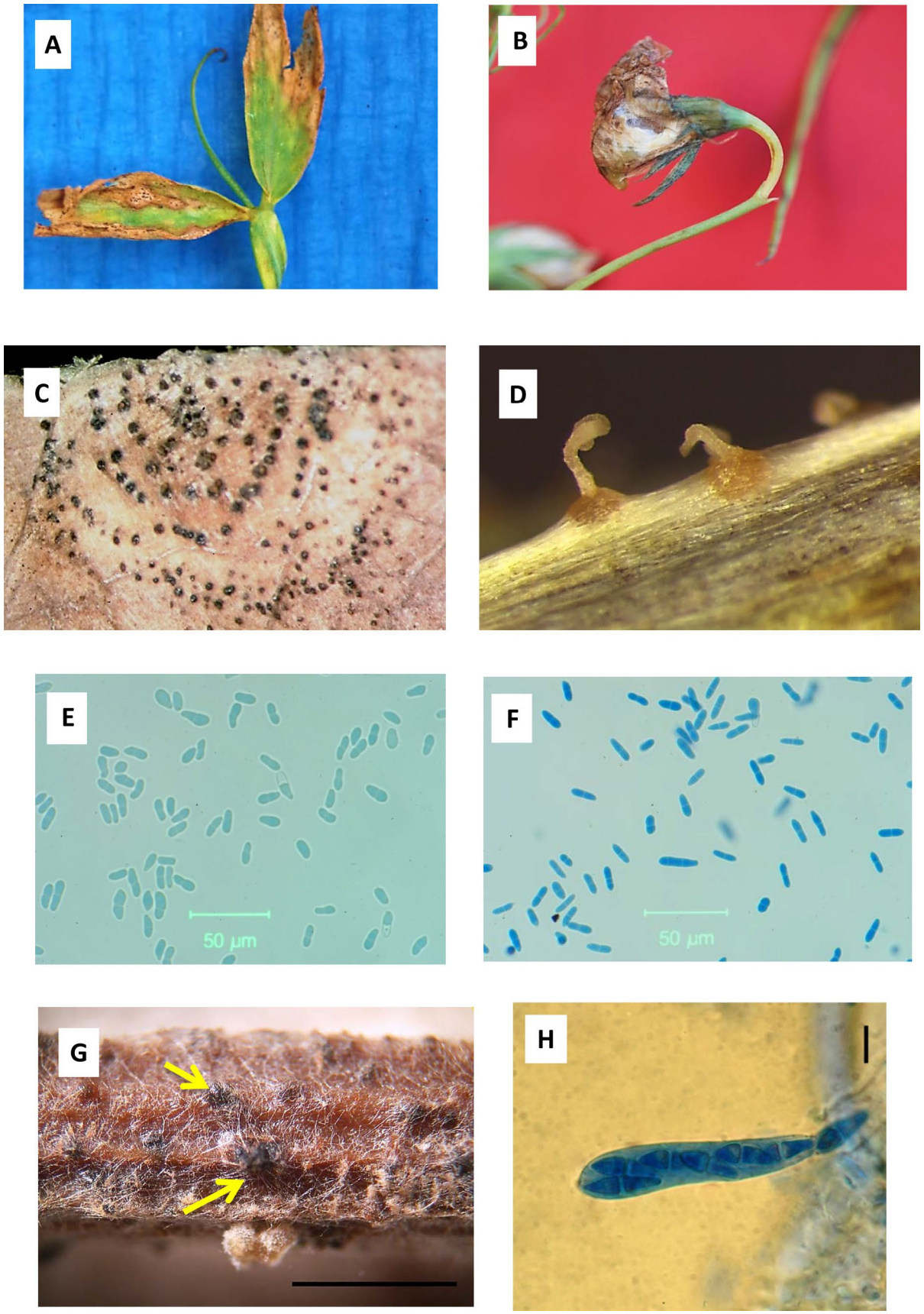

Fig. 1. Symptoms and vegetative and reproductive structures of *A. lentis var. lathyri*: (A) necroses on leaf (B) necroses on flower; (C) pycnidia organized in concentric rings; (D) conidia in cirri erumpent from pycnidia; (E) conidia of *A. lentis.* var. *lathyri;* (F) conidia of *A. lentis*; (G) pseudothecia (arrows) on sterile chickpea stems from crosses between *A. lentis* and *A. lentis.* var. *lathyri* (bar = 1 mm); (H) magnification of an ascus and ascospores (bar = 10 µm).

farm in Battipaglia (Sele Valley, Campania Region), about 65 m above sea level and characterized by annual rainfall of 947 mm and an annual mean temperature of 17°C. The second site was located at the experimental farm of Basilicata University, at Corleto Perticara (Agri Valley, Basilicata Region), about 720 m above the sea level, in a hilly inland region and characterized by an annual mean rainfall of 646 mm and an annual mean temperature of 14°C. Phytopathological surveys were carried out in these plots during 2006 and 2007. On some plants, yellowish necrotic lesions with dark margins and containing black pycnidia were observed (Fig. 1A). Lesions were scattered on leaves, stems and occasionally on flowers (Fig. 1B), confluent or restricted to the margins. Pycnidia were semi-immersed, often organized in concentric rings (Fig. 1C); conidia erumpent from pycnidia as cirri were sometimes observed (Fig. 1D). Symptomatic tissues and later seeds from the same plots at harvest were collected and analyzed in the lab. The objective of this research was to characterize fungal isolates collected on grasspea using biological, molecular and morphological tools. Preliminary results have been published (Infantino *et al*., 2007).

MATERIALS AND METHODS

Isolation of the fungus. Symptomatic grasspea tissues were disinfested with NaOCl (2% active Cl_2) for 2 min, rinsed three times with sterile water (SW) and then placed on potato dextrose agar (PDA) amended with 100 ppm each of streptomycin sulphate and ampicillin. For isolation from seeds, several grasspea seed lots were analyzed. Seeds (100 per lot) were disinfested with NaOCl for 5 min, rinsed three times with SW, and similarly placed on PDA with antibiotic and incubated for 7 days, at 23°C under near ultraviolet (NUV) light (Philips TLD 18W/08 Blacklight Blue Fluorescent Lamp, peak 360 nm) with a 12 h photoperiod. Single conidia were isolated from developing colonies and transferred to fresh PDA plates. For long-term storage, isolates were transferred to PDA in test tubes under mineral oil at 4°C as well as to sterile filter papers at –20°C (Peever *et al*., 1999).

Estimation of optimum growth temperature. Growth rates of three grasspea isolates (ER1415, ER1478, ER1819) were estimated at 7 temperatures (5°, 10°, 15°, 20°, 25°, 30°, and 35°C) by placing 5-mm-diameter mycelial plugs of each fungus from actively growing colonies on PDA face down on fresh PDA plates and incubated at each temperature in complete darkness. Colony diameters, estimated as the average of two perpendicular transects per colony, were measured after 3, 7 and 10 days of growth and expressed as average daily growth in mm.

Molecular identification. For molecular identification, DNA of two grasspea isolates, ER1413 and ER1415, was extracted from powdered, lyophilized mycelium using a Qiagen DNA Easy Kit (Qiagen). Genomic regions used for previous systematic studies of *Ascochyta* fungi (Peever *et al*., 2007), as well as many other plant-pathogenic fungi, were chosen. The nuclear ribosomal internal transcribed spacer (*ITS*), translation elongation factor 1-alpha (*EF*), chitin synthase (*CHS*) and glyceraldehyde-3-phosphate dehydrogenase (*G3PD*) were amplified with primer pairs ITS 5 and ITS 4 (White *et al*., 1990), EF1-728F and EF1-986R (Carbone and Kohn, 1999), CHS-79 and CHS-354 (Carbone and Kohn, 1999), gpd-1 and gpd-2 (Berbee *et al*., 1999), respectively. PCR amplicons were sequenced on both strands, and consensus sequences were assembled for each region. Homology searches were performed in GenBank using the Basic Local Alignment Search Tool (BLASTn) (http://blast.ncbi.nlm.nih.gov/Blast.cgi) (Altschul *et al*., 1990). GenBank matches in excess of 99% similarity were recorded. Sequences were submitted to GenBank with accession numbers KX138467 to KX138470.

Pathogenicity and host specificity. In an initial experiment, the pathogenicity and host specificity of one grasspea isolate (ER-1413) was assessed by inoculating several legume species (Table 1). For inoculum production, conidia were harvested from two-week-old colonies grown on PDA under alternating cycles of 12 h of light/darkness at room temperature, by adding sterile water and gently scraped-off with a glass rod. Conidial suspensions were adjusted at a concentration of 3×10^5 spores ml^{-1} and sprayed to run-off on two-weeks-old seedlings in the greenhouse at 20 ± 3°C. Control plants were sprayed with sterile water. One-hundred percent humidity was maintained by covering plants with plastic bags for the first 48 h. Symptoms were evaluated 15 days post-inoculation. In a second independent experiment, two lentil cultivars (Pardina and Eston) and four grasspea accessions (PI232923, PI209789, PI345525, PI422532) were inoculated with two *A. lentis* isolates: AL1 (ATCC 96419) and AL6 (ATCC 46982), and two grasspea isolates (ER1413 and ER1415). Inoculum was prepared as described above. Plants were maintained in a growth chamber at 20°C under alternating cycles of 12 h light-darkness and approx. 90% humidity. Each treatment (fungal isolate × lentil cultivar or grasspea accession) was replicated four times and the experiment conducted twice. Plants were examined 4, 5, 7, 8, 10, 11, 12 and 15 days post- inoculation. All aerial parts of each plant in each pot were examined. Disease severity was recorded using a parametric rating scale based on percent of diseased plant area and the area under the disease progress curve (AUDPC) was calculated to estimate the aggressiveness of each isolate.

Morphological characterization of conidia using Fourier image analysis. Conidia of two isolates of *Ascochyta* sp. from grasspea (Fig. 1E) (ER 1413 and ER 1478), and

Table 1. List of legume species utilized for host specific determination by artificial inoculation with grasspea isolate ER 1413.

Species	Common name	Cv/ecotype
Lathyrus sativus L	grasspea	Marchigiano; Castelcivita; Calitri; Rufo; Foggia.
Lens culinaris Medik.	lentil	Gaia; ecotipo di Linosa; Miccula; Castelluccio di Norcia; Valle di Nerola.
Pisum sativum L.	pea	Jumbo; Baccarà.
Vicia faba minor Beck	field bean	Scura di Torrelama; Spada; Markur; Prothabat 69.
Cicer arietinum L.	chickpea	Sultano.
Phaseolus vulgaris L.	bean	Lingua di fuoco.
Vicia faba major L.	fababean	Supersimonia.
Lupinus albus L.	lupin	Luteur.
Vicia sativa L.	common vetch	Mirabella.
Medicago sativa L.	alfalfa	(unknown)

Table 2. MANOVA results based on the morphological traits.

Source	DF	exVarSS	nPC	nBu	exVarPC	exVarBU	p-Value
Species	1	0.053976	7	23	0.714	1	0
Staining	1	0.005351	7	23	0.713	1	0.196059
Species × staining	1	0.007352	7	23	0.713	1	0.11264
Error	243	0.933139					

DF – Degrees of Freedom; exVarSS – explained variances based on sums of squares; nPC – number of principal components used for testing; nBu – number of principal components used as buffer components; exVarPC – variance explained by nPC components; exVarBU – variance explained by (nPC+nBU) components; p-Value – the result from 50-50 MANOVA testing.

two *Ascochyta lentis* (Fig. 1F) isolates (ATCC 96419 and ATCC 96420), were collected as described above. In order to evaluate the possible effects of staining on the morphology of conidia, conidia were stained with lactophenol blue and lactic acid, respectively. Thirty to forty conidia per species/treatment were analyzed and images of conidia were recorded using an Olympus Altra 20, 2 Megapixel CMOS digital camera mounted on a Zeiss Axioscope 2 plus microscope (Carl Zeiss, NY, USA). Morphological traits from each image acquired at 40× magnification were automatically extracted using the following image analysis protocol: image segmentation was applied using the moment algorithm (Tsai, 1985) on the Euclidean distance (rescaled from 0 to 255) of each pixel on the RGB channels (Costa *et al*., 2012). The morphological traits extracted were: perimeter, area, major and minor axis lengths, ratio of major to minor axis lengths, and 25 elliptic Fourier coefficients. Overall shape was evaluated by elliptic Fourier analysis (EFA) on the outline coordinates (Rohlf and Archie, 1984). After the binarization (see the image analysis protocol above), 180 points (x, y) equally angularly spaced (one point every 2°) from the centroid were digitized along the outline. Coordinates were aligned by generalized Procrustes analysis (Bookstein, 1991). The 180 aligned coordinates were treated as outline data (Antonucci *et al*., 2012). The Fourier series was truncated at the value of *k* at which the average cumulative power is 99.99% of the average total power (Crampton, 1995; Antonucci *et al*., 2012). Mean outline and standard deviation ranges were graphically reported for each species, together with the basic morphological parameters. Image analysis procedures were developed in the MATLAB 7.1 R14 environment. The multivariate matrix of morphological traits was analyzed with a 50-50 MANOVA procedure (Langsrud, 2002), a generalized multivariate ANOVA method based on principal component analysis (PCA) on standardized data. Adjusted p-values were conducted on a rotation testing based on 99999 simulated datasets. The contribution of the variables to each rotation test was extracted.

Development of the teleomorph and hybrid status of progeny. Mating types of grasspea and *Ascochyta lentis* isolates were determined using a multiplex PCR with primers designed to the idiomorphs of *Ascochyta* spp. (Cherif *et al*., 2006). Either a 450 bp or a 700 bp amplicon was amplified from isolates carrying *MAT1-1* and *MAT1-2*, respectively (Cherif *et al*., 2006). Genetic crosses were attempted between grasspea fungus isolates and *A. lentis* of different mating type following a modified procedure of Kaiser *et al*. (1997). Conidial suspensions (1×10^6 conidia ml^{-1}) were prepared by flooding the surface of 3-week old cultures on V8 medium (Miller, 1955) with sterile water and scraping conidia with a spatula and used to inoculate sterile senescent chickpea stems. Chickpea stems were inoculated with single isolates as controls or with a mixture of conidia of two isolates of opposite mating type by dipping the stems in the conidial suspensions for 30 min (Kaiser *et al*., 1997). Stems were dried in a laminar flow hood for 30 min and then incubated on 10 layers of moistened, sterile filter paper in glass Petri dishes at 10°C for 6-8 weeks. To discharge ascospores, stem pieces were attached to the inner surface of a Petri dish lid, moistened with sterilized water and inverted over a dish containing 3% water agar. Germinated ascospores were harvested after 18 h and

transferred to V8 plates. Mature pseudothecia that did not discharge ascospores using the above method were dissected in sterile water under a dissecting microscope to collect ascospores. Hybrid status of progeny was tested by PCR amplification of the mating type marker (Cherif *et al*., 2006) as well as three microsatellite markers (Ozkilinc *et al*., 2011a) among 20-30 randomly selected progeny from three crosses. The null hypothesis of 1:1 segregation of each marker was tested using chi-square tests.

RESULTS

Fungal isolates from grasspea. Fungi isolated from symptomatic tissues and seeds resulted in cream to brown colonies on PDA with scarce aerial mycelium. Small black pycnidia were present starting from the center of the colony, containing hyaline conidia that resembled other *Ascochyta* fungi. Observations of approximately 600 conidia revealed that 28% of conidia were unicellular, 72% were 1-septate, and less than 1% were 2-septate. Conidia had rounded ends, constricted at the septum, oblong-ellipsoid, sometimes slightly flexuous, with one cell slightly larger and constricted at the apex and measuring 15.5 µ ± 1.6 µ (12-24µ) × 5.7µv± 0.5 µ (2.9-6µ) (Fig. 1E). *Ascochyta lentis* isolates were significantly longer, measuring 17.5 µ ± 3.1µ (11.8-27.0µ) × 5.2µ ± 0.6µ (3.6-6.8µ), straight or slightly curved, 1-2 septate, not constricted at the septum (Fig. 1F).

Estimation of optimum growth temperature. After 10 days growth, the effects of temperature, isolate and temperature × isolate interactions were all significant at $P \leq 0.05$. Average optimum temperature was 25°C (7.0 mm d^{-1}), while no growth was observed at 35°C. Isolate ER1478 grew significantly faster (3.93 mm d^{-1}) than isolates ER1819 and ER1415 (3.22 mm d^{-1} and 2.6 mm d^{-1}, respectively).

Molecular identification. *ITS, EF, CHS* and *G3PD* sequences of grasspea isolates ER1413 and ER1415 were compared to sequences in the NCBI database through BLASTn searches. Isolates ER1413 and ER1415 had identical sequences at all four loci sequenced. Amplification of ITS yielded a 465 bp fragment with similarity greater than 99% to *A. lentis*, *A. viciae-villosae*, *A. pisi*, and *A. viciae-pannonicae.* Amplification of G3PD generated a 516 bp amplicon with 100% similarity to *A. lentis* AL1. Amplification of CHS yielded a 329 bp amplicon with 100% similarity to *A. lentis* AL1. Amplification of EF yielded a 267 bp amplicon with 100% similarity to two isolates of *A. lentis* (AL1 and SAT-AL).

Pathogenicity and host specificity. Inoculation of grasspea with grasspea fungus isolate ER1413 in the first experiment caused disease symptoms similar to those observed on naturally infected grasspea (data not shown). Small necrotic areas were observed on leaves 10 days after inoculation, which were enlarged and merged, sometimes causing necrosis of the entire leaves. No symptoms were observed on any other inoculated legumes. Koch's postulates were fulfilled by reisolation of the same fungus from inoculated grasspea tissues. In the second experiment, two grasspea fungus isolates (ER1413 and ER1415) and two *A. lentis* isolates (AL1 and AL6) were each pathogenic to their respective hosts producing similar symptoms on both stems and leaves. Inoculation of *A. lentis* isolates AL1 and AL6 resulted in modest disease severity on both lentil cultivars and weak disease symptoms on 3 grasspea accessions. In contrast, isolates ER1413 and ER1415 caused much higher disease severities on three of four grasspea accessions with no visible symptoms on lentil (Fig. 2)

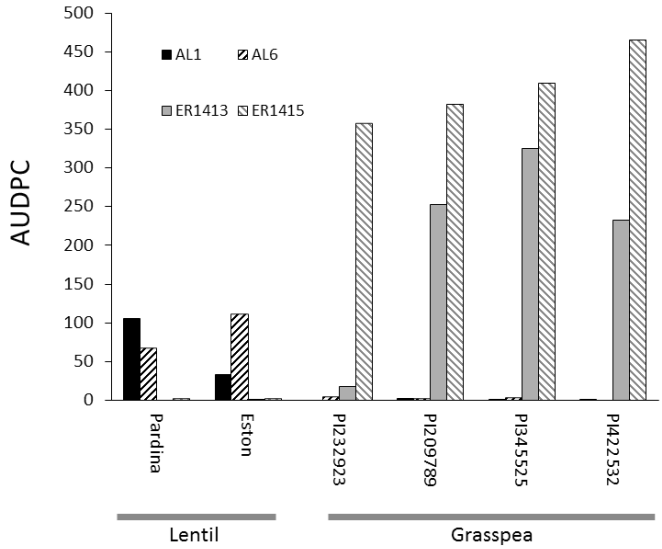

Fig. 2. Area under the disease progress curve (AUDPC) for two lentil cultivars and four grasspea accessions inoculated with two isolates of *A. lentis* (AL1, AL6) and two isolates of the grasspea fungus (ER1413, ER1415). AUDPC values represent the average of four replicate pots for each plant/fungus combination.

Morphological characterization using image analysis. Morphological characters of conidia produced by the grasspea fungus and *A. lentis* were compared using elliptical Fourier analyses. Perimeter, area, major axis length, ratio between major and minor axis lengths and 5 elliptic Fourier coefficients were the most important morphological traits in the species differentiation. Among the observed factors (species and coloration) and their interaction, only species was significant in the MANOVA (Table 2).

Development of teleomorph and hybrid status of progeny. All grasspea fungus isolates screened with the multiplex mating type PCR assay were of mating type 1 (*MAT1-1*) which precluded making crosses between two grasspea fungus isolates. *MAT1-1* isolates of the grasspea

fungus were successfully crossed with *MAT1-2* isolates of *A. lentis* as evidenced by the development of pseudothecia containing ascospores on senescent chickpea stems inoculated with mixtures of suspensions of 1:1 mixtures of *MAT1-1* and *MAT1-2* isolates (Fig. 1G and Fig. 1H). Segregation ratios of idiomorphs at the mating locus and parental alleles at three microsatellite loci among progeny isolates of three crosses between *A. lentis* and the grasspea fungus demonstrated the hybrid status of the progeny and all segregation ratios were not significantly different from 1:1 (data not shown).

DISCUSSION

Isolation of fungi from symptomatic leaves, stems and pods of grasspea (*Lathyrus sativus* L.) grown in experimental plots in Italy yielded uniform colonies on standard media that appeared morphologically similar to *Ascochyta* spp. from other legumes. Molecular identification of two of these fungi using sequence data from four regions of the genome revealed 99-100% sequence identity to isolates of *A. lentis* from lentil. Image analysis has been utilized in taxonomic studies of other phytopathogenic fungi (Andersen *et al*., 2005; Lecellier *et al*., 2014) and elliptical Fourier analysis of conventional morphometric measures and 25 EFA coefficients indicated that conidial dimensions of *A. lentis* and the grasspea fungus were significantly different in this study. Inoculation of several accessions of grasspea and of additional nine leguminous species, and reciprocal inoculation on grasspea and lentil showed that the isolates from grasspea were pathogenic only on grasspea, while *A. lentis* isolates caused only mild symptoms on grasspea. The host specificity of *Ascochyta* species in this clade has been demonstrated previously (Barilli *et al*., 2016; Hernandez-Bello *et al*., 2006). Crosses between grasspea isolates and *A. lentis* were successful and hybrid pseudothecia developed demonstrating that these two fungi have retained the ability to mate despite the morphological and pathogenic differentiation observed. Hybrid pseudothecia formation between grasspea isolates and *A. lentis* suggests a recent common ancestor of these taxa. No significant morphological differences were observed between *A. fabae* and *A. lentis* based on multivariate analyses of morphological and cultural characters but these fungi were host-specific infecting faba bean and lentil, respectively (Gossen *et al*., 1986). Based on these results, two special forms of *A. fabae*, *A. fabae* f. sp. *fabae* and *A. fabae* f. sp. *lentis* were proposed for these fungi (Gossen *et al*., 1986). These taxa were each raised to specific status by Kaiser *et al.* (1997) based on the observations that crosses between these taxa resulted in nonstandard numbers of ascospores per ascus, low ascospore viability, abnormal cultural morphology of progeny and distinct RAPD-PCR fingerprints (Kaiser *et al*., 1997). The systematics of *Ascochyta* spp. and *Phoma* spp. attacking legume crops has never been fully resolved (Kim *et al*., 2016). The classical taxonomy of *Ascochyta* spp. is based on morphological characters such as shape and size of conidia, conidial septation, host of isolation and genetic differentiation based on molecular markers (Peever et al., 2007). Shape and dimensions of conidia are considered to be of major importance compared to other features (Mel'nik, 2000). Our isolates from grasspea and *A. lentis* showed statistically significant differences in conidia dimensions, and strong host specialization, but were identical or nearly identical across four loci commonly used for lower-level fungal systematics (Peever *et al*., 2007). Recently, the metabolic profiles of three grasspea fungus isolates (ER1415, ER1478, and ER1813), were compared with those of other *Phoma* and *Ascochyta* spp. Highly uniform profiles among grasspea fungus isolates and *A. lentis* were observed, suggesting to the authors that these taxa were likely conspecific (Kim *et al*., 2016).

The successful mating and normal marker segregation coupled with the genomic identity we observed between *A. lentis* and the grasspea fungus do not support separation of these taxa as either biological or phylogenetic species. However, the observed differences in conidial dimensions and host specificity clearly demonstrate that these taxa are genetically distinct and are therefore likely in the process of speciation. While isolates from grasspea were specific to grasspea, *A. lentis* isolates were capable of infecting grasspea hosts at a low level which may indicate a host jump of *A. lentis* from lentil to grasspea followed by specialization on grasspea, a pattern which has been observed in other pathosystems (Le Van *et al*., 2012; Stukenbrock *et al*., 2011). Host specialization is predicted to be one of the most important ecological factors driving the speciation of plant pathogens (Silva *et al*., 2012; Ozkilinc *et al*., 2011b; Frenkel *et al*., 2010, Giraud *et al*., 2010) and it seems likely that the host specificity is the major force driving the speciation of the two fungi studied here. Until there is further genetic and morphological divergence between the grasspea fungus and *A. lentis*, we propose naming the former as a specific variety of *A. lentis*. Analyses of whole genomes of these fungi will provide important insights into the genetic divergence between these host specialized and morphologically divergent pathogens. Additionally, genotyping of samples of these pathogens from sympatric populations of grasspea and lentil in Italy or elsewhere will allow the study the speciation process based on gene flow between these closely related fungi infecting each host.

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